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Meet the editor

Bin Wu, Ph.D., HCLD (ABB), is a scientific laboratory director at Arizona Center for Reproductive Endocrinology and Infertility, USA. He received his training in genetics and reproductive biology in China and at Cornell University, New York, USA, and his post-doctoral training at the University of Guelph, Canada. He is an embryologist at the Center for Human Reproduction, Chicago, USA. Dr. Wu is a member of many professional associations, such as the American Society for Reproductive Medicine, International Embryo Transfer Society, Society for the Study of Reproduction, American Association of Bioanalysts, and European Society of Human Reproduction and Embryology. He is the recipient of several research awards from these organizations. Dr. Wu was recognized for his excellence as an embryologist and practitioner in the practice of reproductive embryology by the American College of Embryology in 2014.

Contents

Preface

Embryology is a vast discipline concerned with the study of embryogenesis. It is the branch of animal biology that studies the prenatal development of gametes (sex cells), fertilization, and development of embryos and fetuses. Additionally, embryology encompasses the study of congenital disorders that occur before birth, known as teratology. Human embryology studies began by using human embryo samples derived from maternal deaths, abortion, or surgery in the 19th century. However, human embryology has progressed little during the last 100 years because of ethical aspects and limited human materials. Since the middle of last century, embryo in vitro culture technology has advanced with developments in assisted reproductive technology (ART), including in vitro fertilization (IVF) and its related procedures, including intracytoplasmic sperm injection (ICSI), embryo cryopreservation and frozen embryo transfer, and preimplantation genetic testing. Also, recent studies using both nondestructive and destructive imaging techniques, such as time-lapse imaging, have allowed many morphological measurements of embryos. Embryology has been enriched and developed greatly in terms of its contents and forms. This book summarizes some novel observations and developments in embryology. It includes five sections.

Section 1 discusses gamete collection and cryopreservation technology and theory. The beginning of life starts with the combination of an egg and sperm. The first step in current in IVF practice is the collection of an oocyte and sperm. Important to note is that an individual's body weight has a significant effect on their fertility. Thus, Chapter 1 examines the impact of body mass index (BMI) on oocyte retrieval number in IVF. In the study presented, obese patients had fewer oocytes retrieved.

The cryopreservation of gametes including sperm and oocytes has been widely used in animal breed resource preservation and human IVF clinic practice. Typically, two freezing methods, slow freezing and vitrification, have been used in ART practice. Slow freezing is a conventional cryopreservation method that is based on a slow cooling rate and the use of a low concentration of cryoprotectants. This leads to less toxicity to cells/tissues; however, it can also decrease their survival rates. Nowadays, vitrification has replaced slow freezing to obtain a higher survival rate for sperm, oocyte, and embryo cryopreservation. Vitrification, as a significant stressor, appears to have a significant impact on epigenetic modifiers and maternal transcripts of the oocyte, which ultimately results in lower developmental potential. Thus, Chapter 2 reviews the recent progress of human sperm vitrification technology. Then, Chapter 3 discusses the impact of oocyte vitrification on epigenetic modification and maternal transcripts, providing a theoretical basis for the optimization and improvement of vitrification-warming technology.

ART has been extensively applied for treating human infertility for more than 40 years, but IVF success rates could be further improved.

Section 2 includes two chapters that discuss how to increase IVF outcomes by both enhancing IVF laboratory quality control and improving embryo technique. These approaches include improving the air quality inside the lab to ensure volatile organic compound (VOC)-free air, use of a tri-gas incubator, adequate control of pH and osmolarity of culture media, use of embryo-tested devices and plastics, and strict quality control that allows adequate development of the embryos until the blastocyst stage. Other strategies to improve embryo quality during in vitro culture include volume reduction of drop culture media and individual or group culture of embryos. Embryo transfer is the last step of a series of events in IVF and poor management can jeopardize the entire process. Thus, Chapters 4 and 5 review some key techniques of embryo transfer.

Preimplantation embryo development refers to the maturation of a fertilized ovum to a blastocyst. This process is highly regulated and required for proper implantation of the blastocyst into the endometrium. During this phase, several tasks must be accomplished. The differentiated zygotic genome must undergo reprogramming back to totipotency to generate all the different types of tissue that make a human being. Next, certain cells begin to differentiate to prepare for implantation, which occurs at approximately day 7 post-fertilization. This progression is a result of the careful interplay between maternally persistent RNA transcripts and the activation of the zygotic genome. After the embryonic genome activation, blastomere differentiation begins to occur and cellular polarity has been shown to be the signal transduction that initiates this differentiation. Chapter 6 discusses the molecular and cellular mechanisms regulating preimplantation embryo development and its real effect in ART.

Thousands and thousands of human embryos have been produced by IVF and how to use these embryos has become a heated argument all over the world. The scientific associations of human reproduction experienced fundamental change in the twentieth century, with the development of in vitro fertilization for the treatment of infertility. The separation of sex and assisted reproductive technology treatments led to a revolution in gender selection and similarity relations, while embryo diagnostics led to a shift from scheduling families to planning a child. Furthermore, a fertilized egg outside the womb is a new form of human life that can be conserved and manipulated. The embryo in a petri dish in a laboratory has become the entity of the reproductive market, driven by clients and their claim to a right to reproductive choice. These reproductive technological improvements encounter deep-set moral sensitivities of human self-respect and the relation of human beings to their own nature.

Section 4 (chapter 7) presents these moral approaches, especially regarding interventions in preimplantation-stage embryos in the laboratory, suggesting environmentally suitable laboratory conditions for this entity. Additionally, not only do new suggestions for legitimately suitable regulations takes care of embryo's right, but also embryology laboratory personnel, clinics and parents.

After an embryo is transferred into a uterus, it will begin to grow rapidly and form early organs. With x-ray and ultrasonographic imaging technology innovation, the organogenesis of early embryos has obtained some novel observations and knowledge. Section 5 includes two chapters (chapters 8 and 9) that described some new discoveries in the hepatic fetal venous system and dental crown formation during early embryo development.

This book provides a comprehensive overview of embryology and presents basic theories and techniques for human IVF clinic practitioners and embryologists.

I would like to thank all the contributing authors for their excellent chapters.

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Section 1

Gamete Collection and Vitrification for Freezing

Chapter 1

Impact of Body Mass Index (BMI) on Retrieval of Oocyte Numbers in *In Vitro* Fertilization Women

Linda Wu and Bin Wu

Abstract

Previous research and clinical reports have discovered that body weight significantly affects a patient's fertility status. Underweight, overweight, or obese women may experience reduced fertility. Currently, assisted reproductive technology (ART) is used as treatment for infertile couples to conceive a child. However, whether abnormal body mass indexes (BMIs) affect infertile oocyte production is not clear. The objective of this study is to determine the association between BMI and retrieved oocyte numbers. A total of 136 IVF patient data in 2016 was reported from Reproductive Health Center. The relationship between patient's BMI and retrieved oocyte numbers has been analyzed and their correlation coefficients between patients' age, oocyte numbers, and BMI have been calculated. The results further proved that BMI affects collecting oocyte numbers and oocyte maturation rate. Overweight patients had fewer oocytes retrieved than that of normal weight patients. Likewise, obese patients had even fewer oocytes retrieved than that of both normal-weight and overweight patients. Underweight BMIs seem to have no effect on the number of oocytes collected; however, the oocyte quality and embryo production needs to be further studied. Results from this study may be used by IVF physicians and practitioners when consulting patients for IVF treatments.

Keywords: assisted reproductive technology, body mass index, overweight, obese, oocyte number

1. Introduction

The impact of infertility causes significant mental and physical strain on both men and women. Numerous factors can influence male and female fertility. Common causes of female infertility may include anovulation, hormonal imbalances, structural issues or damage to fallopian tubes or uterus, cervical issues, decreased ovarian reserve with increased age, etc. In the past few decades, one rising factor affecting the fertility status is an individual's body weight. Being severely underweight or overweight can disrupt the process of regular, consistent ovulation in females, leading to anovulation. In 2013, the American Society of Reproductive Medicine (ASRM) released "Optimizing Natural Fertility: A Committee Opinion" in which the society

included recommendations on how to counsel patients to optimize the likelihood of becoming pregnant. ASRM reported that fertility rates are decreased in women who are underweight or obese based on body mass index (BMI).

According to the National Heart, Lung, and Blood Institute (NIH), the body mass index (BMI) is a standardized measure of an individual's body fat percentage relative to their height and weight. A normal range is considered to be anywhere between 18.4 and 24.9, whereas an underweight value is below 18.4. Overweight values range from 25 to 29.9. Obese individuals have BMIs of 30 or greater. BMI can be calculated by an individual's weight divided by their height squared (kg/m^2). In relation to fertility, Hassan and Killick [1] reported that the time to conception was increased by more than twofold among overweight/obese women (BMI >25 kg/m^2) and by more than fourfold among underweight women (BMI <19 kg/m^2). With additional research suggesting the impact body weight has on one's fertility status, the ASRM highlighted several points suggesting women who are obese to receive counseling prior to attempting to conceive in order to prevent potential medical, obstetric, or neonatal complications. In addition, the ASRM indicated that diet and exercise are the first-line treatments for obesity, where weight loss is linked to return of ovulation and decreased miscarriage rates in obese women who were previously infertile. Through pharmacotherapy, such as antiobesity medications, including orlistat, lorcaserin, naltrexone, may be used for obese women, it should only be considered for those who do not respond to a 6-month lifestyle modification. Furthermore, bariatric surgery may be considered to improve obesity-related issues regarding menstrual irregularity and infertility in women, however, pregnancy should not be considered within a year after surgery.

Based on the ASRM's guidelines stated above, it is highly recommended for individuals who are severely underweight or overweight/obese to normalize their weight in order to improve their fertility status. Practitioners may provide patients with appropriate recommendations for weight loss/gain programs, nutritional counseling, dietary modifications, and/or exercise regimens. The goal of physicians should be to allow patients to be in their best possible health condition prior to starting any fertility treatments. This may be done to ensure lower complications of treatment, to better improve infertility treatment success, and to lower complications of pregnancy.

Currently, many infertility patients can be treated by assisted reproductive technologies (ART), in which fertilization of oocytes occurs in a laboratory environment. In vitro fertilization (IVF), the most common ART procedure, involves different stages for conception to occur, including oocyte retrieval from the ovaries, sperm introduction to oocytes, and successful transfer of embryo(s) back into the female for implantation. During the beginning of a cycle, follicle-stimulating hormone (FSH) injections are administered to patients during the follicular phase of the ovarian cycle to maximize the number of developing follicles. On days 12–14, a trigger shot, usually hCG (human chorionic gonadotropin), stimulates the final maturation of oocytes. Then, the matured oocytes are collected for fertilization in the laboratory. Once the oocyte is fertilized by the sperm to create a zygote, the embryo begins to divide. The embryo can be implanted in the uterus by day 3 or 5 after fertilization. Day 3 embryo cleavage comprises 6–9 cells that are in the process of dividing, but the embryo itself will not grow in size. Day 3 embryos can incubate further to day 5 blastocysts containing more advanced cellular structures. In situations where the quality of sperm is not optimal, intra-cytoplasmic sperm injection (ICSI) technique may be used to inject one sperm into the oocyte to increase the chances of successful fertilization.

A significant number of IVF and ICSI babies have been born throughout the world. However, a key factor of assisted reproductive techniques is the reliance on medicine and hormones to stimulate the ovaries to develop multiple follicles per cycle. Nonetheless, some patients may show less than optimal responses to the medication prescribed. One reason may be due to high and low BMIs. As a result, the number of retrieved oocytes, oocyte maturity, subsequent fertilization, embryo quality, and live birth rates may be reduced. Overweight women tend to have lower responses to medication to regulate and/or initiate ovulation, resulting in higher doses. In addition, women who are overweight/obese have a greater frequency of over-response and a higher risk of overstimulation. If a multiple pregnancy occurs, there are greater obstetrical complications in patients with higher BMIs than that of normal range BMIs. Additional complications include fewer eggs retrieved, increased difficulty during the retrieval process, increased risk of bleeding, increased risks of anesthesia, and greater difficulty during embryo transfer when visualizing the uterus.

Likewise, underweight individuals experiencing anorexia nervosa or bulimia are potentially at risk for infertility [2]. In patients who are malnourished or starving, a lowered metabolic rate, along with decreased gonadotropin release, may result in fertility loss. Deficiencies in estradiol, an important player in the female reproductive system that is commonly seen in anorexia is also due to low ovarian stimulation. As a result, patients who are underweight are advised to gain weight prior to starting fertility treatment.

Although previous studies have reported that being underweight or overweight/obese has significant effects on female fertility, there are no reports analyzing the effect of body weight on fertility factors, such as the number of oocytes retrieved and fertilized, embryo quality, and IVF outcomes. Therefore, this study is designed to examine the impact of body weight on fertility treatment outcomes. The goal of this study is to determine if fertility is impacted by individuals who are underweight, overweight, or obese by analyzing IVF outcomes so that practitioners may correctly counsel their patients before undergoing assisted reproductive technology.

2. Materials and methods

This was a retrospective study in an IVF laboratory. The deidentified data was collected from patients who have undergone IVF treatment in 2016 at the Reproductive Health Center in Tucson, Arizona. Information regarding patient IVF procedures includes: patient age, weight, height, number of retrieved oocytes, number of matured oocytes, number of embryos created, number of embryos transferred, pregnancy rates, and live birth.

Data for underweight and overweight patients were compared to individuals within the normal weight range. Body mass index (BMI) of each patient was calculated. The normal range is 18.4–24.9, whereas an underweight value is below 18.4. Overweight values range from 25 to 29.9. Obese values have BMIs of 30 or greater. BMI was calculated by an individual's weight divided by their height squared (kg/m^2). Using these ranges, the infertile patients were grouped into four categories (underweight, normal, overweight, and obese).

Prior to the procedure, various hormones, including gonadotrophin-releasing hormone/follicle-stimulating hormone (GnRH/FSH) injections (250-450IUs depending on patient age and BMI), were given to the patients to target the growth

of follicles during an IVF cycle. Based on follicular size and blood estradiol level, at 36–37 hours after human chorionic gonadotrophin (hCG, 4000 to 10,000 IU) administration for oocyte maturation, the eggs were retrieved from ovaries through transvaginal by our physician standardized procedure. Therefore, the retrieved oocytes were classified as matured oocytes (MII), germinal vesicle (GV), postmatured oocytes, or degenerate oocytes. Only matured oocytes (MII) were used to calculate maturation percentage rate.

2.1 Statistical analysis

The average and standard deviation (means) of all data were calculated by Microsoft Excel. The significant differences between the averages were examined by student t-test statistical analysis, and the difference between the percentages was examined by percentage test method. The differences were considered statistically significant at $p < 0.05$.

3. Results

3.1 The effect of age on retrieval of oocyte numbers

Patient age was plotted as a function of oocyte number for 136 IVF egg retrieval patients, as shown in **Figure 1**.

Figure 1 shows that the number of collected oocytes declines as a function of patient age. Greater number of oocytes were collected from younger patients than for older patients. The negative correlation demonstrates that as age increases, the number of retrieved oocytes is reduced. The correlation coefficient was calculated to be $r = -0.66$, which is also indicative of a negative correlation between patient age and oocyte numbers.

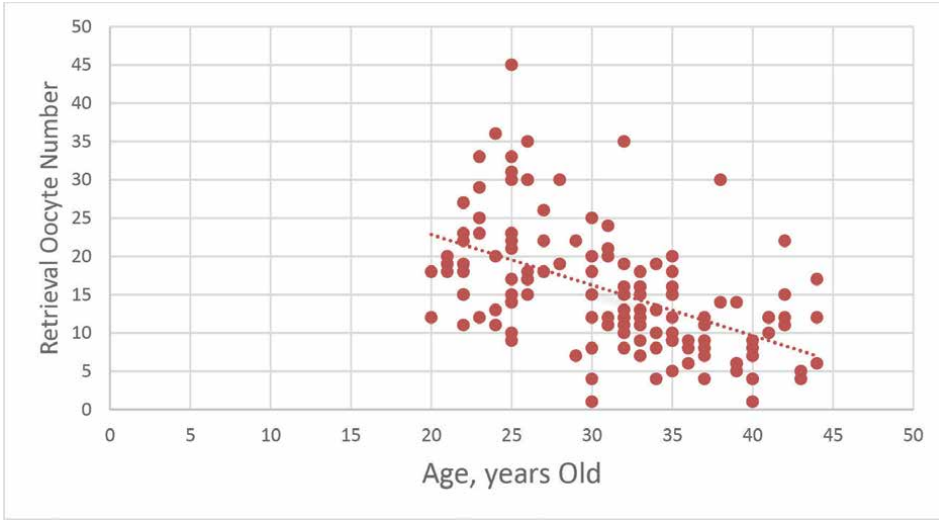

Figure 1.
The correlation of patient's age and collected oocyte numbers.

3.2 Relationship between age and BMI

There was no relationship between patient age and body mass index (**Figure 2**). The correlation coefficient is low ($r = 0.11$) and shows no statistical significance ($p > 0.05$). The averages of patient height, weight, BMI, and oocyte numbers in relationship to age ranges are represented in **Table 1**.

Table 1 shows that there was no significant difference in the patient's height, weight, BMI, and oocyte maturity in various age ranges ($p > 0.05$). However, as age increases, the collected number of oocytes significantly decreased ($p < 0.05$).

3.3 Scatter diagram of patient body mass index and retrieval oocyte numbers

The number of eggs retrieved was plotted as a function of BMI (**Figure 3**). Most patients were in the normal body weight category (18.4–24.9 kg/m^2). There were more overweight patients (24.9–29.9 kg/m^2) and obese patients (>30 kg/m^2) than underweight (<18.4 kg/m^2) patients. The correlation coefficient between BMI and retrieved oocyte number was calculated to be $r = -0.4177$. The negative relationship indicates that as BMI increases, the number of oocytes collected decreased. The statistical test of correlation coefficient shows significant difference ($P < 0.05$).

3.4 The effect of patient's BMI on retrieval of oocyte numbers

According to BMI classification, 136 patients undergoing IVF oocyte retrieval were divided into four groups: BMI < 18.4, BMI 18.5–24.9, BMI 25–29.9, and BMI > 30, representing underweight, normal weight, overweight and obese, respectively. Patient's retrieval oocyte number and oocyte maturation rate have been analyzed (**Table 2**).

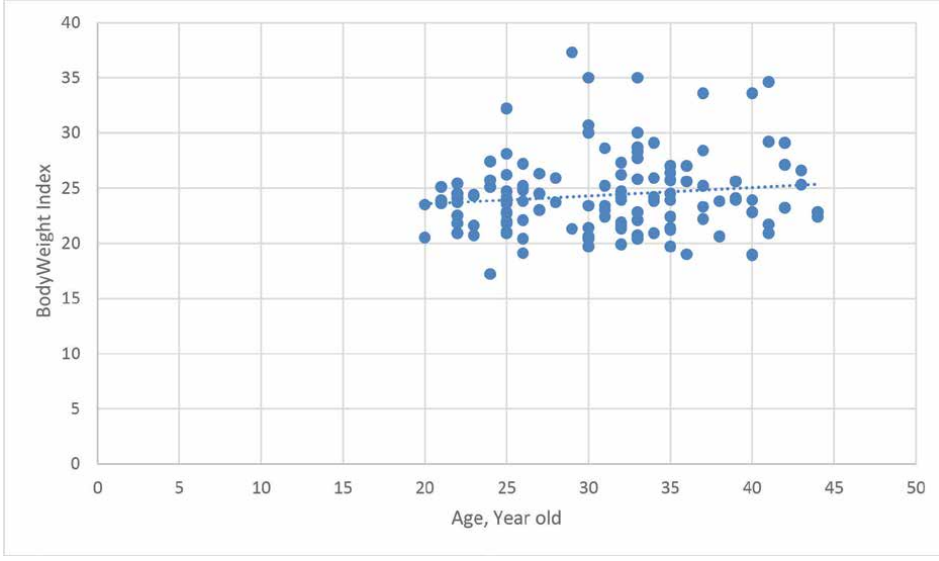

Figure 2.
A scatter diagram for relationship between patient age and body weight index (BMI).

Age (year old)	No. of patients	Height (in)	Weight (lbs)	BMI	Oocyte no.	Maturity
<25	35	64.4 ± 2.1	137.7 ± 14.3	23.4 ± 2.1	20.7 ± 8.3[a]	77.3%
26–30	24	63.9 ± 2.3	146.5 ± 30.1	25.1 ± 4.8	18.7 ± 8.5[a]	72.2%
31–35	42	65.5 ± 2.8	150.4 ± 21.2	24.6 ± 3.2	13.7 ± 5.6[b]	81.7%
36–40	22	63.7 ± 1.5	140.8 ± 22.9	24.4 ± 3.9	8.6 ± 5.7[b]	81.0%
>40	13	64.6 ± 2.5	153.2 ± 27.8	25.7 ± 4.0	12.3 ± 6.3[b]	83.7%
Total	136	64.6 ± 2.4	145.2 ± 22.9	24.4 ± 3.6	15.6 ± 8.1	78.4%

Note: letters a and b indicate significant difference ($p < 0.05$).

Table 1.
Basic information from collected data in relation to BMI and oocyte numbers at different age ranges.

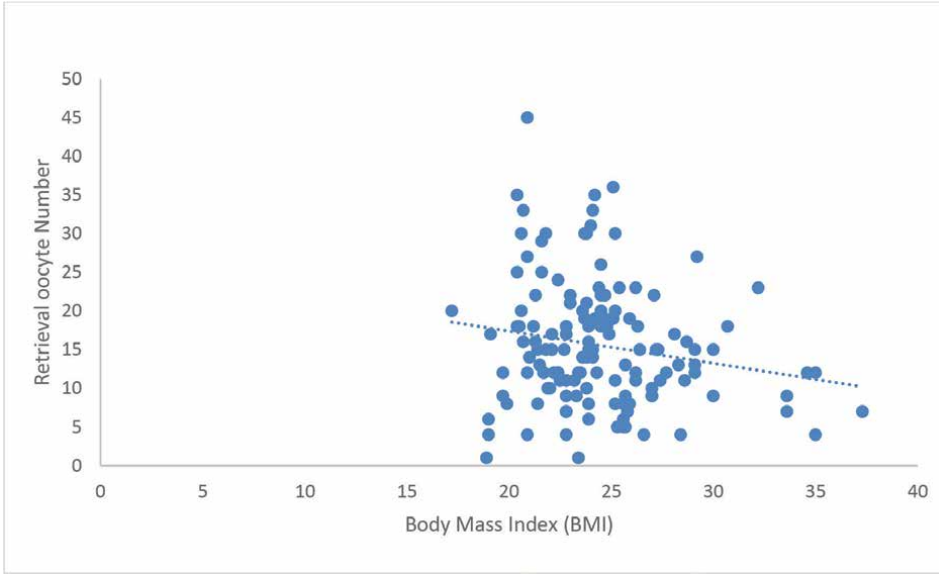

Figure 3.
A scatter diagram of the relationship between body mass index and oocyte retrieval number.

Based on **Table 2**, there is no significant difference in patient's height, in different BMI groups, but as patient weight increases, the BMI shows an obvious big. The overweight patients (BMI 25–29.9) had significantly fewer retrieved oocytes than normal-weight patients. Similarly, obese patients (BMI >30) had significantly fewer retrieved oocytes than normal-weight and overweight patients ($p < 0.01$). Observing oocyte maturity, the number of matured oocytes in obese patients was significantly lower than that of normal-weight and overweight patients ($p < 0.05$). Thus, obese women had lower oocyte maturation rate. **Figure 4** displays the number of retrieved oocytes and maturation rates in different BMI categories. This figure shows that as BMI increases, the numbers of retrieved oocytes and maturation rates are significantly reduced.

BMI	No. of patients	Height (in)	Weight (lb)	No. of retrieved oocytes	No. of matured oocytes	Oocyte maturation rate
<18.4	1*	64	100	20	16	80%
18.4–24.9	85	64.9 ± 2.5	134.6 ± 13.6[a]	16.9 ± 8.5[a]	13.2 ± 7.0[a]	78.4%[a]
25–29.9	40	63.8 ± 2.4	155.0 ± 15.3[b]	13.75 ± 7.3[b]	10.9 ± 5.8[b]	79.7%[a]
>30	10	65.1 ± 1.5	199.6 ± 12.3[c]	11.6 ± 5.7[c]	8.4 ± 4.9[c]	72.4%[b]
P value		P > 0.05	P < 0.05	P < 0.01	P < 0.05	P < 0.05
Total	136	64.6 ± 2.4	145.2 ± 22.9	15.6 ± 8.1	12.2 ± 6.7	77.6%

*Note: letters a, b and c indicate significant difference (p < 0.05). The star * shows only one patient with BMI <18.4 in collecting 136 patients. Although it does not have any statistical significance, it shows that this patient had enough of the number of retrieved oocytes and good maturity.*

Table 2.
The relationship between patient BMI and number of retrieved oocytes.

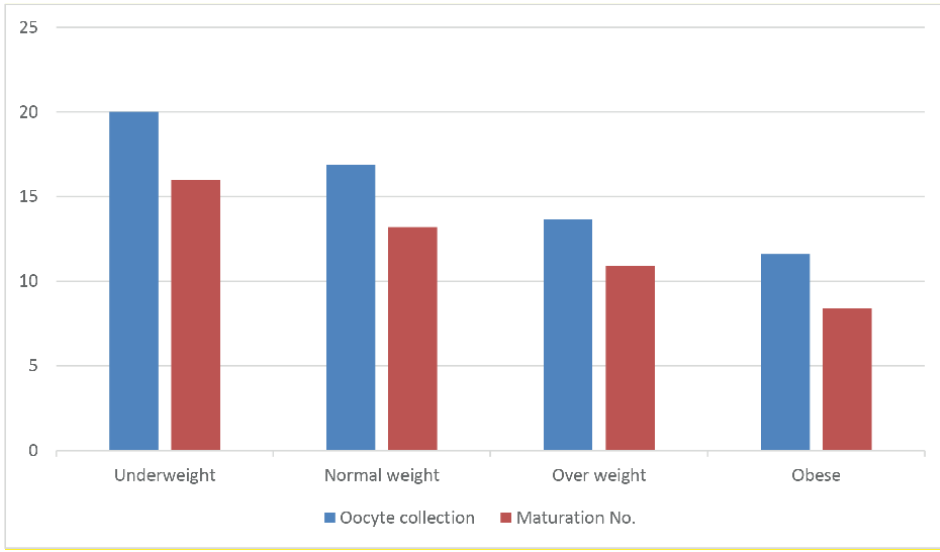

Figure 4.
Numbers of collection oocytes and maturation oocytes in various BMI patient groups. Note: Underweight group just had one patient with BMI <18.4.

4. Discussion

Infertility has affected more than 180 million people worldwide and has become an ongoing critical reproductive issue over recent decades [3]. Currently, the assisted reproductive technologies (ARTs) have been an effective measure to treat infertile couples in conceiving a child. However, the success of ART is closely associated with several demographic characteristics and physical conditions, including maternal age, female body mass index, potential diseases, lifestyle, and various environmental factors [4]. BMI has a significant effect on *in vitro* fertilization outcome in especially women. Women with overweight status and obesity status have been associated with higher infertility rates. In this study, we examined whether BMI affects retrieval

of oocyte numbers and oocyte maturity. Our results showed that BMI significantly affected collection of oocyte numbers and its maturation rate. The overweight patients had significantly fewer oocyte numbers than that of normal weight patients. Similarly, obese patients had fewer oocyte numbers than normal-weight and overweight patients. Obese patients' oocyte maturation rate was also much lower than normal and overweight patients. It has been suggested that obesity could have a detrimental effect on oocytes and endometrium [5] because obese women have a poor response to ovarian stimulation, and thereby need higher doses of gonadotropin hormone injections [6]. Thus, normal doses of medication injection for obese women may result in not enough matured oocytes retrieved.

However, according to the World Health Organization, being underweight is much less common with a prevalence of less than 5%, whereas having excess body weight constitutes around 50% of the adult population in developed societies [7]. As a result, fewer research and publication is generated on the negative impact of being underweight on spontaneous and assisted conception. The greatest concerns for underweight women in trying to conceive include the obstetric complications related to nutrition levels for the mother and fetus. This leads to increased risks of anemia, impaired weight gain, preterm delivery, low birth weight, postnatal complications, etc. In our collecting 136 patients, it is disappointing that there was only one patient in BMI < 18.4 group. Thus, we could do a statistical analysis for underweight group. We just listed this underweight Woman with a total of collected 20 oocytes and 16 matured oocytes. It is very difficult to be able to make conclusions regarding underweight patients and IVF treatment in this study.

The number of oocytes collected from women of different BMIs also depends on the age of the patient. We first analyzed the relationship between patient age and retrieved oocyte quantities. Similar to previous research [8], the number of oocytes retrieved and female age have a close association. This relationship represents a negative correlation coefficient ($r = -0.66$) between patient age and oocyte numbers, thereby indicating that the number of oocytes collected will significantly reduce as a patient is older. For example, although one patient with BMI < 18.4 in our study was underweight, her age was just 24 years old, and she might donate 20 oocytes and had 16 matured oocytes for IVF.

Also, our study indicated that there was no close relationship between age and BMI. The irregular dispersion of the scatter diagraph displays a low correlation coefficient ($r = 0.11$). Conversely, many reports have indicated a positive correlation between age and weight, where an increase in adult age is commonly associated with increased body weight and thus BMI. However, our results did not find any association between the two variables. This may be due to a limited age range of patients in the study. In other words, our study population may have limited generalizability because the infertile patients who have undergone IVF treatment ranged between 20 to 45 years old. Patients' ages outside of this range were excluded from the analysis. Because of the limited age range, it is possible that weight gain is seen among women outside of this spectrum.

The original design of this study was aimed to analyze the effect BMI has on female IVF outcomes, which also included analyzing the quantity of embryo production and live birth. However, due to limitations of data quantity regarding these two variables, we were unable to analyze the numbers of embryo fertilization and live birth rates. Furthermore, embryo formation and patient pregnancy may be affected by numerous factors, including male sperm quality, female endometrium quality, prenatal complications, etc. Therefore, we concentrated on the association between

female BMI and oocyte production and retrieval numbers. Results from this study may be a reflection of current infertile patient population who are seeking IVF treatment.

5. Conclusion

In summary, our study further verified that the quantity of oocyte retrieval and female age have a close association, where the age of a female significantly affects the number of oocytes that can be fertilized for artificial or natural conception. Thus, we further conclude that as age increases, the number of oocytes retrieved during an IVF treatment is significantly reduced. We did not find a close relationship between infertile female age and BMI; however, our results indicated that BMI has a strong influence on oocyte quantities and maturation rates. Overweight patients tended to have significantly fewer oocytes than that of normal weight patients. Likewise, obese patients have even fewer oocytes than that of normal-weight and overweight patients. Conversely, the underweight patient did not seem to have a difference in oocyte number and maturation rate, but we cannot conclude significant findings from this group since a greater sample size is required. Analysis of the study may be used by IVF physicians and practitioners to facilitate an optimal IVF treatment program for infertile patients seeking to conceive.

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Chapter 2

Human Sperm Vitrification: Review of Recent Progress

Feng Gao

Abstract

Sperm vitrification has been used in the field of assisted reproductive technology (ART) for years and has resulted in many healthy live births. Compared to the conventional sperm slow freezing method, vitrification is simpler, quicker, and less expensive, and some vitrification methods are also cryoprotectant free, which has the potential to become an alternative cryopreservation method for human sperm. Human sperm vitrification has been the most commonly used and valuable way to preserve the fertility of males with small numbers of spermatozoa. Recently, new sperm vitrification devices have been developed to help improve volume control. Direct contact during the vitrification process with liquid nitrogen increases the risk of cross-contamination. New strategies have been implemented to minimize the contamination risk. Depending on the variety of semen parameters and patients' purposes at ART clinics, specific sperm cryopreservation approaches should be personalized to achieve the optimal results for each case.

Keywords: assisted reproductive technology (ART), sperm, vitrification, cryopreservation, cryoprotectants

1. Introduction

Traditional cryopreservation techniques are widely used in assisted reproductive technology (ART) programs all over the world; however, vitrification is a novel technique and has become a quickly growing alternative method for the cryopreservation of human spermatozoa in the past decade. Sperm vitrification usually requires a small loading volume of sample to achieve an extremely high cooling rate. Human sperm vitrification has been the most commonly used way to preserve the fertility of males, including those with severe oligospermia or azoospermia patients, who have undergone a TESE/micro-TESE procedure, which has yielded a small number of spermatozoa. However, in the past few years, many new designs of larger volumes have been developed and have displayed promising results. This review summarizes the recent researches on human sperm vitrification, including comparison studies of conventional cryopreservation methods like slow freezing and vitrification, descriptions of different types of cryoprotectants, methods of human sperm vitrification, as well as the warming, storage of vitrified samples, contamination risk and control during sperm vitrification.

2. Principles of human sperm cryopreservation

There are several different cryopreservation methods, including slow freezing (0.5–0°C/min), rapid freezing (50–400°C/min), ultra-rapid freezing (~2500°C/min), and vitrification (~20,000°C/min), applied accordingly depending on the freezing speed and cryoprotectant concentration and temperature reduction [1]. The principles of cryopreservation are based on the laws of thermodynamics. Sperm has been shown to be sensitive to exposure to high concentrations of cryoprotective agents. Freezing media for human sperm vitrification is significantly different from cryopreservation solutions for oocytes and embryos. Contrary to the slow freezing process, the vitrification process is based on an extremely high cooling rate that prevents intracellular ice crystallization and produces an amorphous, glass-like solid state.

The greatest challenge the cells are facing during the freezing and warming processes is cell damage caused by ice crystals forming a temperature range between −15 and − 60°C. At temperatures between −5 to −15°C, extracellular ice formation occurs and develops an extracellular solid phase. Meanwhile, the inside of the cell remains supercooled, which has a high chemical potential and diffuses out of the cell osmotically. Extracellularly hypertonic results in further removal of water from cells causing almost complete dehydration. The composition of the cryopreservation media plays a vital role in the freezing techniques, fast or slow. Permeable cryoprotectants, including glycerol, ethylene glycol, 1,2 propanediol, and dimethyl sulfoxide (DMSO), are typically used in the cryopreservation of human spermatozoa. Due to their lipophilic properties, they cross the cell membrane, creating an osmotic gradient caused by water outside of the cell. Sperm have been shown to be particularly sensitive to high concentrations of cryoprotectants that are routinely used for oocytes and embryos. Non-permeable cryoprotectants, including sucrose, glucose, and trehalose etc., are of high molecular weight, and consequently are not able to penetrate the cell membrane. They are not directly toxic to sperm, but they nonetheless cause damage due to the osmotic shock encountered during addition and removal. Vitrification does not require an osmotic balance during the freezing period of the cells, as fast dehydration occurs with a super high freezing speed and a hyperosmolar medium. During the process, as the viscosity of the solution increases, the molecules are immobilized and the liquid passed to a solid glass-like phase without the formation of ice crystals in both the intracellular and extracellular environment while displacing the water from the cell. For the vitrification of human sperm, the cryoprotectant-free vitrification method has been developed to reduce cryo-injuries [2].

2.1 Comparison of the studies for human sperm slow freezing versus vitrification

Both slow freezing and vitrification have advantages and disadvantages which can be found in **Table 1**. One way to investigate the difference between slow freezing and vitrification is directly compared the two methods using the same semen samples in the same study [3]. With 57 human semen samples, Saritha and Bango et al. discovered no substantial motility differences between the two methods [4]. Zhu et al. used 58 washed human semen samples to compare the efficiency of the slow freezing method versus the vitrification process. No differences in motility or DNA stability were found in this study, however, higher progressive motility, plasma membrane, and acrosome integrity were found in the vitrification group with optimal sucrose concentration than in the slow-freezing group [5]. Recently, Pabon et al. compared

	Slow freezing	Vitrification
Cryoprotectants	Glycerol (5–10%), TEST yolk buffer (12% glycerol), IUI -ready cryoprotectant (HEPES-buffered human tubal fluid with 1% human serum album, 4% sucrose, and 6% glycerol)	Glycerol (5–10%), some vitrification methods are free of permeable cryoprotectants
Standardized protocol	Yes	No
Cryopreservation device	Cryovial	Cryoloop, spermVD, cryotip, cryotube, cryogenic Vial 0.25 ml
Food and Drug Administration (FDA) approved	Yes	No
Validated for human sperm cryopreservation and storage	Yes	No
Current clinically applied populations	Male with normal sperm count (>15 million sperm per milliliter)	Male with severe oligospermia or azoospermia who have undergone a TESE/micro-TESE procedure which has yielded a small number of spermatozoa.

Table 1.
Comparison of slow freezing vs. vitrification of human spermatozoa.

the efficiency of vitrification versus slow freezing. They found that vitrification resulted in better motility recovery and higher mitochondrial activity compared to slow freezing [6]. Karthikeyan et al. conducted a comparison study using 20 severe oligoasthenozoospermia samples and found higher motility and vitality using vitrification than slow freezing [7]. Spis et al.'s study with one epididymal and one testicular sperm sample demonstrated that vitrification had higher mitochondrial membrane potential and motility in both samples than slow freezing [8]. A systematic meta-analysis review study conducted by Li et al. included a total of 2428 published articles and 13 randomized controlled trials containing 486 vitrified and 486 slow-freezing sperm samples [9]. They concluded that although the efficacy of vitrification varied by vitrification protocols and sample quality, the vitrification method was superior to slow freezing regarding the post-thaw total motility and progressive motility [9]. Taken together, human sperm vitrification has shown higher potential compared to slow freezing, although the vitrification procedures need further to be optimized and standardized for a more definitive conclusion.

2.2 Cryoprotectants and methods of human sperm vitrification/warming

A recent review, including all the previous methods, techniques, and devices for vitrification of human spermatozoa, concluded that the universal method/platform has yet to be developed [3]. Novel solutions specially designed to vitrify a small number of spermatozoa needed to be further explored [10]. **Table 2** summarizes

Vitrification		Warming	Reference
Device	Cyrotube 0.85 ml	Warmed at room temperature 30–45 min.	[11]
Medium	Glycerol		
Procedure	Room temperature 10 min, plunged into liquid nitrogen		
Device	Cryogenic Vial 0.25 ml	Warmed in 42°C water bath 1 min, then 37°C water bath till melted.	[5]
Medium	Permeable cryoprotectant free		
Procedure	Room temperature 1 min, plunged into liquid nitrogen		
Device	1.5 ml straw	Warmed in 42°C medium 20 seconds.	[12]
Medium	Permeable cryoprotectant free		
Procedure	Room temperature 10 min, loaded 100 µl in a straw, plunged into liquid nitrogen		
Device	Collector-grid 5–10 µl drop	Warmed in 44°C medium 3 min.	[6]
Medium	Permeable cryoprotectant free.		
Procedure	Room temperature, plunged into liquid nitrogen		
Device	Cryovial.	Quickly submerging the sample in 5 ml G-IVF Plus medium prewarmed to 37°C with gentle agitation. After incubation at 37°C for 5 min, the post-thaw sperm suspension was centrifuged at 300 g for 5 min and resuspended in 100 µl G-IVF Plus medium.	[13]
Medium	Trehalose (0.5 mol l – 1), glycine (100 mmol l – 1) and human serum albumin (1% [w/v])		
Procedure	The vitrification medium was slowly added to human semen sample at a 1:1 dilution, and the resultant suspension was incubated at 25°C for 5 min. Approximately 25 µl of aliquots of the sperm suspension were dropped directly into medical-grade liquid nitrogen free from contaminants which resulting in the immediate formation of a 25 µl floating sphere that solidifies and sinks after about 25 seconds. This process was repeated to obtain a sufficient number of spheres (**Figure 1**). All the spheres were finally packed into a 1.8 ml cryovial and stored in liquid nitrogen.		
Device	SpermVD	Warmed at room temperature, spermatozoa were located in the spermVD droplet, transferred to the collection droplet and used for ICSI.	[15]
Medium	Quinn's Advantage™ Sperm-freezing medium		
Procedure	Spermatozoa were transferred from the collection drop to a 0.8-1 µl droplet in the spermVD, and placed the spermVD inside a cryovial, plunged into liquid nitrogen (**Figure 2**).		

Table 2.
Human spermatozoa vitrification and warming procedure.

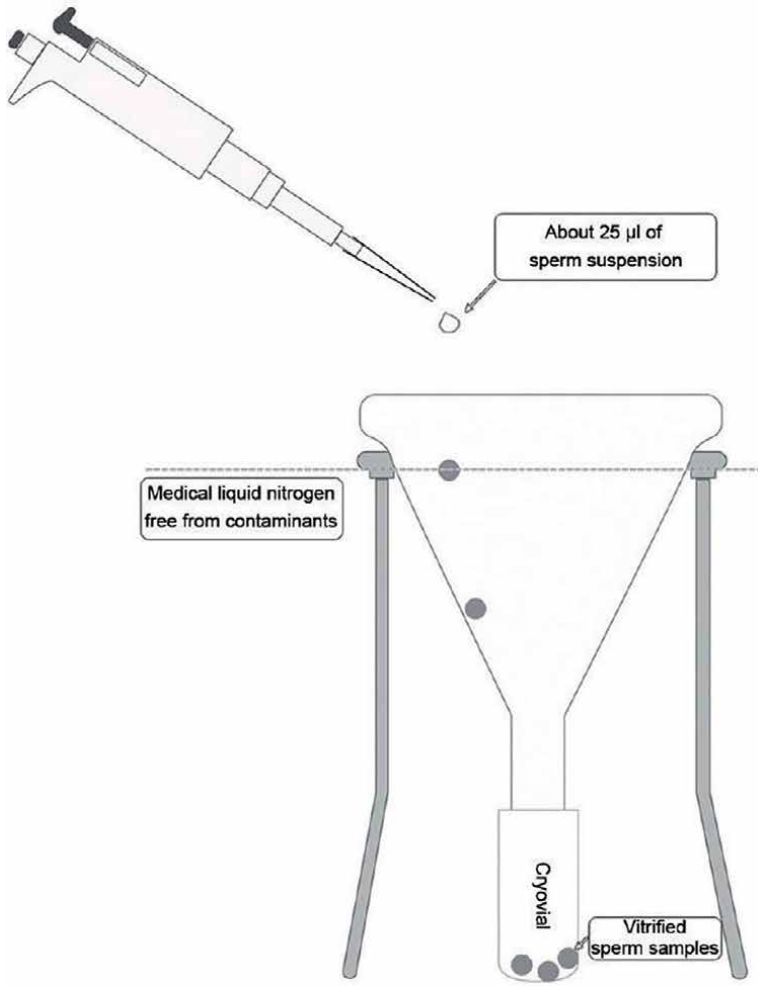

Figure 1.
Illustration of the vitrification process and device by Zhou, D et al. [14]. The vitrification medium was trehalose (0.5 mol/L), glycine (100 mmol/L) and human serum albumin (1% [w/v]). The vitrification medium was added to the semen sample slowly at 1:1 ratio, and was incubated at 25°C for 5 minutes. Then approximately 25 µl of the semen suspension were dropped directly into medical-grade liquid nitrogen-free from contaminants. This process was repeated to obtain a sufficient number of spheres. All the spheres were loaded into a 1.8 ml cryovial and stored in liquid nitrogen for at least 48 h [14].

the various methods of sperm vitrification and warming which have been developed in the past decades.

Vitrification with highly concentrated, permeable cryoprotectants is not suitable for spermatozoa as spermatozoa are osmotically sensitive. Sperm vitrification media is isomolar between 300 and 396 mOsm/L., which can be maintained by non-permeable cryoprotectants or a combination. The most commonly used permeable cryoprotectants include DMSO, glycerol, glycol, and ethylene. Non-permeable cryoprotectants include albumins, dextran, and egg yolk. Isachenko et al. developed cryoprotectant-free vitrification with capillaries method showing higher motility and integrity rates of cytoplasmic and acrosome membranes, and less cryo-injury in human sperm

Figure 2.
Sperm VD: An innovative and efficient medical device for cryopreservation of small numbers of spermatozoa [15]. Vitrification: 1. Place drops of cryoprotectant on the spermVD, then put the spermVD into the culture dish with sperm. 2. Identify sperm and transfer the sperm cells onto the spermVD. 3. Place the spermVD into a cryovial. 4. Plunge the cryovial with the spermVD into liquid nitrogen. Warming: Take the spermVD out of the liquid nitrogen and transfer the cells from the spermVD to sperm washing medium at room temperature [15].

vitrification [2]. They used 52 human swim-up prepared ejaculated samples for vitrification without any permeable cryoprotectants. Some studies reported successful sperm vitrification in straws and cryoloop droplets for small volumes [2]. Azipurua et al. compared a permeable cryoprotectant-free vitrification protocol to the slow freezing method using 18 normal sperm samples and found that improved recovery rates of good quality sperm and better maintenance of sperm quality were generated in the vitrification group compared to the slow-freezing group [16]. Moreover, they demonstrated that vitrification with the cryoprotectant free method produced a higher percentage of spermatozoa, better preservation of acrosomes, and lower DNA fragmentation compared to the slow freezing method [16]. Additionally, they used alpha-tubulin immunochemistry to identify the similar labeling pattern of the sperm skeleton in the tail as fresh sperm, but different from post-thaw sperm from the slow freezing [16]. However, Agha-Rahimi et al. found there was no major difference in post-thaw motility, DNA fragmentation, or hyaluronan-binding potential with or without cryoprotectant for normospermia samples [17].

Regarding nonpermeable cryoprotectants, sucrose (0.25 M final concentration) and serum dextran supplement in a final concentration of 0.1% are generally used for

sperm vitrification base medium. Some modifications have been made to the composition of the vitrification medium, for instance, Butylhydroxytoluene (BHT), a synthetic analog of vitamin E, was used as an antioxidant to improve the cryoprotective effects on human spermatozoa [18]. After warming, higher progressive sperm motility, DNA integrity, and lower reactive oxygen species were observed in the vitrification medium supplemented with BHT. The proposed mechanism of BHT is increasing the fluidity of the cell membrane via incorporation into the membrane [18]. Another study found that post-warming sperm motility using 0.1 mol/L trehalose (69%) was higher than that of used sucrose (0.25 M, 58%) with healthy volunteer semen samples [14]. Similar results were found at 6 and 12 h post-thaw. Furthermore, 0.1 mol/L trehalose improved membrane integrity at 0 h post-thaw. No significant improvements were found at 6 and 12 h in terms of membrane integrity [14]. According to these results, the use of trehalose increases tolerance to hypertonic and hypotonic conditions, preventing cell lysis and death during the vitrification and warming process. Additionally, the study demonstrated that post-thaw spermatozoa maintained at room temperature better than maintained at 37°C up to 4 h in terms of viability and mitochondrial membrane potential [14]. Zhou, D et al. developed a modified vitrification method which has a relatively better recovery rate (65.8%) and improved preservation of several sperm quality parameters compared with slow freezing (**Figure 1**). They used trehalose (0.5 mol/L) glycine (100 mol/L) and human serum albumin (1% w/v) as cryoprotectants to vitrify 28 semen samples from healthy participants [13].

Collectively, some might perform better than others depending on specific situations/concentrations in terms of cryoprotectants. Further research is needed to investigate the optimal permeable and non-permeable cryoprotectants.

The vitrification process is based on an extremely high cooling rate that prevents ice crystal formation. A semen sample can be processed by swim-up and loading in straws or cryoloops, and then rapidly cooled by direct contact with liquid nitrogen (−196°C). After loading, the straws or cryoloops are put into the precooked aluminum blocks for long storage in liquid nitrogen or vapor phase in a liquid nitrogen tank (−180°C). Zhou, D et al. tested different combinations of carriers, including cryoleaf (Medicult, Jyllinge, Denmark), cryoloop (Hampton Research, Orange, CA, USA), and straw (Cryo Bio System, Paris, France). The highest freeze rate (about 10,000°C per min) is achieved by the cryoleaf and cryoloop system, however, they also exhibit the lowest freezing efficiencies (**Figure 1**) [13]. Berkovitz et al. tested a novel vitrification device spermVD and found it is an efficient and simple carrier method for freezing a small number of spermatozoa in low-volume droplets that significantly reduces post-thaw search time from hours to minutes, allowing a 96% recovery rate and leading to successful use of sperm for fertilization (**Figure 2**) [15]. The target populations are patients with a small number of spermatozoa, such as azoospermia patients, who have undergone a TESE/micro TESE procedure or severe oligozoospermia patients.

Besides the high-speed freezing, the warming speed should also be high allowing the water inside spermatozoa to pass from a glassy state to liquid without ice crystal formation. Mansilla et al. tested different warming temperatures and found the progressive motility in sperm samples warmed at 42°C (65%) was higher than at 38°C (26%) and 40°C (57%) and plasma membrane function was better preserved at 42°C [19]. Pabon et al. warmed vitrified spermatozoa micro pills (5-10 μl each) in 500 μl prewarmed medium and maintained them at 44°C for 5 seconds and decent post-thaw motility and mitochondrial activity were observed [6]. Zhou D et al. warmed the sample by submerging the spheres in 5 ml G-IVF Plus medium pre-warmed to 37°C accompanied by gentle agitation for 5 min. Post-thaw sperm achieves a statistically significantly

higher recovery rate, motility, morphology, and curve line velocity than slow freezing ($p < 0.05$) [13]. Furthermore, a lower rate of DNA fragmentation and better acrosome protection were observed in the spermatozoa after vitrification than slow freezing ($p < 0.05$) [13]. Schulz et al. found that 42°C was the optimal temperature to preserve the sperm parameters, including motility and membrane integrity in the warming process [1]. Collectively, the warming process is flexible in terms of temperature.

2.3 Storage of the vitrified sperm samples

Cryopreservation of spermatozoa is the standard of care for fertility preservation in patients who undergo chemotherapy or radiotherapy. There are other reasons why a couple or individual patient needs to cryopreserve the spermatozoa, for instance, before vasectomy or in the case of a traveling husband. The conventional cryopreservation (slow freezing) protocol is standardized and widely used in clinical practice. The spermatozoa being vitrified with non-permeable cryoprotectants reduce the possibility of water inside the cell, allowing storage at lower temperatures. This technique is limited as only small volumes with small numbers of spermatozoa can be cryopreserved. The possibility that vitrified sperm preserve their function at temperature of −80°C could simplify storage, optimizing the space and time as well as the operator's safety [3]. Lyophilization of spermatozoa is another method that requires additional investigation and validation [20]. Since the lyophilized spermatozoa are immobile, they can only be used in intracytoplasmic sperm injection (ICSI). Therefore, further research needs to be conducted on the optimization, safety, and health of the offspring.

2.4 Contamination risk and control using sperm vitrification

Exposing the semen samples directly to liquid nitrogen increases the risk of contamination. A large variety of bacterial, viral, and fungal species have been found in liquid nitrogen [21]. Unsterilized commercial liquid nitrogen could cause transmission and propagation of diseases. On the other hand, the survival of cryogenic pathogens in liquid nitrogen creates the possibility of cross-contamination between stored contaminated semen samples and liquid nitrogen. Bacteria have a higher tolerance than fungi to freezing. Piasecka–Serafin reported bacteria contamination from infected semen samples to sterile liquid nitrogen and then other sterile semen pellets [22]. Within only 2 h of cryo-storage, about 94% of the sterile samples were contaminated with *E. Coli* and *S. Aureus* [22]. Molina et al. compared the contamination risk of bacteria and fungi using open versus closed vitrification devices with human oocytes and embryos [21]. They found the bacteria cross-contamination risk was no greater for open devices than for closed ones in vitrification [21]. But there were *Acinetobacter lwoffii*, *Alcaligenes faecalis ssp*. *Facecalis*, and *Sphingomonas Pauli mobilis* at the bottom of the storage container. No fungi were observed. The source of these pathogens could be from the cryopreservation environment [21].

The basic procedure to avoid contamination is to store contaminated or infected semen samples separately in quarantine to minimize the risk of cross-contamination. Sterilizing the air used to create a small amount of liquid nitrogen by using the air filter with 0.22 μm polytetrafluoroethylene efficiently retained *Brevundimonas* diminutive with extreme temperature, high pressures, and high flow rates. Another way is

using a 0.22 um filter equipped inside the canister to produce sterile liquid nitrogen at a similar temperature so that the samples sealed in the canister are only exposed to sterile liquid nitrogen air [23].

Another basic rule to control contamination is to avoid direct contact with liquid nitrogen. Using liquid nitrogen vapor, instead of liquid nitrogen itself, to store human semen samples can lower the risk of cross-contamination. Ultraviolet (UV) light could also be a possible way to reduce the contamination risk of vitrified sperm samples [24]. Treating a small volume of liquid nitrogen with 8000 us/cm^2 UV light could kill the hepatitis B virus, while 330,000 us/cm^2 destroyed the fungus Aspergillus niger [24]. Most viruses can be deactivated by UV light at a dose of 200,000 uw/cm^2. But it has been reported that the Zika virus may have higher resistance to UV light [25]. The UV light can also possibly induce genetic aberrations in stored spermatozoa. A simple solution is to sterilize liquid nitrogen with UV light before sperm storage. Another concern is the generation of ozone by UV light, which could cause damage to the buffer system in which the sperm samples are stored. However, the formation of ozone from UV light is insignificant as the liquid quid is free from oxygen.

Although there is no way to completely eliminate all the potential risks of cross-contamination in sperm vitrification, it is possible to control the contamination risk of vitrification to the level of slow freezing.

3. Conclusions

In the past decades, slow freezing of human sperm is still the main method used for sperm cryopreservation. However, vitrification provides a simpler, faster, more cost-effective alternative to conventional methods. Major concerns of the vitrification are the size of the sample and cross contamination with open devices. Optimization of the vitrification medium, sample size, and devices are a promising option. Due to different characteristics of spermatozoa species, including normospermia, oligospermia, azoospermia, testicular sperm aspiration, and testicular sperm extraction samples with different parameters, there is no universal vitrification method to serve different cryopreservation purposes at human clinics. Specific sperm vitrification methods should be individually designed to reach the optimal results depending on the personalized purpose at clinics. Future research in human sperm vitrification should include validation of the vitrification methods and whether the vitrification of sperm can improve clinical ART outcomes.

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Conflict of interest

The authors declare no conflict of interest.

Acronyms and abbreviations

ART	assisted reproductive technology
DMSO	dimethyl sulfoxide
ICSI	intracytoplasmic sperm injection
UV	ultraviolet
BHT	butylhydroxytoluene
FDA	food and drug administration

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Chapter 3

Beyond Survival Effects of Vitrification-Warming on Epigenetic Modification and Maternal Transcripts of Oocytes

Yu-bing Liu, Ju Chen and Ri-Cheng Chian

Abstract

Oocyte vitrification-warming is a worldwide used technique for human fertility preservation. The question of whether the potential risk is associated with this specific procedure remains unresolved. As a fundamental factor of development, oocytes play an important role in early embryonic development, including epigenetic reprogramming and maternal-to-zygotic transition (MZT), that can develop to term. Vitrification, as a significant stressor, appears to have a significant impact on epigenetic modifiers and maternal transcripts of the oocyte, which ultimately results in lower developmental potential. Due to the rapidly evolving single-cell multi-omics sequencing, there have been many advances in this field. We will discuss recent progress in the impact of oocyte vitrification on epigenetic modification and maternal transcripts in this manuscript, hoping to provide a theoretical basis for the optimization and improvement of vitrification-warming technology.

Keywords: oocyte, Vitrification-warming, epigenetics, maternal transcripts, epigenetic modification

1. Introduction

Oocyte survival and pregnancy rates have increased significantly since the development of vitrification technology. Many studies have proven that rates of survival, fertilization, and cleavage as well as embryo quality after vitrification are equivalent to those of fresh oocytes [1, 2]. In 2013, the American Society for Reproductive Medicine listed "freezing of human mature oocytes" as a technology that could be widely used in women undergoing gonadotoxic therapy [3]. The scope of clinical application of oocyte vitrification cryopreservation is gradually expanding, including fertility preservation before radiotherapy and chemotherapy for malignant tumors, ART remedy for sperm collection failure on the day of oocyte retrieval, donation of oocytes, and female fertility preservation for non-medical reasons, with an increasing number of babies born through this technology [4–6].

It is acknowledged that oocyte vitrification-warming is still a developing technique, though widely used. Pregnancy outcomes from vitrified oocytes vary from the quality of oocytes before freezing and the operators, who have different knowledge levels and use different technologies [7, 8]. In addition, several studies have reported that vitrification of mammalian oocytes can induce the generation of abnormal reactive oxygen species (ROS), accumulation of histone γ-H2AX, and increase apoptosis rate, as a result of lowing developmental potential of both early embryo and fetal [9–11].

Beyond survival and embryo development, it is essential to study the impact of oocyte vitrification-warming on the long-term safety of offspring from the perspective of epigenetics. Epigenetic modifications during oocyte genesis are crucial in subsequent embryonic development as well as individual development. Epigenetics refers to the process leading to the heritable changes of gene functions and phenotypic changes without modifying the nucleotide sequences, mainly, including DNA methylation, histone modification, and regulation of non-coding RNA, which can directly or indirectly influence the state of chromatin to activate or repress gene expression [12].

In primordial germ cells (PGCs), epigenetic reprogramming leads to germ cell-specific characteristics, such as meiosis, spermatogenesis, and oogenesis. Upon fertilization, the totipotency acquisition and development of embryos also rely on epigenetic reprogramming. In addition, toward the end of oogenesis, condensed chromatin prevents gene transcription, resulting in transcriptional silencing. As a consequence, the oocyte must synthesize and preserve sufficient transcripts to fulfill protein requirements during the period of meiosis completion, fertilization, and maternal-to-zygotic transition (MZT) [13].

Vitrification-warming procedures may affect oocyte epigenetic modifications and the composition of the maternal transcripts, which in turn affect epigenetic reprogramming, may have a significant impact on the biological processes of subsequent embryonic development [14]. This review will discuss the effects of vitrification-warming on epigenetic reprogramming and maternal transcripts of oocytes.

2. Effects of vitrification-warming on DNA methylation of oocytes

DNA methylation is a major epigenetic mark in DNA, which is essentially a covalent modification. DNA methylation is controlled by a family of DNA methyltransferases (Dnmts), which can transfer the methyl group of S-adenosyl methionine (SAM) to the carbon-5 position of cytosine(5mC) of cytosine-guanine (CpG) dinucleotide. DNA methylation can control gene expression by altering chromatin structure, DNA conformation, DNA stability, and the interaction between DNA and protein [15]. In mammals, CpG sites in gene bodies and intergenic regions are generally highly methylated, except for gene regulatory sequences, such as promoters and enhancers, which are usually low or intermediate DNA methylated [16]. Several studies have suggested that DNA methylation in promoter regions plays a regulatory role in the genome by interfering with transcription factor binding, recruiting methyl-CPG binding proteins (MeCP1, MeCP2, and other MBD proteins) and repressor complexes, leading to stable transcriptional repression [17, 18].

Mammals undergo two massive waves of global DNA demethylation and remethylation during the intergenerational transmission of life. The first major wave of genome-wide DNA demethylation occurs in PGCs, which are the progenitors of oocytes and sperm, where the paternal and maternal somatic programs are erased. Subsequent to this, DNA methylation was reestablished during spermatogenesis and oogenesis [19]. Different from male germ cells, which can rapidly initiate and

complete DNA remethylation before birth, oocytes need to gradually remethylate through de novo DNA methyltransferases (DNMT3A, DNMT3L, etc.) over a long period of development, from the primary stage to the antral follicle stage.

During fertilization and early embryogenesis, parental genomes undergo a second wave of demethylation and remethylation for epigenetic reprogramming. It is well known that DNA methylation reprogramming prior to the blastocyst stage is critical for early embryo development and subsequent ontogeny [20]. It is generally believed that the DNA methylation status of oocytes and early embryos is very sensitive to external stimulus, which potentially may lead to the low developmental competence and quality of embryos. Vitrification-warming is such a strong stimulus that its effects on the DNA methylation pattern of oocytes cannot be ignored.

Most research on the effect of oocytes vitrification-warming is performed with murine and bovine animals. It was found that the overall DNA methylation level of bovine oocytes was decreased after vitrification-warming [21, 22]. In mouse oocytes, vitrification-warming significantly reduced the methylation levels in the promoter regions of pluripotency gene Oct4 and Sox2, in addition to the decrease in the methylation levels of imprinted genes H19, Peg3, and Snrpn in blastocysts [23, 24]. However, vitrification-warming did not significantly alter the methylation levels of CpG islands in the promoter regions of Dnmt1o, Hat1, and Hdac1 [25].

Few studies have been conducted on the DNA methylation of vitrification-warming on human oocytes. In 2015, a study analyzed the effects of oocyte vitrification-warming on methylation levels in Day 3 embryos with immunofluorescence staining of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), which showed that there was no significant difference in DNA methylation between embryos derived from young donors vitrified and fresh oocytes [26]. Two other studies analyzed that oocyte vitrification-warming at the GV stage had no significant effect on DNA methylation of oocytes after *in vitro* maturation, using 5-methyl cytosine (5mC) immunofluorescence staining and imprinted genes H19 and KCNQ1OT1 bisulfite sequencing [27, 28]. However, as we know, immunofluorescence staining can only reflect the global DNA methylation level, and cannot measure the methylation levels at single-base resolution. Bisulfite sequencing can allow for the determination of methylation patterns at single-base resolution. However, as it is limited by throughput, only a few genes can be selected for detection.

In conclusion, oocyte vitrification-warming may decrease the DNA methylation level of oocytes or embryos, especially the promoter regions. However, most studies analyze DNA methylation of different gene regions, and lack of relevance between each other requires more experimental studies to be confirmed in the future. Limited by the resources and analytical methods, there are few studies about the effects of human oocyte vitrification-warming on DNA methylation, and the results are not consistent with that in animal models. Furthermore, it remains unclear whether oocyte vitrification-warming has different effects on DNA methylation differ among species. In recent years, the rapid development of whole-genome DNA sequencing of single cells (scWGS) enables the detection of global DNA methylation from the single base level, which makes us believe that more in-depth investigation will soon be available.

3. Effects of vitrification-warming on histone modification of oocytes

Histones are the core components of nucleosomes, the structural unit of chromatin. The nucleosome consists mainly of an octamer of four histones (two copies each

of H2A, H2B, H3, and H4) and 147 base pairs of DNA fragments around the outside. The free N-terminus of histones can undergo a variety of modifications, including acetylation, methylation, phosphorylation, ubiquitination, ADP ribosylation, and so on, which can control the state of chromosomes to produce different biological effects, such as transcriptional repression or activation [29, 30].

Histone modifications often cooperate with DNA methylation to control the state of chromosomes to activate or inhibit genes. Basically, DNA methylation in promoter regions is related to transcriptional inhibition, while histone acetylation is related to transcriptional activation. Actually, when DNA is methylated, lysine 9 of histone H3 is demethylated or trimethylated (H3K9me2/me3), chromatin is compressed, and transcription is blocked. Conversely, when DNA is demethylated, lysine 9 of histone H3 is acetylated, lysine 4 of histone H3 is demethylated or trimethylated (H3K4me2/ME3), chromatin is relaxed, and transcription is facilitated [31].

Similar to DNA methylation, histone modifications are highly dynamic during oogenesis and preimplantation embryo development. For example, in the early growth stage of mouse oocytes, H3K4me3 appears as a canonical pattern (Canonical H3K4me3) at promoter regions, and then in the later stage of oocyte maturation, non-canonical H3K4me3 (ncH3K4me3) gradually replaces canonical H3K4me3, covering broad domains in both promoters and distal regions, which makes up approximately 22% of the oocyte genome. In 2-cell embryos, the coverage of ncH3K4me3 decreases rapidly and is replaced by canonical H3K4me3, which is gradually restricted to the transcription start site regions, instead of being far away from the transcription start site (TSS). It is believed that the broad establishment of ncH3K4me3 in oocytes and timely deletion at the 2-cell stage are essential for embryo development. The broad establishment of H3K4me3 in the early stage can maintain DNA hypomethylation, which is associated with gene silencing, while the gradual removal of H3K4me3 and its restriction to promoters in later stages contribute to the activation of the zygotic genome and embryonic development [12, 32].

In addition, H3K27me3 has been shown to be intergenerationally inherited from the maternal genome during early embryogenesis, involved in regulating the activation of enhancers and lineage-specific genes [12, 33]. During the early embryonic development of mice, H3K9me3 has been found to be mainly enriched in long terminal repeats (LTR), which inhibits the expression of LTR. The abnormal H3K9me3 reprogramming is deemed to directly cause ZGA failure [33].

Therefore, the histone modifications of the oocyte are so vital to zygote and subsequent embryo development that we have to take into account the effect of oocyte vitrification-warming on histone modifications. By now, the studies that analyzed the effect of oocyte vitrification-warming on histone modification have only been conducted in animals. Due to different animal species and histone modifications studied, the reliability of the results needs to be further verified.

The study has shown that vitrification-warming could significantly reduce the H3K9me3 level of bovine oocytes and cleavage-stage embryos, significantly increasing the H3K9 acetylation level of cleavage-stage embryos, while significantly decreasing the H3K9 acetylation level of the blastocyst-stage trophectoderm [22]. Besides, oocyte vitrification-warming has been reported that could increase histone H4 acetylation in porcine and murine oocytes, and increase H3K9 methylation in murine oocytes [34–36]. Overall, oocyte vitrification-warming may lead to significant changes in histone modifications, but the results are inconsistent with each other. As the most commonly used detection method, the immunofluorescence method has the problems of low sensitivity and strong subjectivity, which may be

the reason for the inconsistent results. In addition, current studies mainly focus on histones H3 and H4, while there are few studies on histones H2A and H2B. The application of single-cell epigenetic detection technology in oocytes contributes to a more comprehensive study of the effect of vitrification-warming on histone modifications.

4. Effects of vitrification-warming on non-coding RNA of oocytes

The vast majority of human genes can be transcribed into RNA, but only about 2% of the genome codes for protein. RNA without protein-coding potential is called non-coding RNAs (ncRNAs), and most of the RNA is ncRNAs. According to the number of nucleotides, ncRNAs can be divided into small RNAs (small ncRNAs, less than 200 nucleotides) and long non-coding RNAs (lncRNAs, more than 200 nucleotides), and there is also covalently closed circular RNAs (circ-RNAs) [37].

In mammals, lncRNAs are involved in the regulation of transcription, which is related to cell pluripotency and differentiation. For example, Xist lncRNA and its antisense sequence Tsix, associate with the complex regulation of X chromosome inactivation (XCI), thus silence most of the genes on one X chromosome in female mammals to correct for a double number of X chromosome genes [38]. Small non-coding RNAs as a class of important post-transcriptional regulators, mainly contain micro RNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs), and Piwi-interacting RNAs (piRNAs) [39]. The miRNAs can interact with the 3′ untranslated region of target mRNA to initiate mRNA degradation and inhibit gene expression, which plays an important role in the development of mammalian oocytes and embryos [40]. Studies have shown that aberrant miRNA expression in female germ cells and embryos is associated with infertility and embryogenesis defects [41]. In addition, it has been suggested that RNase III Dicer is important in siRNA processing, and knocking out Dicer in oocytes can lead to meiosis arrest, accompanied by severe spindle and chromosome segregation defects, therefore, endo-siRNAs may be crucial to oocyte meiosis [39].

Non-coding RNAs are so sensitive to the environment that we need to consider whether they are affected by the vitrification-warming procedure. In 2019, a study about the comparison of miRNAs in mouse fresh oocytes and vitrified oocytes found that 22 miRNAs were differentially expressed between the two groups, and most of the target genes regulated by these miRNAs were closely related to metabolic pathways. Real-time quantitative PCR and sequencing results showed that Mir-134-5p, Mir-210-5p, and Mir-21-3p were significantly up-regulated in vitrified oocytes, while Mir-465C-5p was significantly down-regulated [42]. In addition, as a negative regulator of the PI3K/AKT signaling pathway, PTEN plays an important role in coordinating primordial follicular activation and oocyte DNA damage repair [43]. However, the expression of potential target PTEN in vitrified oocytes was significantly decreased at both the transcriptional level and the post-transcriptional level (protein level). Another study on vitrified human oocytes with RNA-seq also found the down-regulation of lncRNAs (CTB-180A7.6, AP000320.7, OOEP-AS1, RP11-59H7.3) [44]. Therefore, oocyte vitrification-warming may result in a decrease in the expression of certain specific non-coding RNAs.

At present, there are few studies on lncRNAs and other types of short coding RNAs in oocyte and early embryonic development, and their functions have not been fully explored. It is also rarely studied whether oocyte vitrification-warming has an impact on these non-coding RNAs.

5. Effects of vitrification-warming on oocyte maternal transcripts

The effect of vitrification-warming on gene expression of human oocytes is still in the preliminary stage. A study using the microarray method to compare gene expression between fresh and vitrified human mature oocytes showed significant differences in gene expression between the two groups, especially the downregulation of many genes in the ubiquitination pathway [45]. Another study, using transcriptome sequencing, also found that vitrification-warming reduced transcription levels of genes closely related to human oocyte genesis and development, involving multiple biological processes, such as cell cycle (NCAPD2 and TUBGCP5 significantly down-regulated), meiosis process (NCAPD2 and TUBB4B significantly down-regulated), multiple metabolic pathways (tricarboxylate cycle, amino acid metabolism, oxidative phosphorylation, etc.), DNA methylation, and DNA damage repair etc., among which HSPA1A and HSPA1B are the most representative ones [44]. HSPA1A and HSPA1B, as members of the heat shock protein family A (HSP70), can reverse or inhibit the denaturation or unfolding of cellular proteins under stress or high temperature, which are an important anti-stress defense system, participating in several cell functions, including regulation of mitosis, ubiquitin-proteasome pathway, regulation of lysosomal membrane stability, etc. It has strong resistance against abnormal apoptosis in cells and plays an important role in immune regulation outside cells. In addition, in early embryo development, the expression levels of HSPA1A and HSPA1B are closely related to the heat shock response ability of the embryo [46]. The downregulation of HSPA1A and HSPA1B gene expression goes against protein synthesis in biological processes, thus lowering the developmental potential of oocytes.

Additionally, one study, analyzing the effect of vitrification-warming on the expression of specific genes by real-time PCR, found that the overall mRNA content of oocytes decreased significantly (63.3% retained) after vitrification-warming, and the expression levels of genes relevant to DNA tissue structure, mitochondrial energetic pathway, cell cycle regulation (NAP1L1, TOP1, H1F0H1, SMC, SCC3, PAD21, SMC1A, SMC1B, etc.) were significantly decreased [47]. In contrast, several studies suggested that vitrification-warming had no significant effect on the expression levels of oocyte cytokinesis-related genes (DCTN1, DCTN12, DCTN13, DCTN16, and PLK1) and oocyte development-related genes (HPRT, GAPDH, CYCLOPHILIN, BMP15, GDF9, etc.) in oocytes [48, 49]. In brief, most studies have concluded that oocyte vitrification-warming results in a decrease in the transcription levels of genes involved in important biological processes in human oocytes.

In animals, there were four studies conducted in murine, bovine, and pig models to investigate the effect of vitrification-warming on gene expression levels, using transcriptome sequencing [50–53]. It was found that vitrification-warming had no effect on the transcriptome of mouse oocytes [50]. On the contrary, differentially expressed genes have been found in bovine and porcine vitrified oocytes, especially genes related to transcriptional regulation, cell division and differentiation, and apoptosis pathways [51–53]. In addition, the expression levels of epigenetic modification related genes (Mad1, Mad2, BubR1, Peg3, lgf2R, Sirt1, Dnmts, CD9, CD81, HMG3a, etc.) were significantly down-regulated after vitrification-warming, using real-time quantitative PCR [24, 25, 54–57]. Vitrification-warming could up-regulate the expression of pro-apoptotic genes BAX and P53 and down-regulate the anti-apoptotic gene BCL2 in bovine and porcine oocytes [58–61].

Vitrification-warming also caused overexpression of Eg5, a gene promoting cell division, in bovine oocytes [60]. It was also found that vitrification-warming decreased the expression of CD9 in mouse oocytes, which may lead to difficulties in fertilization [62].

In conclusion, most studies believe that vitrification-warming can lead to down-regulation of gene expression levels in animal and human oocytes, which may account for the decreased developmental potential of vitrification-warming oocytes.

6. Summary and outlook

Compared with slow freezing, vitrification has become an important means of the oocyte *in vitro* preservation as an important part of assisted reproductive technology (ART), which can improve the survival rate of oocytes. It is worthy of studying and discussing the impacts that oocyte vitrification-warming has on subsequent embryonic development and the later generations. This review mainly summarizes the effects of oocyte vitrification-warming on epigenetic modifications and transcriptional levels.

Generally, vitrification-warming decreased the level of global DNA methylation in oocytes or embryos of the mouse, cattle, and pig models, especially the methylation level in promoter regions [21–24]. Few studies that assessed the effect of human oocyte vitrification-warming on epigenetic modification have found that vitrification has no significant effect on DNA methylation [26–28]. The results are inconsistent with that in animal oocytes. The differences may be due to the low accuracy of immunofluorescence staining, the short sequence length of bisulfite sequencing, or the differences between species, which need to be confirmed by further studies.

In addition, there are a few studies on the effect of vitrification-warming on the transcriptome of human oocytes. Two studies have found no significant difference in the transcriptome characteristics of fresh oocytes and vitrified oocytes [48, 49], while the other three have suggested that vitrification-warming reduces the transcription levels of genes closely related to ovulation and developmental processes, involving multiple biological processes [44, 45, 47]. Therefore, the effect of vitrification-warming on the transcriptome of human oocytes is still inconclusive. The reserve of oocyte maternal transcripts is so essential for fertilization and maternal-to-zygotic transition (MZT) that we cannot ignore the effects of vitrification-warming on human oocyte maternal transcripts, which needs further study.

In animal models, oocyte vitrification resulted in a decrease in the global DNA methylation, changes in histone modifications, differential expression of miRNAs, and a decrease in gene expression levels, thereby reducing the rates of oocyte cleavage and blastocyst formation and the development potential of the early embryo. Another study has found that the maturation rate of vitrified immature human oocytes decreases after thawing, though most people believe that vitrification-warming has little effect on epigenetic modification and maternal transcripts of human oocytes [63]. In addition, there is still a lack of reliable data on whether the epigenetic or expressional changes possibly resulting from oocyte vitrification-warming have any effect on the subsequent offspring, which needs to be further studied.

Mitochondrial activity and redox homeostasis in oocytes are vital for proper embryonic development. It is well known that ROS is an unavoidable byproduct of mitochondrial respiration. Oxidative stress (OS) in oocytes, stemming from the imbalance of ROS production and their neutralization, is related to low rates of

fertilization and embryo developmental potential [64, 65]. Mitochondrial activity and ROS levels are most often affected by extreme conditions imposed by vitrification [66]. Studies found that vitrification significantly affects mitochondrial distribution and mitochondrial potential in bovine, human, rabbit, murine, and porcine oocytes [66–68]. However, it has been suggested that mitochondrial membrane potential reduction caused by vitrification in human oocytes is temporary and would be fully recovered after 4 h culture. In addition, evidence indicates that supplementation of antioxidants in a cryopreservation medium could enhance the developmental competence of vitrified oocytes by preventing mitochondrial damage and reducing oxidative stress [69–71].

In conclusion, there are few studies on the effects of vitrification-warming on epigenetic modification and maternal transcripts of human oocytes, which is still in their infancy. In order to get conclusive evidence, new technologies to analyze the methylation and transcription levels with a small amount of material should be applied. In addition, we need to examine the effects of vitrification variables (type and concentration of refrigerant, cryostorage duration, oocytes at different stages, etc.) on biological processes more thoroughly, so as to continuously optimize the safety and sustainability of the vitrification process.

Conflict of interest

The authors declare no conflict of interest.

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Section 2

Improving IVF Outcome

Chapter 4

Improving Embryo Quality by Strictly Controlling IVF Laboratory Environment

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Abstract

In recent years, several changes have been made in different aspects of in vitro fertilization to improve embryo quality and ultimately the clinical outcomes in assisted reproduction technology (ART). These approaches include improvements in air quality inside the lab to ensure VOCs-free air, use of tri-gas incubator and embryo-tested devices and plastics, adequate control of pH and osmolarity of culture media, and strict quality control that allows an adequate development of the embryos until blastocyst stage. Other strategies to improve the embryo quality during in vitro culture include volume reduction of drop culture media, and individual or group culture of embryos. This work summarizes several strategies to improve embryonic quality during their in vitro culture in assisted reproduction procedures.

Keywords: in vitro culture, embryo quality, culture system, IVF, IVF laboratory environment

1. Introduction

Assisted reproduction technologies allow for better clinical outcomes for infertile couples to achieve a pregnancy and a liveborn baby compared to those obtained in the beginning of in vitro fertilization, and this goal is due a series of innovations made in different aspects of the in vitro fertilization laboratory that guarantee the quality of embryos obtained after an IVF procedure. pH is an essential environmental variable that should be under strict control in the culture media inside the incubators. Gametes and embryos are very sensitive to changes of pH, which calls for special attention to the elevation of the performing IVF laboratory to ensure adequate development and quality of embryos. Furthermore, according to different studies the strategy of physiological culture with low oxygen compared to the atmospheric environment showed excellent quality of blastocysts and higher clinical outcomes. Cultured embryos in atmospheric oxygen systems show damaged inner cell mass and are disorganized, diffuse and with low vacuolated cells compared to those blastocysts with large and compact inner cell mass cultured in lower concentrations of oxygen.

On the other hand, the autocrine and paracrine growth factors are very important and positive to the development and quality of embryos cultured in vitro. Strategies such as reduced culture volume and group embryo culture allow to increase the effect

of embryotrophic factors on embryonic development. During an in vitro culture, a reduced volume allows for a balance between the beneficial effect of growth factors and the negative effect of embryotoxic substances; and similarly, culturing embryos in groups allowed those beneficial embryotrophic factors to positively influence the embryo development via paracrine signals. This review summarizes several strategies to improve embryonic quality during their in vitro culture in assisted reproduction procedures.

2. Oxygen tension in embryo culture

Oxygen during in vitro embryo culture is an essential chemical factor that improves development from gametes to the blastocyst stage, intervening in their metabolism, genetics and epigenetics [1–5]. In some mammals including humans, the oviduct and uterus oxygen concentrations are between 2–8% [6], showing lower concentrations of oxygen in the uterus [7], thus concentrations close to these should represent a more physiological environment. For many years, in most IVF laboratories worldwide embryonic culture has been made in atmospheric concentration of oxygen (20%); however, the current trend has turned to culture at reduced oxygen concentrations (5%) simulating the normal physiological environment.

During in vitro development, the embryos before and after compaction have completely different characteristics and this is one of the reasons why oxygen can affect the culture to a different extent before its transfer to the uterus [8, 9]. The atmospheric oxygen is potentially toxic through reactive oxygen species (ROS) which are a threat to gametes and embryos cultured in vitro, but a reduced oxygen concentration similar as possible to the in vitro environment improves the embryonic development. Bontekoe et al. [10] realized a meta-analysis of four studies analyzing the effect of low oxygen in human embryo cultures showed that a concentration of 5% had a benefit on clinical outcomes compared with those embryos cultured at atmospheric concentrations. Guo et al. [11] evaluating the effect of culturing embryos in 20% or 5 % oxygen concentration from zygotes to blastocyst showed that the low oxygen group had a significantly higher blastocyst formation rate during fresh cycle, and clinical pregnancy and implantation rates significantly higher in the subsequent warming blastocyst-transfer cycles.

Studies of Karagenc [12] culturing embryos in 20% of O_2 showed damage mainly in the embryonic inner cell (ICM) which was morphologically disorganized, diffuse and with few vacuolated cells unlike the blastocysts with large and compact IMC cultured in low concentration of O_2. Rho et al. [13] cultured bovine embryos and showed that low concentrations of oxygen produce higher rates of cleavage and blastocyst stages compared to embryos cultured in 20% of O2. Also, in mice there is a better development to blastocyst stage, bigger number and size of ICM, and a gene expression profile similar to that observed in embryos in vivo [14]. Studies of Adam et al. [15], Meintjes et al. [2], and Nanassy et al. [16] have concluded that culturing embryos in 5% of O_2 is beneficial to preimplantational embryo development especially in patients older than 40 years old, as demonstrated by García-Ferreyra et al. [17]. This last study compared the clinical outcomes in patients older than 40 years old whose embryos were cultured at blastocyst stage under two different oxygen environments (5% vs 20% O_2) and concluded that the women group of 5% of O_2 had implantation and pregnancy rates significantly higher compared to group of 20% of O_2 (**Table 1**).

	5% O_2	20% O_2
Cycles	341	217
Fertilization rate (%)	83.5	81.6
Blastocyst development rate (%)	34.0	31.4
Implantation rate (%)	25.0*	2.7
Pregnancy rate (%)	41.4*	5.6
Transferred embryos (%)	1.93 ± 0.05*	2.06 ± 0.01

**P < 0.05 in relation to 20% O_2 group.*
García-Ferreyra et al. Beneficial effect of reduced oxygen concentration with transfer of blastocysts in IVF patients older than 40 years old. Health 2010.

Table 1.
Effect of atmospheres of 5% and 20% of O_2 on clinical outcomes in older women.

3. Importance of CO_2 to control the pH value in culture media at sea level and high altitude

The control of pH during in vitro culture of human embryos is very important to achieve an excellent preimplantational embryonic development. Intracellular pH of the human oocytes and embryos is ~7.1–7.2 (usually accepted to be 7.12), this value could be affected by changes in the extracellular pH of the culture media, and variations in their values cause cell stress during in human embryo cultures [18]. During the manipulation of gametes and embryos in in vitro procedures, it is crucial to minimize stress factors to achieve adequate clinical outcomes. Intracellular pH regulates cell processes such as enzymatic reactions, cell division, differentiation, membrane calcium concentration, cell communication, protein synthesis, cytoskeleton formation; and slight variations can have detrimental effects on the developing embryo. It is important to highlight that even slight variations in the intracellular pH can significantly impact blastocyst development, alter gene expression profiles, induce relocalization of mitochondria and actin cytoskeletal elements, and alter glycolytic activity and oxidative metabolism [19]. Previous studies showed that changes in the culture media pH (extracellular pH) directly affects the intercellular pH in the embryos during IVF procedures, altering their homeostasis and quality of development [20].

Routinely, a bicarbonate/carbon dioxide (CO_2) buffering system has been employed during in vitro culture to regulate the pH of fluids, and manipulating the concentration of CO_2 and the concentration of bicarbonate is readily feasible to obtain the required pH. This system has the advantage that changing the CO_2 concentration permits the manipulation of the pH value in the culture media. Calibrating the percentage of carbon dioxide gas in the incubators can then allow for precise pH control in the culture medium to adequately support embryo development. Given that factors as altitude will affect the partial pressure of CO_2 in the medium, it is imperative to increase the percentages of gas to reach a physiological pH that allows for an adequate embryonic development in vitro, and IVF labs at high altitude will need higher percentages of carbon dioxide to achieve good embryo development. At high altitude, hypoxia affects male and female reproductive health at hormonal level and gene expression [21], which in turn could affect key events during embryo development [García-Ferreyra, 2022; Personal communication]. However, there are not many reports on IVF and altitude. García-Ferreyra and collaborators carried IVF

procedures in a city at high altitude (Huancayo-Perú; 3300 m.a.s.l.) and needed a minimum concentration of 11,9% of CO_2 in incubators to have a suitable pH to obtain a good development and quality in human embryos compared to those observed at sea level (data unpublished). Finally, it is necessary to measure and calibrate CO2 concentrations to ensure that the pH of the culture medium inside the incubator will be adequate to support the embryonic development, and always keep in mind the height above sea level of our IVF laboratory.

4. Strategies of culture to improve embryo quality in IVF

4.1 Dry versus humidified incubator

The maintenance of stable and optimal conditions during in vitro culture of human gametes, zygotes, and embryos is critical to achieve excellent clinical outcomes; and some variables as the pH of the culture media, temperature, and media osmolality, that directly depend the type of incubator used, could be affected by presence of humidity inside the incubators in the embryology laboratory. Media osmolality, a measure of osmotic pressure of a solution, is one of the main factors in cell volume regulation and osmolality shift during extended culture, as it will affect the embryonic development [22, 23]. In animal models like the mouse, it has been demonstrated that hyperosmotic stress is associated to reduced blastocysts formation rates [24, 25], low cell number, apoptosis [26, 27], and altered epigenetics and gene expression processes [28, 29].

Routinely, humified incubators have been used to culture human embryos, and this has allowed optimal osmolality control, but an important disadvantage of the humidity is risk of contamination by microorganisms which can negatively impact embryonic development. However, in the last years this problem has been overcome with the development of dry benchtop incubators for cultivating human embryos, but the use of dry atmospheres and uninterrupted culture systems leads to continued increases in the media osmolality [30]. A strategy to avoid the significant water evaporation in a dry atmosphere culture, and changes in temperature, pH (by increased concentration of bicarbonate), and osmolality (elevated organic and inorganic osmolytes) involves overlaying the culture dishes with mineral oil. Nonetheless, this plan has not been efficient according several studies published in the recent years.

Studies of Swain et al. [31] that compared media osmolality changes in uninterrupted cultures during 7 days in non-humidified and humified incubators, showed a significative increase of the osmolality over time in dry atmosphere. Osmolality in humidified incubators always remained unaltered and comparable to controls during the experiments. Similar results were observed by Yumoto et al. [29] who investigated the stability of osmolality in culture media microdrops covered with mineral oil incubated for 5 or 6 days incubated in a dry or humidified atmosphere. They observed a significant and linear increase in the osmolality during 5 days of incubation in dry benchtop incubators; concluding that mineral oil alone may not adequately prevent gradual changes in the osmolality of low-volume microdrops during extended in vitro culture of human embryos in non-humidified incubators.

In regards to clinical outcomes, Fazwy et al. [22] compared randomized and prospectively dry versus humid incubators and showed significantly lower blastocyst formation, clinical and ongoing pregnancies rates in the dry culture compared to those observed in humidified culture, indicating that human embryo development

may be compromised in culture without humidity. On the other hand, a study done to investigate whether adding or not outer-well medium to inhibit osmolality changes in culture media during IVF cycles showed higher ongoing pregnancy and low miscarriage rates in embryo transfer at day 3 and similar pregnancy rates but significantly lower miscarriages in day 5 transfers [32]. Additionally, Mestres at al. [23] in an excellent work analyzed which culture system factors affect the media evaporation and osmolality, concluding that humidity levels inside the incubators, the volume of mineral oil, and the type of culture media, play a crucial role in osmolality stabilization. Finally, since osmolality is one of the critical parameters of in vitro culture systems that can affect embryonic development, the use of humidified incubators, and adequate techniques (preparation speed and preparing one dish at a time) are highly recommended to avoid evaporation and shifts in osmolality.

4.2 Volume of culture media drop

One of the conditions to achieve an excellent human embryo culture system is to provide the optimal medium to the embryos, guaranteeing accumulation of autocrine factors that act upon the embryo itself or upon neighboring embryos, without affecting an adequate blastocyst development by embryotoxic metabolic products as ammonium; in such a way the medium volume will be an important factor in a successful blastocyst culture program.

Routinely, culture human embryos in larger medium volume have been the standard practice in worldwide clinics, but is expected that nutritional and autocrine factors will be diluted in these culture methods. Studies of O′Doherty et al. [33], and Gopichandran and Leese [34] using murine and bovine models suggested that culturing embryos in a reduced volume can increase the blastocyst development rates, and Lane and Gardner [35] showed that decreasing the volume from 320 to 20 μL had a positive effect on cell number of morulae and blastocysts. Studies by Gardner and Lane [36] in humans, suggested that the minimum amount of medium should be 6.26 to 12.5 μL per embryo as a way to avoid the depletion of nutrients and possible effects of negative factors. However, it is important to consider the embryo density by media drop because culturing embryos in droplets of small volumes could lead to the accumulation of detrimental factors such as ammonium and/or oxygen-derived free radicals [37, 38]. Carolan et al. [39] cultured individually bovine embryos but did not observe blastocyst development in 1, 2 or 5 μL drops covered with oil, requiring a minimum of 10 μL medium to achieve the full developmental potential in vitro. In mouse embryos, 2μL drop medium may provide appropriate conditions for individual cultures and less than 1 μL is enough for two mouse zygotes to achieve blastocyst stage [40].

Rijenders and Jansen [41] cultured individual human embryos in two medium culture volumes (160 μL vs 5 μL) and showed improvement, albeit not significant, in the blastocyst formation rate when the embryos were cultured in small volumes. A study performed by Melin et al. [42] showed that reducing culture volume from 20 to 5μ L affected the mouse embryo development with a reduction of blastocyst development rate from 86.6% to 50%. On the other hand, Minasi et al. [43] considered that a "reduced volume" of 5 μL adopted by previous studies is not an ideal balance between the negative effect of toxic metabolites and the positive effect of beneficial autocrine factors; and in this way, they decided increase to 7 μL as "reduced volume" sustaining that 2 μL may be important to prevent evaporation and changes in osmolarity which can significantly affect embryo development, and when they reduced the medium

volume from 35 to 7 μL they observed a significantly higher blastocyst development rate at day 5 (50.5% vs 70%, respectively).

Therefore, it is important to consider media volume and embryo density during an in vitro embryo culture system to achieve that the autocrine and paracrine factors act on embryonic development and allow an increase in the embryo quality and finally the clinical outcomes.

4.3 Individual versus group embryo culture

Routinely, individual culture of human embryos has been standard method in fertility clinics around the world mainly as a way of evaluating the embryo development during the extended culture to blastocyst stage; however, this strategy is not necessarily related to an improvement in development and quality in embryos, or to the clinical outcomes.

It is important to highlight that in vivo, early embryonic development occurs in the absence of signalling factors because mammalian zygotes develop to the blastocysts stage free-floating in a dynamic fluid environment and without direct cell to cell contact; however, Lonergan [44] and O'Neill et al. [45] suggested that signaling factors are necessary to modulate cell growth and cell division, or have anti-apoptotic functions, during preimplantation embryo development in vivo. When embryos are cultured in group, developmental rates and embryo quality are improved compared to those embryos individually cultured [46, 47], and this beneficial nature is related to embryotrophic factors that support or promote their development in vitro, such as insulin-like growth factor-I [48], insulin-like factor-II [49], platelet-activating factor (PAF) [50], phospholipase C (PLC), protein kinase B/Akt, and 3-phosphoinositide-dependent kinase 1 (PDKS1) [51]. Cultured embryos in group produce and release trophic factors that act on the embryo itself and neighboring embryos through paracrine/autocrine actions improving their development, quality, and their implantation probability in the uterus [50].

Several authors have evaluated the effect of group embryo culture strategy compared to individual culture on several parameters of preimplantational embryonic development and clinical outcomes. In regard to embryonic parameters, Ebner et al. [52] prospectively studied the effect of individual or group embryonic culture and showed that the group culture strategy was superior in terms of compaction, development and quality of blastocysts; and Tao et al. [53] evaluated the influence of the same culture strategy but grouping embryos after day 3 according on embryo quality, showing that culturing embryos in group promoted blastocyst development and blastocyst utilization rate compared to those embryos individually cultured. Similar results were observed by Ruíz et al. [47], Glatthorn et al. [54], and García-Ferreyra, 2022 [personal communication].

With regard to clinical outcomes of group and individual strategies, some studies (Ebner et al. [52], Tao et al. [53], and Glatthorn et al. [54] did not observed significant differences in the pregnancy and implantation rates between group and individual culture. However, Ruíz et al. [47] carried out a prospective study to evaluate the effectiveness of group embryo culture using undergoing IVF one hundred forty-eight women data and the result showed a significantly higher implantation rate and live birth delivery rate with the first fresh embryo transfer. Our team used donor oocytes cycles to analyze the effect of group culture on embryo development and clinical outcomes and our results showed significantly more blastocysts, higher pregnancy, implantation ans live birth rates and lower miscarriage rates compared to individual culture (**Table 2**) [García-Ferreyra, data unpublished].

	Group culture	Individual culture
Cycles	70	70
Blastocyst development rate (%)	51.6*	46.7
Pregnancy rate (%)	88.6*	62.9
Implantation rate (%)	64.4*	45.9
Live birth rate per embryo transfer (%)	84.3*	54.3
Miscarriage rate (%)	4.8*	13.6

**P < 0.05 in relation to the individual culture*
García-Ferreyra, personal communication.

Table 2.
Effect of group culture compared to individual culture strategies on clinical outcomes in oocyte donor cycles.

At present, most laboratories worldwide using the individual embryo culture method as a manner to assess the embryo development and principally induced by the freeze-all and PGT-A practice, nonetheless this strategy has a detrimental impact on some embryonic parameters and inclusive on clinical outcomes as has been shown by several authors. Therefore, culturing embryos in group will be a good strategy because effectively improves the embryonic development to blastocyst stage, increasing the embryo utilization rate, and principally arise the clinical outcomes as pregnancy, implantation, and live birth rates.

5. Conclusions

Strategies like cultivating embryos in a humid atmosphere, decreasing the volume of culture medium, and group embryo culturing are appropriate to increase the effect of autocrine and paracrine factors that promote the quality and development of embryos cultured in vitro and increase overall success of clinical outcomes. Additionally, strict pH control to maintain intracellular pH levels during embryo culturing, and maintaining oxygen levels to mimic physiological conditions are crucial to increase success rates in infertile patients, especially those patients are 40 years or older. Finally, it is of utmost importance to highlight that any strategy to be used in an embryo culturing system must be adapted correspondingly to the performing laboratory in order to increase the pregnancy, implantation, and live birth rates.

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Chapter 5

Embryo Transfer in In-Vitro Fertilization: Factors Affecting Successful Outcome

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Abstract

Embryo transfer is the last component of series of events in the process of in vitro fertilization treatment. Events happening at the embryo transfer stage if not well managed could jeopardize the entire process with unpleasant consequences. It is considered to be a critical determinant of clinical outcome in the entire in vitro fertilization procedure. Consequently, factors affecting its efficiency are very vital in the determination of clinical pregnancy, implantation, ongoing pregnancy and live birth rates. Some factors have been clearly seen to be beneficial to the positive outcome of in vitro fertilization, while the benefit of the other factors is still very questionable or have been dismissed. The most important of all factors are ultrasound guided embryo transfer and the use of soft flexible catheters for embryo transfer. Others are removal of cervical mucus before the transfer process, accurate placement of the embryo within the endometrial cavity and immediate ambulation after embryo transfer.

Keywords: in vitro fertilization, embryo transfer, implantation rate, pregnancy rate, live birth rate

1. Introduction

Among all the events involved in the process of In Vitro Fertilization (IVF) treatment, embryo transfer (ET) is considered one of the key steps. It is in deed the last procedure in the process of IVF. In vitro fertilization success rate is largely dependent on three main factors viz.: the embryo quality, the development and receptivity of the endometrium and the efficiency of the transfer process i.e. the embryos transfer technique [1]. It is estimated that 30% of in vitro fertilization failures, can be attributed to poor embryo transfer techniques [2].

Embryo transfer has evolved over the years both in timing and technique involved. At the inception of IVF treatment, embryo transfer was done on day 2, later day 3 embryo transfer gained prominence and now the blastocyst [day 5] transfer has dominated the IVF practice world over.

The original position for embryo transfer as described by Steptoe was in the knee-chest position [3]. However, the supine/dorsal lithotomy position is now preferred. Blind embryo transfer was the order of the day till ultrasound-guided transfer started

gaining prominence. Patients were kept on bed rest for hours and days post ET however the usefulness of this practice is now highly questionable.

The procedural technique of embryo transfer has also seen some changes with associated practices which may or may not be beneficial. In recent times the embryo transfer technique varies. American Society for Reproductive Medicine [ASRM] practice committee in 2017 outlined four transfer modalities [4].

*Direct transfer***:** The catheter is loaded with the embryo(s) and without any form of immediate preceding trial, the transfer is performed.

Trial followed by transfer: A trial or regular embryo transfer catheter (completely connected outer and inner sheath) is used immediately before the real transfer. At the immediate trial, the catheter is passed to and just through the internal os ensuring no resistance and withdrawn thereafter. The embryo transfer catheter is then loaded and the actual transfer is performed.

Afterload transfer: The outer sheath of the embryo transfer catheter is separated from the inner catheter. The inner catheter is pulled back to the extent that only about 1 cm of its tip is protruding through the outer sheath. The two (outer and inner) are held together while advancing through the cervical canal until the inner catheter passes through the internal os of the cervix. At this point, the outer sheath is positioned at the top of the cervical canal and stabilized there while the inner catheter is withdrawn. The embryo is loaded into the inner catheter which is threaded into the endometrial cavity through the outer sheath for the expulsion of the embryos.

Trial transfer converted into an after-load transfer: This is applicable when trial transfer is difficult. In a difficult trial transfer, once the inner catheter passed the internal os, the outer sheath is separated from the inner catheter and moved forward to the top of the cervical canal at the same time the inner catheter is gradually withdrawn. Just like the after-load transfer, the inner catheter is now loaded with the embryo(s) and threaded through the outer sheath for expulsion into the endometrial cavity. Embryo transfer is done usually without any form of analgesia or sedation and it's done in an atraumatic manner. Difficulty at embryo transfer is to be avoided as much as possible. The proficiency of the personnel carrying out the embryo transfer is also important.

Embryo transfer efficiency is a major rate-limiting step to IVF success [2]. Consequently, performing a smooth and technically sound embryo transfer is paramount. A host of factors have been associated with successful embryo transfer, some of them have been proven to be beneficial while others are of questionable importance. We shall take a look at some of those factors and review their importance in a successful embryo transfer process. Factors for consideration are**;** Ultrasound-guided embryo transfer, The type of transfer catheter used, proficiency of personnel carrying out the embryo transfer, Site of embryo placement within the endometrial cavity, loading discharge interval, Mock/trial embryo transfer, full bladder, flushing or removal of cervical mucus, bed rest, embryo glue and other factors.

2. Factors affecting successful outcome of embryo transfer

2.1 Ultrasound scan guided embryo transfer

Traditionally embryo transfer was done blindly placing the embryo at an imaginary depth within the endometrial cavity. It is thought that blind embryo transfer could

have a limitation in that, there is a great variation in uterine size and depth between patients. Therefore, using a standard depth of 6 cm as is the case in most situations may not be very representative. The blind embryo transfer could also be fraught with the possibility of coil back of catheter tip resulting in depositing the embryo in the cervix.

Over the years, ultrasound scan was introduced to aid the process of embryo transfer. Initially, it was difficult to see the tip of the catheter during the transfer process. This led to the development of the echogenic tip catheter.

There is great debate on the usefulness of embryo transfer under ultrasound guidance. The majority of these studies looked at the trans-abdominal ultrasound scan [5–9]. Other ultrasound techniques used are the transvaginal ultrasound scan (TV—US) and more recently the three-dimensional ultrasound imaging (3D—US) and uterine length measurement before transfer (UL MbET). In UL MbET transvaginal ultrasound scan is used to measure the cervical length and the distance between the internal os and fundal endometrium, then using the clinical touch (CT) method the operator discharges the embryo about 1.5 cm short of the fundus.

The advantages of ultrasound-guided embryo transfer are related to the relatively low cost of the technology, the likelihood of accurate placing of the embryo in the desired location within the endometrial cavity and the opportunity for patients and operators to watch the entire process, possibly reducing anxiety levels [10, 11]. On the other hand, the disadvantage of using ultrasound guidance during embryo transfer include; The need for additional manpower, prolongation of embryo transfer duration and possible inconvenience to the patient of filling the bladder [8].

Some earlier studies showed that ultrasound-guided embryo transfer does not offer any advantage in the hands of experienced operators compared with blind transfer. That ultrasound-guided embryo transfer may be of probable benefit in the early training period [6, 7]. However, Sallam [12] accorded the Drakeley outcome to the fact that different catheters were used for the USS embryo transfer and clinical touch embryo transfer, thus the bias in outcomes.

Tang et al. [5] reported a significant improvement in implantation rate following ultrasound-guided embryo transfer, however, they noted that the extent of improvement in the pregnancy rate may depend on the specific techniques and methods of embryo transfer, used in individual centres. A meta-analysis of eight randomized trials [13] showed that ultrasound-guided embryo placement improves implantation and pregnancy rates.

Cozzolino et al. [14] did a systematic review and meta-analysis of 38 eligible studies on ultrasound-guided ET and came to the following conclusion;

Meta-analysis of RCTs comparing trans-abdominal ultrasound (TA—US) guided embryo transfer versus clinical touch: Analysis of five thousand, five hundred and three (5503) patients showed a significantly higher pregnancy rate in the trans-abdominal ultrasound embryo transfer group compared to the clinical touch group. The ongoing pregnancy and live birth rates were also significantly higher in the trans-abdominal ultrasound embryo transfer group. There was no significant difference between the two groups in ectopic pregnancy rate and miscarriage rates.

Meta-analysis of RCTs comparing Transvaginal ultrasound (TV-US) vs trans-abdominal ultrasound (TA-US) guided embryo transfer: Four studies were included in this analysis with a total of six hundred and thirty-five (635) patients. There was no significant difference between the two techniques in clinical pregnancy rate, ongoing pregnancy and live birth rate, ectopic pregnancy rate and miscarriage rate.

- Transvaginal ultrasound (TV- US) guided embryo transfer was compared with clinical touch embryo transfer in an observational retrospective study involving eight hundred and forty-six (846) patients [15]**.** This study found significantly higher implantation and pregnancy rates in the former group compared with the latter.

- Two dimension was compared with three-dimension trans-abdominal ultrasound-guided embryo transfer by Saravelos et al. [16] in a randomized controlled trial and found no significant difference in ongoing pregnancy rates, implantation rates, clinical pregnancy rates, ectopic pregnancy rates and miscarriage rates. Similarly, Li et al. [17] did not find any difference in pregnancy rate using either of the techniques in a single-blind, single-centre RCT.

In a large randomized controlled trial involving one thousand six hundred and forty-eight (1648) patients, Revelli et al. [18] compared TV UL MbET with transvaginal ultrasound (TA—US) guided embryo transfer (ET) and found comparable implantation rate, clinical pregnancy rate, and ongoing pregnancy rates between the two groups. However, there was a significant reduction in discomfort intensity score and proportion of patients with moderate to severe discomfort at embryo transfer in the UL MbET group.

In the evaluation of patient discomfort; comparing transvaginal ultrasound with transabdominal ultrasound-guided embryo transfer, Bondri et al. [19] reported uterine cramping rate to be comparable in the two groups. This study also noted 41% of light, 16% of moderate and 6% of severe discomfort associated with bladder distension in the TA US group, but Porat et al. [20] reported no significant difference between the two groups with regards to the degree of uterine cramping and pain during ET.

In recent times Karavani et al. [11] noted that transvaginal ultrasound scan (TV US) use was significantly associated with better visualization of the endometrial cavity and location of embryo transfer. There was also a significant reduction in anxiety, discomfort and pain using the transvaginal ultrasound (TV US) method.

Hassan et al. [21] compared TV US versus TA US-guided ET in obese women with BMI ≥ 30 Kg per sqm in a randomized controlled trial involving 800 participants and found significantly higher clinical pregnancy rates in the transvaginal ultrasound group compared to the trans-abdominal ultrasound group (37.8% vs. 30.8% P = 0.044). However even though the live birth rate was higher in the transvaginal ultrasound group (50.2% vs. 44.8%), the difference was not statistically significant. Pain associated via the visual analogue scale (VAS) was significantly less for the transvaginal ultrasound group compared with the trans-abdominal ultrasound group. Likewise abdominal discomfort.

The use of transabdominal ultrasound scan to assess the endometrial cavity and other pelvic structures and for the guidance of the embryo transfer procedure has been recommended by the ASRM as there is good evidence based on several RCTs that it improved clinical pregnancy and live birth rates [4, 22].

2.2 Type of transfer catheter used

There are generally two categories of catheter used commonly for embryo transfer, the soft and the stiff (rigid) catheter. Theoretically, it is thought that the stiff catheter is more likely to cause trauma during embryo transfer, while the soft catheter

can easily meander its way through the curve of the cervix into the curvature of the endometrial cavity in an atraumatic fashion. This translates to less risk of plugging the catheter tip with blood, mucus or endometrium. The drawback for use of soft catheters is that sometimes they are more difficult to insert and could require assistance with the stylet.

To improve the ease of the ultrasound-guided embryo transfer, the echogenic tip catheter was introduced in clinical practice. A comparison of the Wallace soft echogenic catheter (sureview catheter) and the conventional soft Wallace catheter revealed comparative implantation and clinical pregnancy rate. However, the sureview catheter simplified the ultrasound-guided transfer process [23, 24].

Studies have compared the soft and rigid catheters in embryo transfer. The superiority of the soft catheter over the rigid catheter in terms of success rates of in vitro fertilization was demonstrated [25]. Many studies have favored the use of soft catheters over firm catheters [26–29]. The use of soft catheters has relegated firm catheter use. Consequently, firm or rigid catheters are not used any longer as the first choice in modern-day practice.

The ASRM practice committee [22] has concluded that there is good evidence to recommend the use of soft embryo transfer catheters to improve in vitro fertilization embryo transfer pregnancy rates. However, there are limited data on live-birth rates and specific types of soft catheters used (**Figure 1**).

2.3 Proficiency of personnel carrying out the embryo transfer

The proficiency and clinical experience of the operator carrying out the embryo transfer may be of great value in the success of embryo transfer.

Angelini et al. [30] reported a significant variation of 36.1% versus 20.6% [$P \leq 0.01$] clinical pregnancy rate between two operators all other conditions remaining the same. It is a common place in medical practice that perfection comes with clinical experience consequently the outcome of embryo transfer is expected to be better with experienced clinician than trainees.

2.4 Site of embryo placement within the endometrial cavity

The actual portion of embryo deposit within the endometrial cavity has been a subject of scientific debate. Even though there tends to be a gradual resolution based on scientific evidence, continued research on the subject matter is still very valid. Embryo transfer site has been imperative in the determination of pregnancy rate in In Vitro Fertilization. The catheter must not touch to fundal endometrium as this could irritate the endometrium and cause contraction.

The position of the embryo deposited during ET has been measured largely in two categories viz.: Absolute position measured the distance from fundal endometrium and relative position according to endometrial cavity length. Controversies exist regarding which measurement to adapt universally for the absolute position. Many studies including a meta-analysis have shown that embryo placement at 2 cm distance from the fundal endometrium was associated with higher pregnancy rate, ongoing pregnancy rates and live birth rate [31–33]. The relative position of the catheter tip that is favorable for a higher success rate of embryo transfer was discovered to be close to the mid-point as reported in a randomized controlled [34]. Comparing the site of embryo deposit using the absolute or relative distance measurement Kwon et al. in a

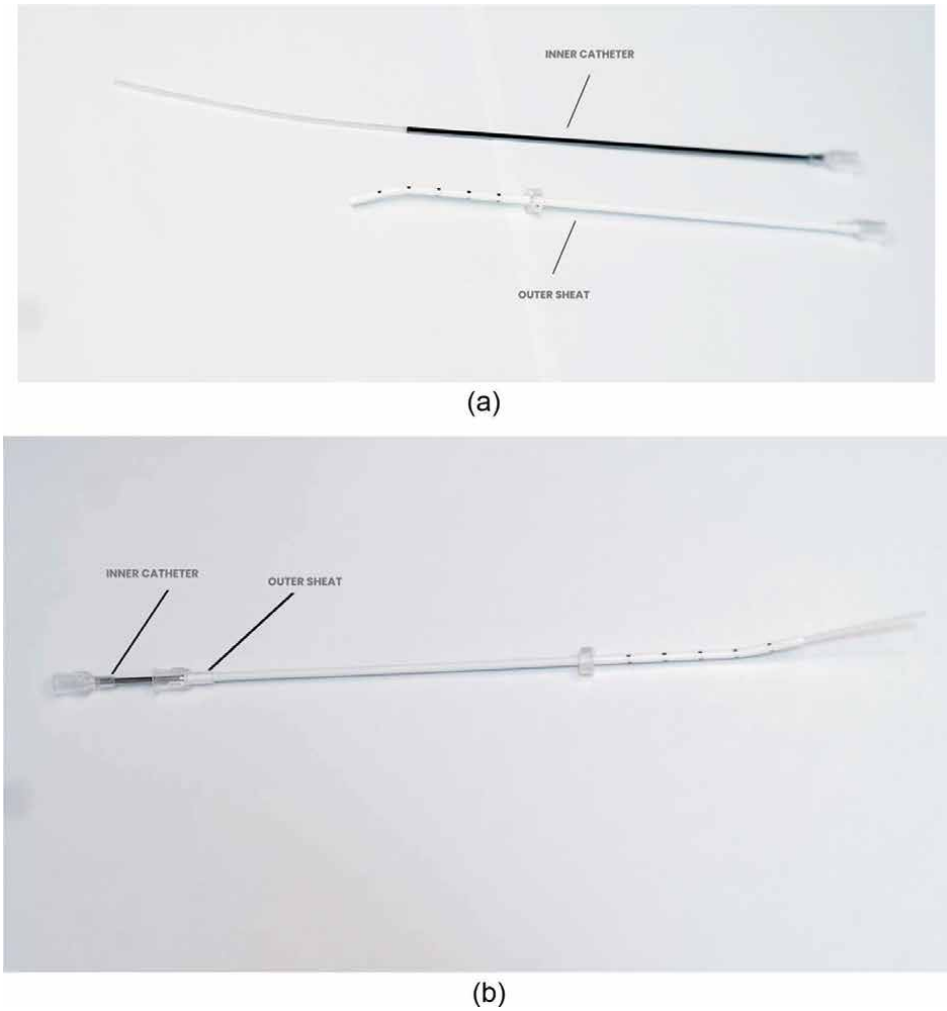

Figure 1.
(a and b) soft tip catheter.

randomized controlled trial found no difference in pregnancy and implantation rate when the site of the embryo transfer was fixed at 2 cm to fundal endometrium or transfer at mid-point of the endometrial cavity length (**Figure 2**) [35].

2.5 Embryo loading and discharge interval

The time between embryo loading into the catheter and discharge of the embryo could be a factor in outcome parameters. Anything that creates difficulty at the time of embryo transfer could elongate the embryo transfer time. Lee et al. in a retrospective cohort study found that longer transfer time does not negatively influence clinical pregnancy rate, implantation rate or live-birth rate [36]. This study however noted that difficulty in transfer negatively affects the clinical pregnancy rate. Some other studies however reported a significantly lower pregnancy rate when there is a longer loading discharge interval [over 60 seconds in some cases] [37, 38].

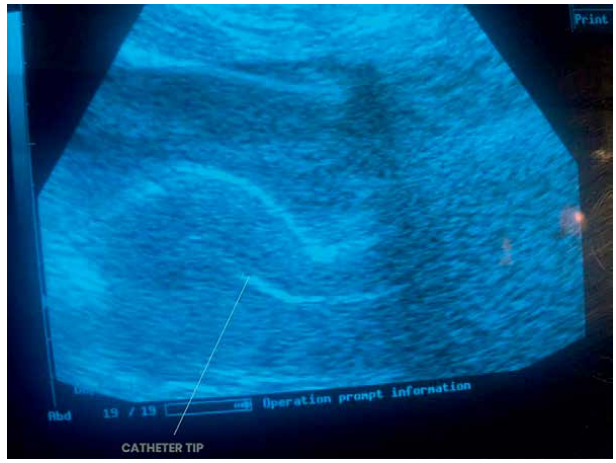

Figure 2.
Catheter tip placement.

Ease of the transfer procedure which culminates in less procedure time has been associated with higher pregnancy rates compared with difficult transfers [39, 40].

2.6 The time interval between the discharge of the embryo and withdrawal of the catheter

Variability in the practice of time interval between embryo expulsion and catheter removal exists. While some practitioners remove the catheter immediately, others traditionally wait for 30–60 seconds before the withdrawal of the catheter. There are few documented studies on this subject. Two studies [a randomized controlled study and a follow-up cohort study] have compared immediate withdrawal versus delayed withdrawal [up to 60 seconds] and found that delayed catheter withdrawal was of no benefit in increasing pregnancy and live birth rate [41, 42]. ASRM practice committee [22] concluded that there is fair evidence to recommend the withdrawal of the embryo transfer catheter immediately after embryo expulsion.

2.7 Mock/trial/dummy embryo transfer

Some practitioners practise mock/dummy embryo transfer in the cycle preceding the in vitro fertilization cycle or sometimes at the time of oocyte pick up. Mock transfer aims to estimate cavity depth, determine the direction of the cervix and uterus, and establish if there is any cervical stenosis. It is used to determine the anticipated difficulty in embryo transfer and correct the obstacles before the actual transfer and in the situation of extreme cervical stenosis plan alternative routes of embryo transfer such as trans-myometrial embryo transfer.

Very few studies have been conducted on trial/mock embryo transfer. Two notable studies showed clear benefits of mock embryo transfer [43, 44]. One study expressed concern that the position of the uterus could change between the time of mock and real embryo transfer, they observed some retroverted uterus at the time of mock transfer changing to anteverted at the time of real transfer [45]. They, however, advise that patients with retroverted uterus at mock embryo transfer should still present

with a full bladder for real embryo transfer since a significant proportion will be converted to an anteverted position.

2.8 Full bladder

Just like many other factors in embryo transfer, the benefit of a full bladder at the time of embryo transfer has been a subject of controversy. The anatomical portion of the uterus in most cases is that of anteversion with slight angulation at the internal os of the cervix. A full bladder is thought to passively straighten the uterine curvature. Straightening of cervico-uterine angle allows easy entry of the catheter into the uterine cavity [46, 47]. Bladder distension could create some level of discomfort to the patient and in the presence of retroverted uterus catheter visualization may be difficult.

Many clinicians would prefer to perform embryo transfer under ultrasound guidance with a partially filled bladder and some empty bladder. Their main reason is to avoid immediate post embryo transfer micturition. Lorusso et al. [48] in RCT concluded that bladder distension has not shown any possible impact on the in vitro fertilization success rate. Abou- Setta in a systematic review and meta-analysis, observed significantly higher clinical pregnancy and ongoing pregnancy rates with a full bladder [49]. The study observed that the need for instrumental assistance and incidence of difficulty was significantly greater with an empty bladder.

2.9 Flushing or removal of cervical mucus

Controversy exists about whether or not to remove cervical mucus from the ectocervix before embryo transfer. The presence of cervical mucus has been thought to interfere with the embryo transfer process by obstructing the passage of the embryo through the catheter tip. It may also pull the embryo back from the expulsion site. There is also the possibility of contaminating the uterine cavity with cervical microbes. Conversely removing the cervical mucus could be detrimental as it may cause bleeding in the cervix and may stimulate uterine contraction.

There has not been extensive research on this subject matter. However, of the few studies, published, majority favor removing cervical mucus before embryo transfer as this has been associated with a higher success rate at embryo transfer [50, 51].

The ASRM practice committee, therefore, concluded that there is fair evidence of the beneficial effect of cervical mucus removal at the time of embryo transfer in improving clinical pregnancy rate and live birth rate [22].

2.10 Bed rest after embryo transfer

Traditional practice in In Vitro Fertilization programs established bed rest for a variable duration following embryo transfer [up to 2 weeks in some instances]. It is presumed that the supine position and bringing physical activity to the barest minimum is likely to reduce the chances of embryo expulsion from the uterine cavity post embryo transfer. However, over the years the role of bed rest became questionable. This prompted a lot of research to determine the value of bed rest in the successful outcome of embryo transfer. The etiology of implantation failure in most cases has no relationship with physical activity. It can therefore be inferred that bed rest may not have a positive impact on pregnancy rate following embryo transfer.

One study followed via an ultrasound scan of the air bubble within the endometrial cavity in a patient who stood immediately after embryo transfer and discovered

that the position of the air bubble before and after standing did not change [52]. Hence it was concluded that standing immediately after embryo transfer has no significant impact on the eventful position of embryo-associated air, consequently not associated with embryo expulsion.

The association between pregnancy rate following embryo transfer and physical activity level was assessed in a prospective observational cohort study which demonstrated that ambulation following embryo transfer has no adverse effect on pregnancy rate [53]. They, therefore, recommended immediate resumption of regular activity following embryo transfer. In the same light a meta-analysis by Cozzolino et al. [54], concluded that immediate mobilization after embryo transfer does not have any negative influence on the success of in vitro fertilization.

Some studies have shown a harmful impact of bed rest on the success of embryo transfer following in vitro fertilization [55, 56]. Garkwad et al. in a randomized controlled trial (among oocyte recipients in IVF donor oocyte cycle) showed a statistically significant live birth rate in the non-bed rest group and recommended a further study to evaluate the anatomical/physiological or psychological reason for the positive effect of physical activity on success rates post embryo transfer [56]. A study was abandoned in clinical trial for ethical reasons due to poor results in the group observing bed rest [57]. Waterstone et al. noted that the supine position makes the anteverted uterus, become vertical therefore predisposing its content to the action of gravity. As such bed rest could be seen as detrimental to the success of embryo transfer [55]. A series of systematic reviews and meta-analysis on randomized controlled trials concluded that bed rest following embryo transfer did not improve clinical pregnancy and live birth rates, instead, there was a reduction in implantation rate [58].

A Cochrane review did not provide any sufficient evidence to support the usefulness of bed rest following embryo transfer [59]. The ASRM practice committee summarized that there is good evidence not to recommend bed rest after embryo transfer [22].

2.11 The use of embryo glue

There is no robust evidence in favor or against the universal use of embryo glue in improving clinical outcome of embryo transfer. Most of the studies on this subject are limited by sample size and study design. Most studies have failed to demonstrate the universal benefit of embryo glue hence only showed that it could be beneficial in selected groups of patients particularly patients with repeated implantation failures [60, 61]. Yung et al. in a randomized, double blind controlled trial involving 550 women concluded embryo glue use does not improve live birth rate following frozen embryo transfer [62]. In a review article Atkinson and Woodland concluded that the use of embryo glue may be justified for selected group of patients since there is no evidence to show any harmful effect [63]. However, higher pregnancy and live birth rates using embryo glue (hyaluronic acid) were observed in a Cochrane review of 16 randomized controlled trial [64]. In this review, except for a higher rate of multiple pregnancy, there was no other complication associated with the use of embryo glue.

2.12 Acupuncture

The role of acupuncture in IVF-ET outcome remains controversial. Wang et al. in 2021 [65] took a critical review of the role acupuncture in IVF-ET outcome. In their review, 312 original randomized control trials and 65,388 participants were included.

They concluded that even though acupuncture seem to provide beneficial effect in increasing clinical pregnancy rates, there were significant limitation to the studies, which weaken the power of the observed outcome. They further suggest that primary studies of high quality are in high need in this subject area.

While some meta-analysis showed positive efficacy of acupuncture in improving ET outcome [66], some have shown no benefit in outcome, particularly live birth rate [67–70]. Despite the observation that the problem with the efficacy of acupuncture could be with the failure of actual protocol tested rather than acupuncture itself, the ASRM practice committee [22] concluded that there is fair evidence that acupuncture performed around the time of the embryo transfer does not improve live birth rate in IVF.

2.13 Other factors

Transcutaneous Electrical Acupoint Stimulation (TEAS): This involves placing electrodes over the acupoints instead of needles. There are limited data in literature on the role of TEAS in IVF outcome. A randomized controlled trial showed a significant increase in clinical pregnancy and live birth rates with TEAS performed on embryo transfer date [71]. However due to paucity studies on this subject the ASRM practice committee concluded that no recommendation for or against TEAS can be made with regards to improving ET outcome [22].

Use of sterile Latex-free gloves: The nature of the gloves used during embryo transfer has been thought to affect the outcome of the embryo transfer. Powdered latex gloves particularly have been taught to be detrimental to outcome of ET as the [power is thought to escape into the air or be in direct contact with the embryo, which is toxic to the embryo. However, in a randomize control trial involving 712 women comparing effect of powdered versus non-powdered gloves on ET outcome, the pregnancy rate was similar in both groups. The author therefore pointed that the key factors was avoidance of direct contact of the gloves or powder with the embryo [72]. ASRM practice committee [22] suggested no recommendation for any specific gloves to be used, as more research is needed to make any definitive inference.

Massage: Body massage is a non-invasive therapeutic modality employed to alleviate both psychological and physical stress and discomfort. It is thought to be of benefit in IVF ET outcome. There is however paucity of research to elucidate its actual effect on IVF ET [22]. One observational retrospective analysis however showed its benefit increased pregnancy and live birth rate [73]. More robust clinical studies is required in this area to ascertain the usefulness of massage in IVF outcome hence the ASRM practice committee submitted that there is insufficient evidence to recommend for or against massage therapy to improve IVF—ET outcome [22].

ASRM practice committee [22] looked at some other factors in patient preparation before and at embryo transfer. These factors include: the use of *Analgesics, Anesthesia, Prophylactic antibiotics and Whole-System Traditional Chinese Medicine (WS-TCM)*. It was concluded that there is insufficient evidence to recommend for or against analgesics and WS-TCM to improve In Vitro Fertilization embryo transfer (IVF-ET). In the case of anesthesia, there is insufficient evidence that it improves outcomes. Therefore, knowing the possible risk of anesthesia and unconfirmed benefit, routine anesthesia is therefore not recommended to improve outcomes. Prophylactic antibiotics has shown no clear benefit and as such not recommended.

3. Conclusions

Embryo transfer is critical in the overall process of IVF. Consequently, adequate measures must be put in place to ensure its efficacy. Some factors have been associated with improvement in the outcome of embryo transfer procedures while other factors may have no value at all in outcome determination. It is also reported that some of these factors could have a negative impact on the outcome. Intervention supported by literature for improving pregnancy rates are [22]

- Abdominal ultrasound-guided embryo transfer.
- Removal of cervical mucus.
- Use of soft embryo transfer catheters.
- Placement of embryo transfer tip in the upper or middle central area of the uterine cavity greater than 1 cm firm the fundal endometrium, for embryo transfer procedure is completed.
- Immediate ambulation after embryo transfer procedure is completed

While some other interventions would require further research to authenticate their usefulness, others have been proven to be ineffective.

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Section 3

Embryo Development

Chapter 6

Molecular and Cellular Mechanisms Underlying Preimplantation Embryo Development

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Abstract

Preimplantation embryo development refers to the maturation of a fertilized ovum to a blastocyst. This process is highly regulated and required for proper implantation of the blastocyst into the endometrium. During this phase, several tasks must be accomplished. The differentiated zygotic genome must undergo reprogramming back to totipotency in order to generate all of the different types of tissue making up a human. Next, certain cells begin to differentiate to prepare for implantation which occurs at approximately day 7 post-fertilization. This progression is a result of a careful interplay between maternally persistent RNA transcripts and activation of the zygotic genome. After the embryonic genome activation, blastomere differentiation begins to occur. Cellular polarity has been shown to be the signal transduction that initiates this differentiation. Understanding the molecular and cellular mechanisms regulating preimplantation embryo development is of fundamental importance for reproductive science and has numerous applications in fields such as assisted reproductive technology and stem cell therapy.

Keywords: development, implantation, fertilization, embryo, zygote, blastocyst

1. Introduction and overview

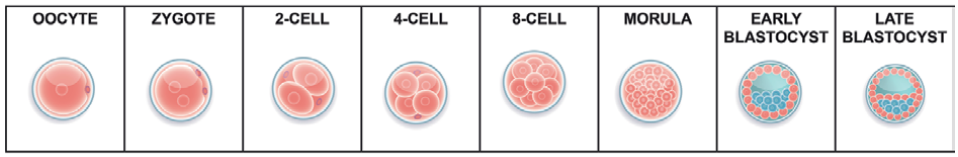
Until recently, most analysis of human preimplantation embryo development has focused on morphological changes, and little was known about the cellular and molecular mechanisms regulating the process. What was known was mostly derived from analysis of embryo development in fish, amphibians, mice, and other animals; however, recent research has examined this process in greater detail in humans. Across species, there are many general similarities in embryonic development. A major commonality is the transition of embryonic developmental control from the maternal genome to zygotic genome. This is commonly referred to as the maternal to zygotic transition (MZT). The MZT requires two major steps: the degradation of persistent maternal RNA and the activation of the zygotic genome (ZGA) through transcriptional and epigenetic mechanisms.

To examine the mechanisms of preimplantation development, it will be useful to provide a brief morphological overview of the process starting with fertilization.

All times referenced will use fertilization as the zero point. Sometimes the specific time frame may be referred to using the number of cells present e.g., 8-cell stage (**Figure 1**).

Human-specific differences in embryonic development are exhibited in the timing of the ZGA and epigenetic modifications [1]. The ZGA is not a discrete event but occurs over a continuum with two waves of increased transcriptional activity. In humans, a minor wave occurs around the 4-cell stage (~48 hours), and a major wave occurs around the 8-cell stage (~72 hours), whereas in mice the minor wave occurs at the late zygote (~24 hours) and the major occurs at the 2-cell stage (~36–48 hours) [2, 3]. As embryonic gene transcription increases, maternal factors regulating development decrease. After the ZGA, the embryo continues division and transitions from a loose cluster of cells to a densely packed ball known as a morula (~day 4). This process is known as compaction. After formation of the morula, divisions continue, and the first cellular differentiations begin causing cavitation. The resulting embryonic structure is known as a blastocyst (~day 5). Cells along the outer edges of the embryo differentiate in trophectoderm (TE) and the inner cells become the inner cell mass (ICM), which are the cells that will become the fetus. The blastocyst is what will then implant into the uterine wall for continued embryogenesis. For this to occur properly, the blastocyst must "hatch" from its protective outer coating, the zona pellucida (~day 6). The final step is implantation of the embryo into the uterine wall (~day 7–8). Cells of the trophectoderm begin to differentiate into syncytiotrophoblast and cytotrophoblast. Syncytiotrophoblast

Stage Specific Molecular and Cellular Events During Preimplantation Embryo Development



Hours (Post Insemination)

0 hours	~18 hours	~42 hours	~54 hours	~66 hours	~78 hours	~90 hours	~102 hours

Primary Events

Fertilization	Syngamy		Embryonic Genome Activation		Cell Determination	Initial Cell Differentiation	Secondary Cell Differentiation

Gene Expression

Maternally Derived:							
TLE6 PLK4 PADI6 REC114 KHDC3L REC8 TUBB8 SMC1-beta			DUX4 OCT4 SOX2	E-Cadherin AMOT YAP1 TEAD4 LATS		GATA3 (TE) CDX2 (TE) OCT4 (ICM) NANOG (ICM)	NANOG-epiblast KLF17-epiblast GATA4-hypoblast

Figure 1.
Stage-specific molecular and cellular events that take place during preimplantation embryo development. Genes listed under each developmental stage are associated with elevated expression at that time of development.

mediates attachment to the uterine wall. Cytotrophoblast can be thought of as stem cells that produce more syncytiotrophoblast or invasive cytotrophoblast, which directly invade the endometrium allowing for implantation.

In summary, the first step of embryo development is fertilization. For this to occur, the ovum and sperm must fuse their nuclear material generating a zygote. After fertilization, the zygote undergoes several divisions, and around the 8-cell stage, heterogeneities in cell types begin to emerge. By approximately day 4–5, the first morphological differences are seen with the formation of a blastocyst. Cells begin to differentiate into the ICM and trophectoderm. After blastocyst formation, a second wave of differentiation occurs forming the epiblast and hypoblast which will form the embryo and primitive endoderm, respectively. Around day 7–8, the embryo will implant into the uterine wall via trophectoderm-derived syncytiotrophoblast and cytotrophoblast which mediate the attachment.

2. Preimplantation development

2.1 Fertilization and zygote genome activation

2.1.1 Fertilization

Fertilization is the first step in embryo development after the entry of the sperm into the oocyte and is generally regarded as the zero point in describing the timeline of development. Following the cortical reaction, the ovum is stimulated to complete its second meiotic division and release the second polar body. This trigger is mediated by the sperm causing an increase in calcium within the ovum via the DAP/IP3 pathway. After the completion of the second meiotic division, the female pronucleus is formed. The male pronucleus rotates 180 degrees positioning the centrosome between itself and the female pronucleus [4]. The centrosome's microtubules function to draw both of the pronuclei toward each other [4]. As the pronuclei approach, they concurrently move toward the center of the cell [4]. Once sufficiently close, each pronuclei membrane ruptures and fusion occurs resulting in the formation of a diploid zygote [4].

2.1.2 Reprogramming zygotic genome

Post-fertilization, the zygote genome must be transformed back into a totipotent state to generate the variety of tissues that will eventually make up the human fetus. Initially, the zygote genome is relatively inactive while maternally derived transcripts and proteins are the major effectors of development and are responsible for reprogramming the zygote genome [5]. Reprogramming is almost entirely dependent on maternal factors. This phenomenon is exhibited by the fact that a terminally differentiated cell nucleus can be reprogrammed into a pluripotent state by maternal factors within an oocyte [6]. Due to the dependence on the maternal genome, certain maternal genes have been implicated in arrest of the early embryo. Specifically, genes involving the subcortical maternal complex (SCMC) have been linked with early embryo failure [7]. The subcortical maternal complex is a recently discovered maternally derived multiprotein complex that has several important functions in sustaining early embryo development. The functions of the SCMC include organelle distribution, translational regulation, and epigenetic reprogramming. **TLE6**, **PADI6,** and **KHDC3** are all genes that code for components of the SCMC, and each has been associated with early embryo arrest [7].

This transition to a totipotent genome and beginning of ZGA is mediated by DNA and histone modifications [5]. Paternally and maternally derived genes are differentially modified due to the asymmetric epigenetic modifications present in the terminally differentiated egg and sperm. During spermatogenesis, sperm histones are replaced with protamines which must be replaced with maternal histones. Maternally derived histone H3.3 replaces the protamines, which yields an increase in the transcriptional accessibility of genes required for pluripotency. Nucleoplasmins are chaperones thought to play a role in replacing protamine with H3.3. In frogs, nucleoplasmin 2 (NPM2) was shown to be required for embryo development [8]. Additionally, knockout of H3.3 in mice causes embryo arrest and shows decreased levels of gene expression associated with pluripotency [9].

The parental genome undergoes active demethylation through the action of 10–11 translocation (**TET**) methylcytosine dioxygenases prior to cleavage, and this demethylation continues throughout the preimplantation period [10]. During spermatogenesis, the parental genome is highly methylated, so this active demethylation is required for totipotency. Additionally, the maternal pronuclear DNA has also been shown to undergo active demethylation as well [8]. The demethylation process is critical for proper embryo development. In mice, lack of TET3 causes increased methylation of the paternal genome which resulted in increased incidence of embryonic failure [8]. Additionally, Cullin-ring finger ligase-4 (CRL4) ubiquitin ligase has been shown to upregulate TET3 activity and play a role in female fecundity. In mice, deletion of CRL4 component results in embryo lethality [11]. Additionally, increased levels of DNA methylation have been linked with aneuploidy and negative embryo quality [12].

2.1.3 Zygotic genome activation

As the zygote genome is being reprogrammed, there is a concurrent shift in regulatory control over development. There is a decrease in maternal transcript activity and an increase in embryonic transcripts. In addition to their role in establishing totipotency, histone modifications also play a major role in ZGA. In humans, there is an increase in trimethylated histone H3 lysine 4 (**H3K4me3**) which is associated with gene activation, and a decrease in histone H3 lysine 27 (**H3K27me3)** which is associated with gene repression [13]. Several mechanisms exist to ensure proper timing for transcriptional activation of the zygote genome. One mechanism is the titration of maternal repressors. During the first few cellular divisions, the volume of the embryo does not change. As divisions progress, there is an increase in the nucleus to cytoplasm ratio which dilutes maternally persistent repressors [2]. Another method regulating ZGA timing is the synthesis of transcription factors. The embryo lacks key transcription factors, but translation of maternally supplied mRNA leads to their synthesis [2]. These factors are synthesized and eventually, their accumulation will reach a threshold leading to transcriptional activation of the zygote genome. One such transcription factor is **OCT4**. **OCT4** is a transcription factor that is responsible for stem cell self-renewal, and it is used in the induction of pluripotent stem cells. Binding regions for OCT4 are upregulated in accessible regions during the MZT [2]. In mice, OCT4 knockout embryos do not gain totipotency and are non-viable. These embryos develop to the blastocyst stage, but the entirety of the embryo differentiates into trophoblast, and none of the cells maintain pluripotency required for formation of the ICM [14, 15]. In humans, **OCT4** seems to play an earlier role directly associated with zygotic genome activation. Human **OCT4** expression begins as early as the

cleavage stage. Lack of **OCT4** in humans results in failure to form a viable blastocyst [16, 17]. Not surprisingly, upregulation of **OCT4** plays a role in the tumorigenesis of several cancers [16]. **SOX2** is a transcription factor that forms a complex with **OCT4** to co-bind the regulatory region of DNA [18]. Another transcription factor that is vital to human ZGA is **DUX4**. **DUX4** has been shown to have increased expression before the ZGA and bind to promoters of ZGA genes, increasing their transcription. Additionally, knockout of the murine analog for Dux halts embryo progression at the 2-cell stage [19].

The progression from maternal to embryonic gene activation is a major regulator of early embryogenesis. Progression through each stage of development is associated with specific transcriptome activation derived from a combination of maternal and embryonic RNA. In early embryogenesis, this combination is almost entirely maternal RNA, and later it is entirely embryonic. Oocytes up to the 2-cell stage embryo show high levels of maternal mRNA. Two cell stage embryos show low levels of RNA from both maternal and embryonic sources. It is not until the 4–8 cell stage that embryonic RNA levels begin to increase. Maximum zygotic gene expression does not occur until the blastocyst stage. This variation in gene express allows for a stage specific gene expression profile to be created. Examination of gene expression patterns at different stages of development could provide insight into preimplantation embryo viability [20].

2.2 Heterogeneity leads to differentiation and blastocyst formation

2.2.1 Generation of asymmetry between blastomere

Molecular and morphological blastomere asymmetries are what will eventually result in differentiation. The first cleavage introduces variability between blastomeres. Partition error and transcriptional noise (fluctuations in gene expression) are two processes that contribute to blastomere heterogeneity. These chance fluctuations can be enough to determine a cell's lineage specification. Partition error is the unequal distribution of cellular contents (such as maternal mRNAs) during cellular division. Both phenomena increase heterogeneity between cells; however, in the early embryo, partition error likely contributes to this variability the greatest due to low levels of transcription [21]. Once transcription begins to increase, the heterogeneity initially caused by partition error can be amplified leading a cell to differentiated fate. Lineage analysis has shown that cell fate bias may begin as early as the 4-cell stage in mice [5].

2.2.2 First differentiation and the morula to blastocyst transition

In humans, blastomeres begin to undergo compaction followed by the first signs of polarization between days 3 and 4 post-fertilization (8–16 cell stage) [22]. Compaction refers to the transition of the blastomere arrangement from a loose bundle of cells to a tightly packed mass increasing the area of cell-to-cell contacts. Polarization of the blastomeres is critical to establishing distinct cell lines. The first polarized cells along the outer rim of the embryo will generate the trophectoderm (TE), and the central nonpolar cells will form the pluripotent inner cell mass (ICM). **OCT4** and **NANOG** are genes with localized expression to the ICM. **OCT4** was discussed earlier in its relation to the ZGA where it is vital in maintaining pluripotency and is thought to function in a similar manner for cells of the ICM [23].

As compaction occurs, organelles, cytoskeletal elements, and cell adhesion molecules begin to preferentially localize in different regions of the cell, generating the apicobasal polarity. A lack of compaction will result in no blastocyst formation and early arrest of the embryo [24]. Several adhesion and cytoskeletal proteins have been shown to play key roles in the process of compaction and polarization in the morula stage. In mice, a lack of E-cadherin, α-catenin, and β-catenin causes embryo arrest [24]. Specifically, **E-cadherin** and **β-catenin** have been shown to play a vital role in humans as well [25]. **E-cadherin** is a cellular adhesion molecule, and α-catenin links the **E-cadherin/β-catenin** complex to the intracellular cytoskeleton composed of actin. The localization of these adherens junction (AJs) is critical for initiating a signaling cascade generating cell heterogeneity and differentiation [25]. Additionally, phospholipase C (**PLC**) has recently been shown to contribute to apicobasal polarity [22]. The mechanism involves recruiting actin–myosin complexes to the apical membrane [22]. RNA interference repressing **PLC** demonstrated that a lack of **PLC** leads to a lack of cell polarity [22]. The establishment of cell polarity is critical for Hippo pathway signaling which stimulates cell fate differentiation.

In mice, the Hippo pathway is central in the first differentiation of blastomeres to either the trophectoderm or inner cell mass. Hippo signaling inhibits two major transcriptional activators of a trophectoderm fate program, YAP1 (Yes-associated protein 1) and TEAD4 (TEA-domain family member 4) [25]. YAP1 is a transcriptional cofactor that localizes to the nucleus and interacts with transcription factor TEAD4 to promote differentiation to the trophectoderm. The Hippo pathway results in phosphorylation of *YAP1* which prevents its localization to the nucleus. Without the YAP1 nuclear localization, TEAD4-mediated transcription of GATA3 and CDX2 does not occur [5, 25]. GATA3 and CDX2 are both transcription factors that promote the trophectoderm lineage [5]. Thus, activation of the Hippo pathway leads to an ICM fate, and inhibition of the Hippo pathway leads to a TE fate. Hippo pathway signaling is modulated via angiomotin (AMOT). The major regulator of AMOT is cell polarity and adherens junction localization [26]. In inner cells, AMOT binds to AJs via an E-cadherin and NF2 complex [24]. When bound to AJs, AMOT is phosphorylated by large tumor suppressor kinase (LATS) which acts as the molecular switch to turn on the Hippo pathway preventing TE differentiation[24]. In outer cells, atypical protein kinase C (aPKC) sequesters AMOT to the apical membrane. Apical cellular domains are rich in f-actin which binds AMOT sequestering it from adherens junctions [26]. Without AMOT activating the Hippo cascade, YAP1 translocates to the nucleus activating transcription of the TE fate program. Altogether, Hippo pathway signaling in mice is modulated by cell polarity. The presence of an apical domain suppresses Hippo signaling resulting in a trophectoderm fate, whereas lack of an apical domain maintains pluripotency required for the formation of the ICM. The Hippo pathway's role in embryo differentiation was first discovered in mice, but evidence are emerging to suggest a conserved role in cows and humans [25].

2.2.3 2nd differentiation

As the blastocyst develops, the second round of cell differentiation occurs. The ICM differentiates into either epiblast or hypoblast. The hypoblast is what will eventually form the lining of the yolk sac. Epiblast will give rise to the primary germ cell layers which are the endoderm, mesoderm, and ectoderm. Epiblast must maintain a pluripotent state to generate such diversity of tissue. Several genes in mice and humans have been linked to formation of the epiblast. In mice and humans, the

genes **GATA4**, GATA6, and **NANOG** have been associated with the second cell fate decisions. **NANOG** is associated with epiblast formation while **GATA4** and GATA6 are associated with hypoblast lineage. In mice, FGF/MAP Kinase signaling modulates the transcription factors NANOG and GATA6 Interestingly, human epiblast is also associated with increased **NANOG**, but it is not dependent on FGF signaling as in mice [27]. **TGF-β** is another marker highly associated with the epiblast. Inhibition of **TGF-β** leads to decreased amounts of **NANOG** suggesting it is also required for pluripotency of the epiblast [28]. **KLF17** is a protein that has been observed to colocalize with **NANOG** within the epiblast [28]. Recently **KLF17** has been shown to stimulate pluripotency which is not surprising given its relative localization within epiblast [29].

3. Early embryo pathology

3.1 Cellular and molecular changes

3.1.1 Overview

Human pregnancy loss is much more common than in other species [30]. Estimates of natural human pregnancy loss frequency have varied greatly over the years. This is due to the difficulty in the availability of data on fertilization efficiency. Some sources have estimated the pregnancy loss rate to be as high as 70% from fertilization until birth, but recently a range of 40–60% seems to be more widely agreed upon. Of these, 10–40% are due to preimplantation embryo loss [31]. Furthermore, assisted reproduction technology (ART) has a live birth rate of ~23% [32]. Understanding the pathologic mechanisms for these failures has critical significance for the field of reproductive science. Generally, these failures are due to cellular or molecular etiologies.

3.1.2 Cellular Pathology

Fragmentation, also known as blebbing, occurs when a portion of an embryo's cell breaks off from the rest of the cell resulting in a membrane-bound cell fragment. Fragmentation may occur as early as the first embryo cell division [33]. Fragmentation is a common occurrence in embryos; however, high levels of fragmentation are associated with embryo loss. Specifically, embryos with less than 10% fragmentation have the highest likelihood of implantation. Fragmentation has been shown to be a dynamic process in that cytoplasmic fragments can be resorbed back into blastomeres. There is considerable variability in the size and cellular and molecular composition of these fragments [33]. Several etiologies of these fragments have been proposed.

Blastomere exclusion is one phenomenon that could generate these fragments [33, 34]. Recent time-lapse monitoring of embryo development has shown that blastomere exclusion is a common occurrence (64%) [34]. Chromosomal analyses of these excluded blastocysts show a higher frequency of aneuploidy which is consistent with the hypothesis that this could be a mechanism of self-correction [33].

Chromosome-containing micronuclei are another potential origin of embryo fragments. Micronuclei are nuclear membrane-bound structures located in the cytoplasm which contain damaged chromosomes or chromosome fragments [35].

Apoptotic bodies are also the possible origin for embryo fragments. Apoptosis has been suggested as playing a key role in preimplantation development. During apoptosis, the cell shrinks as plasma membrane blebs to form apoptotic bodies which break off from the cell [36, 37].

Persisting polar bodies also may make up some embryonic fragments. Normally polar bodies undergo apoptosis within 1 day of formation. However, evidence exist for their persistence. DNA analysis of some fragments has shown that all of the fragment DNA is maternally derived, specifically from the second polar body [38].

Another potential source of embryonic fragments is extracellular vesicles (EVs). Recently, EVs have been identified as being released from blastomeres of the preimplantation embryo and within blastocoel fluid. These vesicles have been implicated in cell-to-cell communication within the embryo [33, 39, 40].

Abnormal cytokinesis has been shown to be one cause of fragmentation [41]. Therefore, it is not surprising that genes regulating microtubule organization have also been linked to embryo fragmentation and arrest. One such gene is *Tubulin B 8 class VIII (TUBB8)* **TUBB8** codes for a β-tubulin specifically in oocytes as a component of the meiotic spindle. In humans, loss of **TUBB8** impairs meiotic divisions that are critical for fertilization and post-fertilization cell divisions [42]. In mice, mutations in TUBB8 result in embryos with high degrees of fragmentation [43].

Fragmentation is theorized to be associated with reduced implantation rates for multiple reasons. One possibility is due to loss of important cytoplasmic contents, such as regulatory proteins, mitochondria, and mRNA [33]. In fact, mitochondria are the most commonly isolated structures in embryo fragments [44]. Another mechanism proposed for impairing embryo development is disruption of the spatial arrangement of blastomeres [33]. As previously discussed, proper topographical positioning is crucial for proper embryo development.

Fragment removal has recently garnered interest as a technique to improve implantation outcomes. Data supporting this therapy is mixed. Some studies have shown a beneficial effect and others have shown no difference [44, 45]. This variation could be due to different etiologies of fragmentation. If the fragments contain essential organelles such as mitochondria, then removal could deprive blastomeres of vital ATP. In contrast, if the fragments did not contain essential structures, then removal could be beneficial. The most recent study analyzing fragment removal showed a benefit of fragment removal at day 2 which is the safest for the developing embryo. Day 2 embryos have larger blastomeres with more accessible perivitelline space making removal easier and decreasing the time spent outside of the incubator. In addition, day 2 embryo cell membranes are the most resistant to the mechanical stress from manipulation. As time progresses *in vitro* culture media is not able to supply the necessary membrane components and they become stiff and inelastic [45]. More research must be done to evaluate this potential tool for improving pregnancy outcomes.

3.1.3 Molecular Pathology

The major molecular etiology of embryo arrest is aneuploidy. Aneuploidy refers to a cell having an abnormal number of chromosomes. Aneuploidy is the most common cause of pregnancy loss [46, 47]. Aneuploidy is estimated to affect 4–5% of clinical pregnancies and has been shown to have an even higher incidence in preimplantation embryos [46]. The most common cause of embryonic aneuploidy stems from errors meiotic division of oocytes [47]. At 40 years old, approximately 74% of oocytes that

have completed both meiotic divisions are aneuploid. In females less than 36 years old, errors in meiosis I are most common. In females greater than 36, errors in meiosis II are most common [46]. At the cleavage stage, around 83% of embryos are aneuploid. This increase is thought to be due to mitotic errors since aneuploidy is only seen in approximately 5% of sperm [46]. Interestingly, by the blastocyst stage, only 58% of embryos were chromosomally abnormal [46]. The prevailing thought is this is due to apoptosis of nonviable cells. Additionally, some embryos may have completely arrested and not progressed to the blastocyst stage.

Aneuploidy has a very high association with increased maternal age. Several mechanisms such as recombination errors, cohesion dysfunction, spindle assembly failure, and mitochondrial dysfunction are all implicated in the decline of oocyte quality with age.

Evidence shows that the ability to repair recombination errors decreases with maternal age [48]. Experiments on several genes required for recombination such as meiotic recombination protein **REC114** and synaptonemal complex protein SCP3 highlight the importance of the process in preventing aneuploidy. REC114 is a maternally derived gene that encodes a protein that forms double-stranded breaks initiating homologous recombination, which is critical for proper segregation of homologs in meiosis. Mutations in the gene **REC114** result in multiple pronuclei formation and have been linked to early embryonic arrest and female infertility [49]. SCP3 is a component of the synaptonemal complex formed between homologous chromosomes during the prophase of meiosis. In mice, loss of SCP3 results in aneuploidy due to abnormal chromosomal segregation during oocyte meiosis [50].

Cohesins are protein complexes required for meiosis and mitosis via holding sister chromatids together. In mice, knockouts of cohesin subunit proteins Smc1β or Rec8 result in aneuploidy and lethality in early embryos. In humans, levels of **Smc1β** and **Rec8** have been shown to decrease with age [48]. Additionally, the intrakinetochore distance (iKT) of sister chromatids increases with age. As the distance increases, the sister chromatids no longer function as a single unit which results in abnormal separation and increases the likelihood of aneuploidy [48].

The spindle assembly checkpoint (SAC) is another maternal factor thought to decrease in quality with age. The SAC functions to ensure proper chromosome segregation by preventing separation of chromosomes until each is properly attached to the spindle apparatus. The SAC is made up of multiple proteins, and one of the most studied is mitotic arrest deficient protein 2 (MAD2). MAD2 specifically inhibits the anaphase-promoting complex (APC/C). The APC/C is a ubiquitin ligase that normally degrades cyclin B1 stimulating the metaphase to anaphase transition. MAD2 associates with APC/C preventing it from degrading cyclin B1 and thus halting the cell in metaphase. In mice, RNAi inhibition of MAD2 led to increased levels of aneuploidy along with increased levels of chromosomal misaggregation and reduced fertility [48]. In a related manner, genes regulating microtubule organization have also been linked to aneuploidy and early embryo arrest. PLK-4 is a serine/threonine kinase that is a major regulator of centriole assembly. In humans, mutations in **PLK-4** are associated with abnormal mitotic spindle formation and embryo cleavage. Specifically, **PLK-4** mutations result in tripolar mitotic spindle assembly which causes abnormal chromosome separation and aneuploidy [51].

Mitochondrial dysfunction also plays a role in the age-related decline of oocyte quality. Meiotic errors have been the most implicated so far in this discussion of aneuploidy. It should be no surprise that mitochondria have responsibility as well

since they are the key energy providers for the meiotic processes such as microtubule assembly, chromosome segregation, cytokinesis, etc. Mitochondrial damage from reactive oxygen species (ROS) is thought to play a major role in this dysfunction. Mitochondria are the major generators of ROS but also have the least defenses against these ROS due to limited DNA repair mechanisms. Thus, ROS damages mitochondrial DNA (mtDNA) which causes mitochondrial dysfunction which then causes an increase in ROS and ultimately creates a repetitive cycle leading to more mitochondrial dysfunction. Mitochondria DNA mutations are estimated to accumulate at a rate of 25 times more than nuclear DNA [52]. Antioxidant levels have been shown to decrease with maternal age. In mice, the antioxidants peroxiredoxin 3 (Prdx3), thioredoxin 2 (Txn2), glutaredoxin 1 (Glrx1), glutathione S-transferase mu 2 (Gstm2), and superoxide dismutase 1 (Sod1) have all been shown to be decreased in advanced age [53]. Mitochondrial dysfunction leads to ATP depletion which ultimately causes failure of meiotic spindle assembly resulting in aneuploidy [53].

A recently discovered phenomenon that is closely related to aneuploidy is the relatively high incidence of mosaicism in the embryo. Mosaicism is defined as the presence of more than one genetic cell line. In contrast to the meiotic errors causing aneuploidy in all cells, mosaicism typically arises from mitotic errors after fertilization. The two most common etiologies of mosaicism are nondisjunction and anaphase lag [54]. Importantly, mosaicism and aneuploidy are not mutually exclusive, and mosaicism is associated with a spectrum of aneuploidy.

Embryonic mosaicism has important ramifications for preimplantation genetic testing used in assisted reproduction technology (ART). Preimplantation genetic testing for aneuploidy (PGT-A) is currently a widely used screening test for embryo selection in *in vitro* fertilization (IVF); however, its validity has recently been called into question for several reasons. PGT-A relies on a single trophectoderm biopsy which some argue cannot be representative of the entire embryo genetic profile since mosaicism is common [55]. In addition, there is evidence of self-correction of aneuploidy in preimplantation embryos. A recent study showed that transfer of aneuploid embryos can result in euploid pregnancies at delivery. Interestingly this study also showed that aneuploid cells were preferentially localized within the trophectoderm [56]. This phenomenon supports the theory that blastomere exclusion takes place and could serve as a self-corrective mechanism. Additionally, the presence of a corrective mechanism implies that PGT-A testing may result in a disproportionate number of false-positives since the aneuploidy may have been corrected later in development.

4. Conclusion

The development of the embryo from fertilization to implantation is a highly regulated and incredibly complex phenomenon. A zygote resulting from the joining of two terminally differentiated cells must be able to give rise to the diverse groups of cell types and tissues making up the human. For this to occur, the zygotic genome must be reprogrammed back to a state of totipotency. As this is happening, there is a shift from maternal to zygotic genome control. As the zygotic genome becomes the dominant effector, molecular heterogeneities begin to appear which will eventually result in the first cell fate decisions. Cells will differentiate to either the trophectoderm or the ICM, resulting in the formation of a blastocyst. The trophectoderm is

required for implantation and some parts will eventually become the placenta. Cells of the ICM will maintain their pluripotency and continue to develop as the embryo.

Understanding the cellular and molecular mechanisms controlling this process has important implications in the field of assisted reproductive technology, especially IVF. IVF generally produces 6–10 embryos in a cycle, and a major challenge of the process is deciding how to select the best embryo for transfer. A more complete knowledge of what makes a preimplantation embryo viable would allow for better selection and improve IVF rates. This would dramatically lower costs for patients by preventing the need for additional cycles of therapy. Additionally, it would decrease the psychological toll of repeated failed pregnancies despite IVF treatment.

Conflict of interest

The authors declare no conflict of interest.

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Section 4

Embryo Application

Chapter 7

Perspective Chapter: What about Embryo's Rights?

Bahar Uslu

Abstract

The scientific associations of human reproduction experienced fundamental change in the twentieth century, with the development of in vitro fertilization for the treatment of infertility. The separation of sex and hi-tech reproduction treatments led to a revolution of gender and similarity relations, while embryo diagnostics led to a shift from scheduling families to planning a child. Furthermore, the presence of fertilized eggs outside the womb is a new form of human life which can be conserved and manipulated and the embryo in the petri dish in laboratory has become the entity of reproductive market, driven by clients and their claim to a right to reproductive choice. These improvements encounter deep-set moral sensitivities of human self-respect and the relation of human beings to their own nature. The conclusion is that the prospect of a posthuman future sounds for an ethic of care and responsibility. This chapter firstly presents these moral approaches briefly, especially regarding interventions in preimplantation-stage embryos in the laboratory, suggesting environmentally suitable laboratory conditions for this entity. Additionally, new suggestions for legitimately suitable regulations takes care precisely embryo's right, but also embryology laboratory personnel, clinics and parents.

Keywords: human embryo, embryo right, embryo ethic, reproductive legitimacy and regulations, clinical embryologist, embryology laboratory directorship

1. Introduction

Ever since the birth of the world's first test-tube baby in 1978 the altering scenery of human reproduction has produced public debate, moral questions and legal problems. From a realist point of view which pays close attention to the societal and cultural results of technological innovations three main issues can be distinguished: the shifting nature of medical practice, the creation of 'the embryo' as a new entity, and the differentiation of parenthood. All off these progresses are accompanied by shifts in roles and responsibilities which create new moral questions and problems.

These treatments were that the previously private sphere of proliferation became, in an awareness, public. The involvement of professionals, doctors, hospitals, laboratories and pharmaceutical industries in the formation of human life; the request for public funding of IVF research and IVF therapy; the need to regulate law and to develop public policy; and the media attention for all kinds of new reproductive developments and 'idiosyncrasies'—these all-sketched breeding from the dark

shelter of the private realm into the bright light of public attention and scrutiny. Furthermore, the participation of various authorities, institutions and public bodies raised the question of expert and responsibility of reproductive matters. Moreover, as a result of scientific progress in embryology, the discussion on the ontological status of the human embryo has also been raised to a more sophisticated level, enabled by new factual data provided by biomedical and social researches.

The application of Human reproduction treatments is complete with ethical problems and legal regulations. Several topics are defined in which some morally relevant developments have occurred in recent years. These advances, and the perennial conflict about the moral status of the embryo, is crucial for the development of the technology in general.

The past 30 years have seen the rapid evolution of many other assisted reproductive technologies (ARTs), which have evoked a variety of social, cultural, legal, and ethical responses. An ethic of care would necessitate the individual to performance as a moral agent, transcend self-interest, acknowledge and understand the relational background of his or her repro choices, care about embryo and for the others whose collaboration is crucial to comprehending the desire to give birth to a child, nurture getting of limitation of human control over nature, and assume responsibility for the embryo and children who are born, loving them as they are.

The possibility to create an embryo in a petri-dish, and to keep it there for a few hours up to a few days, made the early embryo much more of an 'individual entity'. The status of the embryo also became an issue in a quite different debate, that about the property rights over gametes and embryos. Currently, however, a human embryo can be created not only by fertilization, including fertilization with technological assistance prior to implantation for reproductive purposes as well as IVF for both reproductive and research purposes. Their research signifies both the destabilizing and the generative effects of ARTs at the interface between science and society.

Different combinations of gametes, wombs and background parents concentrate many different parenting-arrangements. While some of these preparations may sound unusual or strange, many of them have been brought into practice. In short, the new reproductive technologies have increased the number of parties involved in the manufacture of a child. Not only appointing parents, donors and surrogates, but also physicians, lab-technicians and lawyers, and institutions like surrogacy agencies, sperm- and embryo-banks, infertility centers or laboratories are involved.

Furthermore, proliferation of the technology has been driven in large part by private markets, which are increasingly global, rather than being guided by public policy, ethical and legal regulations. Reproductive ethics cover a broad range of issues and concerns about the potential effect of individual choices on the very nature of the human species.

To obtain some basic answers related to reproductive ethics to direct questions, in this article I will devote myself to studying two challenging points of view on the start of human life, the dignity of the human embryo, and while doing that I will disclose the views of those in favor and those against the theory that human life starts with beginning, and with that about the dignity of human person.

The reasons for more discussion about the everyday publicity of western society currently can be found in the fact that between physicians, theologists, philosophers, politicians, ethicists and sociologists and others there is still no agreement on when human life truly begins.

The remarkable progress of biomedical science and medical technology has added new scopes to the problem of the biological, ontological and moral status

of the human embryo. Two polar moral positions can be distinguished in the Embryo debate. First, those who completely oppose research with stem cells obtained from human embryos. In their opinion, the embryo has the same moral status as a person and consequently, must be (legally) protected as a person. Another, there is the perspective that young *in vitro* embryos, regardless of their origins, may be used for scientific research on the condition that the embryos used in the experimentations will not be implanted in the womb afterwards Most countries adopt an 'middle view'. They search for a 'happy medium' between these two polar views.

Owing to different countries of the world different legal regulations exist on protection of human life, more specifically, in different countries different regulations exist on legal consequences. Considerate reproductive medicine as a new practice implies a need to create new concepts or a new vocabulary, to define new (social) roles and responsibilities and to cultivate new rules guiding this practice.

I shall focus on those issues that seem to raise certain concerns but so far have met with relatively less interest. Specifically, I shall concentrate on a prospective parent–future child perspective to examine: the embryo's right to an open future; and the parents' reproductive autonomy.

2. Text

2.1 The definition of the early human embryo

Before exploring the bioethical debate about the embryo, it is important to select how this paper defines the embryo. We do not follow the description of Merriam-Webster's Dictionary of English stating that an embryo is "the developing human individual from the time of implantation to the end of the eighth week after conception." (Merriam-Webster, n.d.), as we only focus on preimplantation-stage embryos to be used or to undergo interventions in the lab [1].

The definition of human embryo used to refer to a prenatal human entity in the first weeks of pregnancy: "a developing unborn human during the first 8 weeks after conception." This definition was considered plausible and sufficient for the practical purposes in the era when embryos could have come to existence in only one way, that is by natural reproduction.

Some rather favors a definition of the embryo as "a totipotent single cell, group of contiguous cells, or a multicellular organism which has the inherent actual potential to continue species-specific i.e., typical, human development, given a suitable environment." [2].

Later, when the era of ART commenced, the definition was broadened, so that it could cover also assisted reproductive treatment strategies. Furthermore, assisted reproductive technologies raise intrinsic questions about the definition of embryo outside the womb besides the moral status of embryo. Even the etymological mirrors the mystery of our moral relation to this new form of human life: some call it a "pre-embryo," to characterize it an "early embryo" in the womb, while others prefer the term "fertilized egg cell".

The use of different definitions and terminology, in scientific and other literature, is often confusing. It is to be expected that as reproductive strategies progresses, more accurate nomenclature will develop.

2.2 Can we reflect embryos only as biological material?

Some view is that this entity deserves the full- fledged status of "human being" and recognition as a subject of human rights. Another view is that there is a gradation in the acquisition of moral status that parallels the development of the fertilized egg from a preimplantation embryo, to one that implants in the womb, differentiates into placenta and fetus, grows to become viable independent of the womb, and finally is birthed as an infant.

Jurgen Habermas, an opponent of genetic modification of embryos, considered the idea of one human being determining the genetic makeup of the other as a challenge to "the moral self-understanding of the human species" [3]. Others justify their objection to the prospect of designer babies in terms of the child's right to an open future. On the other hand, proponents emphasize the benefits of scientific progress and the individual's right to reproductive liberty and choice.

Dutch Embryo Act [4] and is articulated by the Health Council of the Netherlands as follows: "since it is human in origin and has the potential to develop into a human individual, the embryo has intrinsic value on the basis of which it deserves respect" [5]. The French National Consultative Ethics Committee supports the position that "the embryo or fetus has the status of a potential human being who must command universal respect" [6]. These advisory figures defend to use spare and research embryos, and both believe the embryo has essential value because it is a *potential human being*, a potential person. Others say that because the embryo is only a *potential* person it does not merit full respect and have used the potentiality argument *in defense* of human embryo research [7].

Most ethical philosophies, nevertheless, hold that whereas preimplantation embryos do earn protection, they need not be valued as persons [8]. Those in favor of this fact consider that a human being should be protected in his biological attribute, no matter which one of the attributes and capabilities he possesses.

Regarding the 'embryo as a potential person', the internal factors are the features of the embryo itself (e.g. its genetic structure, its growing potential).

Belgian Senate's Bill on Embryo Protection describes an embryo as a "cell or cohesive system of cells with the capacity to develop into a human being". Because of this potential, the argument goes, the embryo deserves special respect [9].

2.3 Some views and discussions on embryo research

In the beginning of this century, egg cells became an entity of demand for embryonic stem cell research for the purpose of personalized regenerative medicine. And the argument of the usage of embryos for research was reawakened by the discovery of the potential of embryonic stem cells. The question has superior importance because of the large possible therapeutic profits that might result from the ability to regulate and manipulate human embryonic stem cells.

Before the end of the first quarter of the twenty-first century, scientific advances are emerging at a unprecedented pace, challenging our traditional approaches to pregnancy, parenthood, and life itself. Since IVF was incorporated into reproductive clinics, it has been possible to create an embryo in a petri dish, implant it in the womb, freeze it in a nitrogen tank, discard it, or use it for scientific research [10].

The fundamental problem is that once one allows the generation and use of embryos for beneficial purposes, it becomes very hard to justify a ban on generating and using embryos for morally equivalent purposes.

Researchers' interest shifted to a certain degree from embryonic to adult stem cells following investigation of a scandal surrounding embryonic stem cell research, in Korea in 2005, which found both fraud in the reporting and ethical misbehavior in the appropriation of the human egg cells that were used. However, egg cells remain an important raw material currently for research in reproductive and regenerative medicine. Embryonic stem cells (the origin of all other types of cells in the body) may also be obtained by other means, by 'reprogramming' differentiated cells of various tissue types (skin, muscle, heart, etc.), which makes them turn 'backwards' into stem cells (so-called induced pluripotent stem cells) [11].

Newly, researchers around the world have been asking for permission and state funding for stem cell research with a fundamental explanation that it is necessary to spend the excessive number of embryos. Specifically, they would like to use unused, at conception unspent, embryos. Such embryos could be used for treatment of some incurable diseases. Many state that this is prohibited, because it disobeys the fundamental right of integrity of the human body, with more than a pure request to use it in therapeutical purposes to help a third party. Adrian Holderegger, considers that the "embryo becomes a foreign body" [12].

This view raises serious doubt about the validity of restrictions combined in regulation like the restriction in the United States that only stem cell lines derived before a certain date can be used for federally funded research or the German rule that embryonic stem cell lines created before a certain date in another country can be imported even though embryo research is prohibited.

IVF embryos that are to be used in research deserve some sort of respect, comparable to that given to human corpses serving educational purposes. Most doctors supported for legalization of embryonic stem cell manufacture from spare embryos. Ethicists were opposed to every kind of donation and research on surplus embryos. Most human geneticists and obstetricians approved egg, but not embryo, donation to other couples.

In terms of embryo ethics, pressing questions include, when does life begin—namely, does an embryo constitute a human life [13]. Are all embryos created equal, or can/should some be culled before embryo transfer for the purposes of research, disposal, or stem cell manufacture [13]. These questions are beginning to be studied in earnest by anthropologists, particularly in the new era of so-called reproductive medicine [14].

Examination of the use of human embryos for research will be structured around two ethical principles, specifically the subsidiarity principle and the separation principle. The subsidiarity principle states that no research on organisms with a high moral status should be performed if the same results can be obtained by research on organisms with a low moral status. The separation principal cuts the link between the wrongful act (the destruction of the embryo) and the subsequent use of the material that is obtained as a result of the wrongful act [15].

Steinbock also states that "a human embryo is something special, and a source of awe…" and deserves moral respect that imposes some restrictions on our use and disposal of embryos [1].

Preclinical embryo research is highly debatable, mainly because the embryos involved are used 'instrumentally'. The important controversy is whether preimplantation embryos have the same moral status as children or adults, who are to be protected from 'damaging' research:

The "embryo is a person in the making", states the embryologist and philosopher, Guenter Rager, Director and the embryo has its own dignity so therefore should not

be instrumentalized or used in therapeutical or research purposes. Prof. Rager reflects that for the purpose of therapeutic one disease embryos should not be sacrificed, because an embryo is a human person in the making. He therefore considers that the order of things should be respected, and the fact is that a person in the making is more important than curing one of the many different degenerative diseases and states of individual persons. Additionally, he considers, that concerning ethical boundaries does not mean the culmination of science itself [16].

Habermas stated "Embryos should never be formed for the purposefulness of research, and this would comprise the predictable surplus of embryos from IVF treatments" [3].

Raymond added "Creating embryos for research is being examined out of concern for the independence and the interests of the women donating oocytes for research" [17]. After all, women must undergo invasive processes, which has some health risks. Additionally, there is a risk of abuse, in view of the temptation to withhold detailed information on risks for anxiety of losing 'willing' candidate donors [17, 18].

Outka and Zoloth suggested a new method: Embryos that are considered to have a low chance of surviving freeze-thaw cycles or of developing to term are not cryopreserved or replaced. Since these embryos are intended for destruction, it is more suitable to use them for research. This indication has been expressed as the 'doomed embryo' rule [19] or the 'nothing is lost' rule [20].

Recently, A specific and comparatively rare application of PGD, i.e. typing embryos on their HLA type, is analyzed. However, this application, like social sexing, indirectly traces upon the original justification of performing PGD, i.e. preventing the birth of handicapped offspring. Progressively, the indications are widened to include transitional and social reasons. It can be predicted that gradually complex cases will present themselves. A nuanced ethical attitude is needed to do justice to such cases.

Whatever the moral perspective, whether the human embryo is viewed as the subject of rights, research in human embryos and their genetic manipulation for reproductive purposes are ethically sensitive activities, due to concern about the imaginable impact on future generations. For the time being, there is an international consensus that reproductive cloning and inheritable genetic modification are impermissible. Yet these conventions are challenged of late by advocates of research into mitochondrial replacement (three-parent babies), as well as by consumerist demand to select and enhance embryos based solely on personal preference.

In the United States, embryo disposition decisions (i.e., to discard or to donate to other infertile couples or to stem cell research) appear to be emotionally significant for couples and are frequently unresolved [13]. And, research in the private sector is restricted only by state laws prohibiting embryo research. In states that have no laws against it, privately funded embryo research is essentially unregulated.

Respective groups have recommended several steps to preserve the separation between the decision to donate embryos for research and the research itself [21].

UNESCO Universal Announcement on the Human Genome and Human Rights, 1997 releases the declaration that the human genome underlies the essential unity of all members of the human family and is, in a symbolic sense, the "heritage of humanity." This and other international human rights tools also make it clear that the manipulations of genetic science should request to improve the health of humankind. But the spread of Assisted Reproductive Treatments has been led in unlimited part by markets and by incentive for personal prominence and profit rather than by a public awareness in health [1].

We only focus on preimplantation-stage embryos to be used or to undergo interventions in the lab. It rather favors a definition of the embryo as "a totipotent single-cell, group of contiguous cells, or a multicellular organism which has the inherent actual potential to continue species-specific i.e. typical, human development, given a suitable environment." [2].

Although Steinbock proceeds from Sandel in her proposal that respecting embryos does not mean we can treat them as inviolable or we should prohibit embryo experimentation (as the all-approach suggests), we should still inaugurate some regulations and restrictions for the embryo manipulations and embryonic microsurgery that will help alleviate a wide range of human sufferings [1].

2.4 Are embryos complete humans who have full moral status? Or nothing but accumulations of cells, and do not have any moral status?

Conventionally, bioethical debates about embryo research tend to resort to moral status arguments and both subjects are deeply tangled. Bioethicists arguing in favor of embryo research who embrace what we call the "none- approach" use psychological or physiological arguments to claim that embryos do not have any moral status. On the other hand, major participants in the debate arguing against embryo research usually adopt what we call the "all-approach," using development-based and metaphysical moral arguments to defend the claim that embryos have a moral status [1].

These discussions, while pushing the limits of our imagination, bring up new moral questions within the discipline of bioethics regarding the embryo. it is important to elaborate not individual arguments but their common point of origin: moral status. Therefore, the discussion will (i) intricate the im/practicality of the all-or-none approach to moral status in the lab; (ii) support the unequal consideration of moral status approach developed by ref. [22] with the notion of "moral respect" borrowed from ref. [1] and (iii) outline what moral respect for embryos entails in practice.

The all-or-none approach to the moral status of the embryo includes two poles. The first one, which we call the *all-approach*, claims that "embryos are complete humans who have full moral status." By contrast, the second position, which we call the *none-approach*, asserts that "embryos are nothing but accumulations of cells, and do not have any moral status." Next, we will examine these two viewpoints and then challenge the alleged practicality of these two majors polarized traditional approaches to the embryo debate [1].

It seems that a vital issue is from when the human embryo matters morally, and if it matters *per se*—as such, because of its inherent characteristics as a being—or if it requires moral respect simply because of its value to other people.

Additionally, reproductive technologies increase central questions about the moral status of the human embryo outside the womb. Even the etymological imitates the mystery of our moral relation to this new form of human life: during laboratorial manipulations, some call it a "pre-embryo," to distinguish it from an "early embryo" in the womb, while others prefer the term "fertilized egg cell." The Catholic view is that this entity earns the full- mature status of "human being" and recognition as a subject of human rights. The Jewish view is that there is a gradation in the acquisition of moral status from pre-embryo to infant.

Ontological and moral status of human embryo matters *per se*—as such, because of its inherent characteristics as a being—or if it requires moral respect simply because of its value to other people.

Living entities regarded as valuable by the involved moral subjects. According to Matthew Liao, the Some Assisted Reproductive manipulations like enucleation or genetic editing are of such nature that both eggs, or both embryos, cease to exist and a *third* egg, or embryo, is created. In order to understand Liao's argument, we must bear in mind that an egg, or embryo, is an organism. An organism, *as a kind of thing*: (1) starts to exist when the capacity to regulate and coordinate the various life processes (respiration, absorption, metabolism and so on) is there; (2) it persists if there is a continuing ability to regulate and coordinate the various life processes and (3) it ceases to exist when the capacity to regulate and coordinate the various life processes is permanently gone [23].

Related to this, 4 facts were made by editors of the "Moral status of the human embryo" (Der moralishe status menschilcher Embryonen), G. Damschen, scientific collaborator at the Institute of philosophy of the Martin Luther University in Halle Wittenberg and D. Schoenecker, science assistant at the same university. That specification characterized the situation:

1. *Fact of species:* as members of *Homo sapiens* sapiens species embryos are humans and with that, they have dignity.

2. *Fact of continuity:* embryos are, unless no morally relevant procedures are made, constantly developing into fully grown humans which have dignity.

3. *Fact of identity:* embryos are from the morally relevant point of view identical to fully grown humans who have dignity.

4. *Fact of potential:* embryos have the potential to become fully grown humans and that potential requires full protection [24].

From a virtuously biological point of view, this statement is completely correct, because the embryo belongs to the human species (Species *H. sapiens sapiens*).

2.4.1 Embryo manipulations and morality

It has been generally acknowledged that regardless of the way they have come into existence, human embryos are considered valuable by the people who have some connection to them. These are not only parents or gamete donors (nowadays not necessarily the same people) but, as we argued, "embryos created by IVF of an egg by a sperm and allowed to develop to the eight-cell stage before implantation, with the intention to create a pregnancy, have always been treated with respect by doctors and scientists."

To intend by supposing the relation to the human embryo to involve a slight second-person point of view, and not just a third- person, scientific point of view push court, is intended as a way of avoiding instrumentalizing or commodifying views on human embryos.

Suchlike the moral standpoint, whether the human embryo is viewed as the subject of rights, research in human embryos and their genetic manipulation for reproductive purposes are ethically sensitive activities. It is because of concern about the possible impact on future generations. For the time being, there is an international agreement that reproductive cloning and inheritable genetic adjustment are unallowable. Yet these conventions are challenged of late by advocates as well as by

entrepreneurial request to select and enhance embryos based solely on personal preference.

Jurgen Habermas, an adversary of genetic modification of embryos, considered the idea of one human being determining the genetic makeup of the other as a challenge to "the moral self-understanding of the human species" [3]. Others defend their opposition to the viewpoint of designer babies in terms of the child's right to an open future. Instead, proponents highlight the benefits of scientific progress and the individual's right to reproductive liberty and choice.

Unfortunately, the assessment of an embryo is bestowed on it by those who choose its destiny: whether it is to be implanted and given the chance of further development into a living human, or it is to become a research embryo allowed only limited growth *in vitro* (approximately 14 days) and preserved as a source of research (in the future, also therapeutic) material.

Furthermore, genetic manipulation of embryos may produce unintended, unwanted, and irreversible side effects that are passed on to future generations. These practical oppositions to genetic engineering noise for the exercise of precaution and limit.

Also, there is a deontological dispute that genetic manipulation and enhancement should not be allowed, because when existing persons make decisions about the genetic arrangement of another, they generate a relationship of producer-produced which undermines the very possibility of mutual social relations and hence the initial evidence of equality between human beings [3].

2.4.2 Embryo deserves only moral respect or full moral status?

There are several issues implied by the concept of morally significant interests that are especially relevant to the question of the moral status of the human embryo. Should we say that only self-conscious beings can have a moral status? This seems not to be the case. If we agree that the absolute value presented by an embryo to others seems a rather uncertain basis for any justification of its own moral value, we should analyze other possible practicalities for its moral status.

The key idea forward-thinking by the defendants of the time of fertilization view is that because an embryo has its unique genotype, even "the early human embryo is one and the same individual human being from the start." means that even at the early stage it is the same individual as later, at the stages of being a fetus, newborn, infant, child, and an adult. Admittedly, the early embryo's identity with the adult may seem reasonable.

Referring this problem to the human embryo, DeGrazia frames it as the question of whether we can ascribe morally relevant interests to the pre-sentient human being based on what it becomes—or rather what it can become, because the concept of potentiality is involved in his reasoning, as well. If we adopt the stance that only those beings that are, not merely potentially, sentient can have morally relevant interests, the obvious conclusion could be drawn that not only human embryos but also fetuses before late gestational age do not have any moral interests [22].

Of course, we cannot say that "Sentient creatures that are able to feel both pain and pleasure and as such can be harmed or benefitted, respectively, we can talk about their welfare and their quality of life, and that is a sufficient basis for them to be granted a moral status."

It is maintained that the embryo is an individual 'from the moment of fertilization' or, while not yet an actual person, should be preserved and valued as a individual

because of the inherent potential to progress into a person the 'strong' version of the so-called potentiality argument [25, 26].

2.5 When the life begins? What is the relations with identity and human essence?

The most extreme approach is to endowment it such status from the very beginning, that is from the moment when the zygote appears, which traditionally is referred to as conception, but which presently does not essentially mean natural fertilization. Embryological sign shows that the embryo—or pre-embryo, to use the scientifically suitable term for the entity at this stage of expansion, that is in the first 14 days. At this stage "the embryo functions less like a single integrated, energy-using unit of the sort we call an organism than like a collection of single-cell organisms contingently stuck together" [22].

Also, another widespread attitude that from the very beginning the zygote demonstrates polarity and has directional orientation—meaning that it is inclined to develop in a certain direction [22].

Additional shared opinion, often pointed to by advocates of the zygote approach, that conception is the moment when the egg's and sperm's respective sets of chromosomes unite [22].

The key idea advanced by the defendants of the time of fertilization view is that because an embryo has its exceptional genotype, even "the early human embryo is one and the same individual human being from the start" [1].

The most noteworthy biological detail in this regard is the totipotency of the early embryo's cells, which means that a human organism can develop from each of them. Up to the eight-cell stage, if one of the cells is separated from the rest, it can develop into a distinct human being. Furthermore, not all the early cells will develop into parts of the embryo. Some will shape the placenta, but which ones is not decided before 14 or 15 days of the early embryo's development, when some middle cells begin to differentiate. Early embryo is that until the primitive streak, which is the predecessor to the spinal cord, is formed on around the 14th day of its development, it can undergo processes known as twinning and fusion. From a biological point of interpretation, the possibility of division or fusion challenges the opinion that the early embryo is one integral organism. We can also study the reverse situation, when there are two early embryos, coming from two eggs fertilized by two sperms at the same time (the fraternal twins), which fuse into one entity, biologically called a chimera, that then progresses into one human being. In this case, genetically, the chimera has two different sets of chromosomes, however it is one individual. In discussing the problem of identity. Aaron Simmons has offered an interesting challenge to the view that human identity must be transitive [27].

Simmons noticed that the recognition of the principle of transitiveness in human identity required: not only physical or psychological stability, but also *the nonexistence of any 'branch-line cases'*—that is, it cannot be the case that there exist two numerically distinct humans who are both physically or psychologically continuous with one and the same human organism [27].

All living creatures, regardless of their respective levels of biological development, have morally significant interests because they have specific needs that must be addressed to keep them alive. It seems reasonable to argue that to have morally significant interests, a living being must be sentient, that can have conscious sensations and experiencing feelings.

Single oocyte, sperm cell or somatic cell could be considered a potential embryo. Consequently, the pre-sentient human being is a creature whose award naturally inclines it to develop in such a way that it "will eventually accomplish sentience".

Individuals with means would want to improve their embryos themselves; genetically enhanced individuals would marry one another; the gen-rich and gen-poor would not intermarry; the human species would split into humans and posthumans [28].

2.6 Who would be the embryo assigned authority? Can embryo choose its own fully authorized microsurgeon?

The embryo is a highly ambiguous entity in both moral and biological context. For this reason, bioethical discussions over embryos seem never to reach a mutual conclusion from a theoretical point of view. Yet, embryo manipulations need to be regulated more clearly, given that embryos are expected to become part of reproductive clinics once genetic intervention finds a way to overcome moral, theological, and legal obstacles. It is thus significant to find a practically viable common ground in between all- and none-bioethical approaches [1].

The assistants, biologists, or technicians are not considered to be embryologists proper, but their responsibilities can, under some circumstances, be carried out by embryologists, so there can be some confusion between the roles. Numerous titles are presently used for clinical embryology positions. This leads to confusion about the roles and responsibilities for each [29].

I personally partly agree with this view: The question was not 'what are we allowable to do with embryos' but 'are embryos pre-born children, property, or something else' and 'who has authority over extracorporeal embryos?' [1].

In such unclear situations it is logical to ask: Who has the right, or, in what way does someone have the right to decide on when human life starts? In human rights law, the accepted view is that human beings become the subject of rights from the moment of birth onward neglecting embryonic life in laboratory.

While being the embryo's right, it is simultaneously a duty for a clinical embryologist who ought to be a guardian keeping all options open until the embryo becomes autonomous and will have the capacity.

2.6.1 Who is the fully authority for embryo microsurgery?

Of course, "embryo development in natural way" view may change should we observer the growth of artificial wombs such that *in vitro* embryos were able to be established into babies in them.

All the training programs that currently exist involve the review and/or evaluation of both theoretical knowledge and practical know-how and skills but not involve medical hierarchy, full medical authority or meaningful regulations to keep not only embryo, also parent's, additionally institution's rights.

Clinical Embryologists do not fit precisely into any of the outdated categories of laboratory workers, such as technician, biologist or technologist.

This is because the work of the Clinical Embryologist is typified by a high degree of technical skill and experience, extensive medical knowledge, and day-to-day responsibility for making many of the routine, but crucial, decisions that directly affect patients' treatment, like a Medical Director. The most other 'medical

laboratories' that are already directed by a physician with specialist training in specific areas of laboratory medicine.

Some authorities state that "if we are saying "clinical" meaning carries medical capacity and responsibility"; If we are talking about embryo has an individual capacity; if we are mentioning that embryo manipulations are namely "microsurgery"; it would not be surprised choosing a laboratory director among medical doctors received a special education from authorized institutions carrying a clinical embryologist specialist diploma. Because The role of the embryologist typically encompasses clinical treatment, microsurgery, clinical laboratory testing and laboratory management. They are hierarchically overing nurses, technicians, biologists and technologists. Within these broad categories, the Clinical Embryologist makes treatment decisions, reviews records and consults with other members of the ART team as well as with patients [30].

Many Clinical embryologists tend to think the problem is one of *design*, of specifying the best types of persons so that they can proceed to produce them. Thus, they worry over what sort of individual there is to be and who will control this procedure. They do not tend to think, perhaps because it weakens the importance of their role, of a system in which they run a "reproductive bazaar," meeting the individual specifications of prospective parents. The term *reproductive bazaar* can deal with many reproductive processes and services, such as preimplantation genetic screening both for disorders and abnormalities and for positive traits or sex; sale/donation of mitochondria, of female and male gametes and of embryos; surrogacy; or the use of germline reproductive technologies.

Kevles stated that: Without any regulation or medical authorizations, some embryonic manipulations like eugenics fell into disgrace in the rouse of the Nazi policy of "racial hygiene" because of the painful and inhumane methods it used to "purify" the Arian race and rid it of evidently mediocre individuals, including mentally retarded individuals [31].

2.6.2 Current regulations

Regulatory authorities and professional associations do not always appropriately recognize the meaning of Clinical Embryologist as a title or key role of the Clinical Embryologist as an organization's scientific professional, or their roles in effective ART laboratory hierarchy, direction and management. International agreement on the character, role and hierarchal place of the Clinical Embryologist was currently absent in terms of medical law or ethical regulations. Embryo has a part of legitimate, moral and ethical right like.

The progress that has taken place in clinical embryology has given rise to the need for new conceptualizations of the human embryo.

The role of Clinical embryologist as a reproducer, the physician as a co-reproducer, which has been neglected in the bioethical debate, can be questioned, as can the role and responsibilities of other professional or institutional co-reproducers: the lab, the sperm-bank or the infertility center.

In many countries, like physicians or mimicking physician's role, the embryologist is responsible for communicating with patients about laboratory procedures and the progress of their embryo development in the laboratory.

The value they accord to the embryo is variable and depends also on criteria external to the embryo and related to intentions of people. At present, for example, only some countries had officially recognized certification.

Similarly, It is a virtually unknown qualification in the 'outside world'. In France, only specially qualified clinicians can run ART laboratories, everyone else is a technician. In the USA, for example, the American Board of Bioanalysis has defined educational, and training requirements for five different levels of certification (including Technical, Supervisor, Embryology Laboratory Director, and High-complexity Clinical Laboratory Director). Another good example, Only qualified specialists are running ART in Turkish Governmental Hospitals. Turkish medical faculties have specific laboratory medicine physician program namely Medical Histology and Embryology Specialization program to be good candidate to manage not only Embryology laboratories also Tissue and Cell Laboratories with full authority. But Turkish private clinics still prefers to keep non-physician individuals stating some excuses financially or lacking number of Specialist related to this field. Personally, I would like advocate Turkish governmental system as a good hierarchical model in terms of embryo ethics, embryo moral right and legitimacy of reproductive medicine. Also, this model protecting institutions from insurance companies of parents blamed lab personnel stating "what happened to my baby" If something happens their embryo. I am presenting this model as a "Turkish medical reproductive management model" in some seminar and congress [30].

2.6.3 Preparing external conditions for embryo

If we evaluate the to prepare convenient environment discussion on embryo manipulations from the point of view of the new and innovative character of reproductive technologies, at some points this debate has been underdeveloped.

Human embryos deserve moral respect because soon they may become part of clinical procedures in reproductive clinics. Thus, to adopt such a significant future application, we need to use the transitory time period we are currently in to discuss and regulate such interventions in advance.

The embryo as a new entity created by these techniques has been discussed at length, but the creation of new relationships and responsibilities has received rather fragmented and one-sided attention, while the shift in medical practice itself (from therapeutic to productive) has gone largely uncommented. We must now analyze to takes seriously the technological character of our present culture.

In the years subsequent the first IVF-birth, many governments, institutions and authorities started to make a start in the path of regulating the new reproductive techniques. According to Walters, the period 1979 till 1988 can be considered the "heyday of guideline writing" [32]. Many ethical and advisory committees were appointed to issue statements and references that were tried to be both ethically defensible and politically acceptable. Thus, these statements form a special kind of bioethical discussion directed towards agreement and public policy creation. Over 85 committee statements were prepared in 25 countries. Walters' review of the 15 major reports issued between May 1979 and December 1986 shows that IVF was unanimously considered ethically close to be acceptable but not enough in terms of new social necessities or hi-tech development of this field.

A large part of the literature in this third period does not actually discuss normative problems surrounding IVF. Others compare the several reports, laws and regulations issued in various countries and some even discuss the role of law, philosophy and ethics in public policy making. Other authors, especially those with a legal background, make suggestions or issue proposals for new laws or regulations themselves.

Equally important principles the instruction suggest the right of every human being to life a physical integrity from the moment of fertilization; the right of the family as an institution; and the right of the child to be conceived, brought into the world, and brought up by his parents [1].

Given the wide acceptation of 14 days limit among ethicists and committee members, there may be some difficulties in justifying this limit "philosophically" it clearly works in practice.

The concept 'pre-embryo' for the embryo during these first 14 days can be characterized as a realistic intervention in the argument. It presents a new term to designate a new entity, it makes it easier to speak about questions surrounding this new entity, and it combines the idea of gradual protectability. The new term 'pre-embryo' both enables and expresses a consensus on this point. New perspective and technique of embryo manipulations opened or created a new practice, different from traditional medicine. While IVF used to be described as a therapy for infertility, it can just as well be described as a "service to bypass childlessness".

Once it was possible to cultivate the embryo outside a body, and especially after it had become possible to preserve the embryo almost open-endedly by freezing it, it also became imaginable to ask to whom this (frozen) embryo belonged (to the providers of the gametes, to the embryo-bank, or to the lab?); and once it was possible to operate or extinguish the embryo at will, it developed imaginable to ask what one was allowed to do with it.

We consider that with the act of merging the oocyte and the spermatozoid a long-lasting procedure. Any effort at ending the process of development would be a criminal act. Because what would be the difference in guarding dignity, we can ask ourselves, between a newborn of a few weeks and a newborn of a few seconds. Therefore, the human embryo should be protected from its very beginning. This point of view is also shared by L. Honnefelder, who states that the description of the embryo's dignity is done by relocating the moral status of the born to the unborn human based on identity and continuity of development, which principals from the unborn to the born human [33].

The Assisted Reproductive Treatment market is motivated for the most part by for-profit motivation. New reproductive technologies generate new markets of consumers; supply generates demand. Immediately a new market is established, the hunger of medical entrepreneurs is complemented by a consumerist address of desire. The language of "would like" or "want" translates into "need," which then turns into a sense of claim, the declaration of a "right to use the technology", and a demand for instant satisfaction [34].

Leist reflects that with this fact is based on the belief that there is a general prohibition on killing any human being where a human being is considered that which is alive and belongs to the human species, a category in which the human embryo falls. Appropriately, the conclusion could be made that "human life starts with the fertilized egg cell" [35].

Almost every country has similar article says, "Every human being has the right to live" or "A person's liberty and personality are paramount" and "No-one should be subjected to any kind of abuse or, without consent, medical or scientific experimentation. Forced and obligatory labor is forbidden" From these totally well-defined constitutional principles follows the conclusion: the question of protection of human life is existential question of a human community, one human species which supports the dignity of a human being.

3. Conclusions

Most authorities are seeking competency-based framework factors on consideration for Direction level Clinical Embryologists. These manuscripts are mostly mentioning "Ideally, earned doctorate in biomedical science", but they are forgetting doctorate degree is an academic degree not clinical.

Rather, Clinical Embryologists are more like 'practitioners' in the general sense of the word, rather than the specific sense used in the Turkey in the definition of hierarchical roles, responsibility in clinical embryology and reproductive medicine.

We determined that, while embracing diverse moral thoughts and religious beliefs about the embryo, we can use the potential of embryo manipulations to help human beings.

We can discriminate firm factors playing a part in the probability that an embryo develops into a person: (1) the amount of resemblance to the human bodily form, (2) the degree of independence or self-sufficiency with respect to a particular environment (e.g. the endometrium), and (3) the fact that the embryo has passed some critical and easily identifiable markers in its development (individuality, development of the spinal cord, brain stimulation, becoming a responsive being, etc.).

Although I personally consider that human life begins with fertilization, that moment when the possibility that a living human being develops from an embryo.

In this chapter I have searched for an answer to the question: when does human life begin, and answer to the question who has the right to decide that life begins with fertilization or in some other phase of growth of the human embryo.

In this respect I have revealed numerous and conflicting opinions of view about when human life begins, listing different arguments, arguments of those who are in favor of human life beginning with fertilization and consequently the need to protect the dignity of the human embryo, and arguments of those who consider that the dignity of a human person is not the same as the dignity of the human embryo.

I personally respect their intuition that embryos should not be treated merely as any other type of body tissues and there should be some limits to the uses of human embryos.

Consequently, the fundamental question is asked: when does human life accurately begin, i.e. when does a human being undertake the right not to be destroyed? Nevertheless, without clear legal regulations there will always be the possibility to perform morally questionable procedures on the human embryo. In the case of embryos, this respect-commanding quality is connected to the embryo–child's potential to become a person consistent with its consecutive phases.

Picking the future child's height, hair or eye color, sex and, possibly at some point, cognitive skills, artistic or sporting capacities may seem instinctively wrong, as it implies conditional acceptance as well as rejecting the idea of the child as a gift. It also involves designing and shaping—in other words, interfering with— the child's future, which may lead to violating her autonomy.

Embryology laboratory is a tentative mother for embryo. This means that if something is human, then it should be sheltered, and that should be the value of all actions. Embryos have human dignity and need protection.

According to our proposal, the concept of moral respect involves that embryo should be handled by a physician/ specialist trained in embryology. If we argue that respect to embryos can be shown by leaving their handling to embryologists, a reform in postgraduate medical education may become necessary. To our knowledge, clinical embryology training as a postgraduate medical specialty for medical doctors exists only in a few countries.

Consistent with the accepted legislation, in order to be an embryologist in Turkey (similarly some part of Dutch, France and Belgium), it is necessary to be a reproductive specialist medical doctor who fully authorized for microsurgery and intra cytoplasmic micro injections alike manipulations [30]. Additionally these individuals must pass the specialty examination in medicine. This specialty program is called Medical Histology and Embryology belongs to Medical Histology and Embryology Department belongs to Faculty of Medicine. This Medical Clinical Embryologist Group also have an international organization open access is called Society of Histologists and Embryologists SoHE/ hemud [36].

Throughout this chapter, we defend the view that preimplantation embryos used in the lab deserve moral respect in some point, because (i) they have an inherent potential to become "one of us"; (ii) they are human organisms in a biological sense, and (iii) they will soon be considered as patients in reproductive clinics. We also suggest that if embryos soon are to be considered as patients being subjected to genetic intervention in a petri dish, then they need to be handled by a physician/specialist trained in microsurgery and has full authorization to deal with embryos: a medical clinical embryologist.

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Conflict of interest

The authors declare no conflict of interest.

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Section 5

Organogenesis of Early Embryo

Chapter 8

The Hepatic Fetal Venous System

Dominic Gabriel Iliescu, George-Lucian Zorila, Roxana Cristina Dragusin, Marius Cristian Marinas, Maria Cristina Comanescu, Alexandru Cristian Comanescu, Pana Razvan Cosmin, Ovidiu Costinel Sîrbu, Sidonia Catalina Vrabie, Sidonia Maria Sandulescu and Rodica Daniela Nagy

Abstract

The vascular architecture of the human liver is established at the end of the 10th week of gestation as a result of a complex process. Recent developments in ultrasonographic imaging facilitate the prenatal evaluation of this system. However, many of the involved mechanisms are poorly understood. The hepatic primordium is in contact with the vitelline veins and the umbilical veins, and by the end of the 6th week, the afferent venous system of the liver is acquired giving rise to the portal vein, the portal sinus, and the ductus venosus. The only afferent vein of the liver that remains open at birth is the portal vein. Also, the efferent venous system of the liver is formed and emerges from the vitelline veins.

Keywords: fetal venous system, embryology, hepatic vasculature, liver vascular anatomy, ultrasonography

1. Introduction

Intensive research has been done in the evaluation of the fetal venous system to recognize the normal and abnormal sonographic appearance. Furthermore, prenatal diagnosis of fetal venous system anomalies requires knowledge of its embryology and physiology. The information currently available regarding the etiology, importance, and prognosis of these abnormalities enables caregivers to provide appropriate parental counseling when an anomaly is encountered. Still, the sonographic evaluation of the fetus's venous system remains largely undetermined and needs more studies.

2. Fetal circulation

The fetal circulatory system functions differently than after birth and has certain properties that are present only prenatally and are very important since the fetus is

completely dependent on the mother's circulation. Thus, ductus arteriosus, ductus venosus, foramen ovale, and placental-umbilical flow are vital to fetal life. The placenta oxygenates the deoxygenated blood, and the three shunts allow umbilical blood to bypass specific organs (liver and lungs). As a result, oxygenated blood is delivered via the umbilical vein (UV) to the liver (70–80%) and directly to the fetal heart, across the DV (20–30%).

Roughly 60% of the fetal cardiac output is pushed through the umbilical arteries and back to the placenta. The placenta's blood returns to the fetus via the umbilical vein, which has an oxygen saturation of about 80%. The DV rises from the umbilical and portal system connection, as a continuation of the UV, bypasses the liver, and joins the inferior vena cava at the level of sinus venosus, located at the base of the right atrium. Although DV oxygenated flow joins venous blood from the lower trunk and extremities, as well as blood from the liver, because of the DV direction and flow velocity, it is preferentially directed from the right into the left atrium via foramen ovale.

The UV, DV, foramen ovale, left atrium and ventricle, and the aorta form the so-called via sinistra, while the superior and inferior vena cava, the right atrium and ventricle, the pulmonary artery, and the ductus arteriosus form the via dextra. The majority of blood from the superior vena cava enters the right atrium and ventricle and exits via the pulmonary artery.

Because the collapsed lungs have high resistance and the pressure in the pulmonary artery is higher than in the aorta, most of the blood in the pulmonary artery travels via the ductus arteriosus to the descending aorta. The fetus's head receives better-oxygenated blood from the left ventricle and aortic arch [1].

3. Fetal venous system – embryology

The vascular architecture of the liver is very complicated, and understanding the embryology and pathophysiology of the fetal venous system is critical for a more effective approach.

The cardiovascular system is the first organ system to develop. In a 4-week embryo, three symmetric paired veins flow to the right and left horns of the sinus venosus into the heart: the umbilical veins (UVs)—which drain the chorion, vitelline veins (VVs)—which drain the yolk sac, and cardinal veins (CVs)—which drain the embryo's body.

The septum transversum development of the fetal liver allows the connection of the vitelline and umbilical veins with the sinusoids, resulting in changes in the VVs and UVs: the left UV becomes the dominant pathway of blood from the placenta, while the right UV and the left cranial segment of the left UV become atrophic and disappear. The left umbilical vein receives practically all of the placental blood drainage and will route itself through the hepatic sinusoids that develop from the vitelline veins [1, 2].

A large channel develops and shunts blood from the left umbilical vein to the right cardiac channel and then to the sinus venosus. This is ductus venosus, the functional shunt that allows oxygenated placental blood to bypass the liver, with a relatively direct path to the heart. The establishment of a DV as a "critical anastomosis" between the umbilical and vitelline venous systems, followed by increased hepatic perfusion, appears to be important for the successful development of an intrahepatic portal venous system.

The growing sinusoids, on the other hand, will form the right and left hepatic veins, which will all drain to the developing inferior vena cava, at the site of the right cardiac channel. The primitive vitelline veins create the future portal venous system within the liver. The vitelline veins will degenerate proximally on the left side, and more distally on the right side, resulting in a convoluted S-shaped path along the back and front of the growing gut. Around the same period, the superior mesenteric vein and the splenic vein will develop from the VVs, and eventually converge to form the hepatic portal vein.

The common cardinal veins, with anterior and posterior cardinal veins draining the embryonic cranial and caudal segments of the embryonic body, comprise the third venous system that enters the sinus venosus. The posterior branches of the CVs are severed, but the caudal portion remains, giving birth to the common iliac vein and the caudal part of the IVC. The CVs are eventually replaced by subcardinal veins, which drain the kidney and gonads, and supracardinal veins, which drain the thoracic wall and iliac veins.

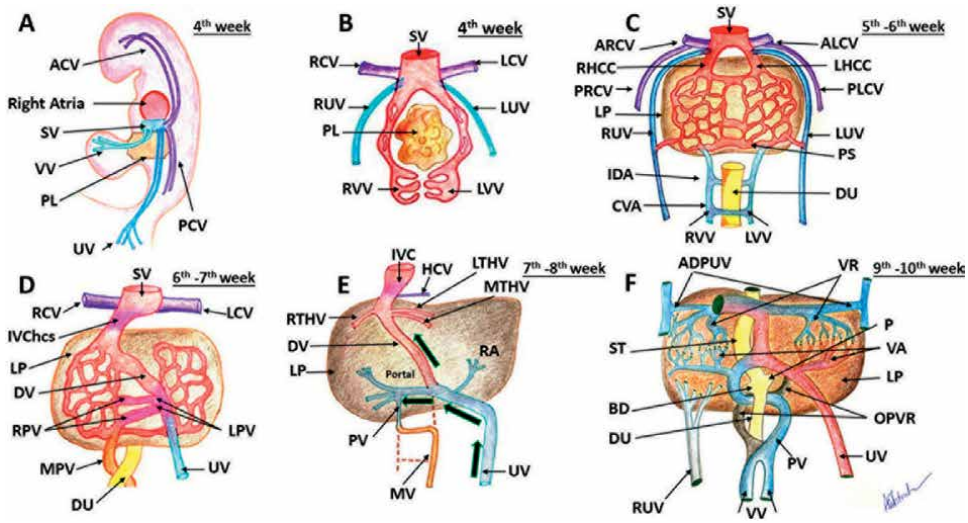

Figure 1.
Embryological development of the human venous system. A: The embryo demonstrates the development of paired sets of "vitelline" and "umbilical" veins in its fourth week, which initially drain the yolk sac and allantois. B: At the 4th week, there are three symmetric paired veins: the umbilical veins, vitelline veins, and cardinal veins. All three systems converge into the sinus venosus. C: In 5–8 weeks, the liver cords develop into the septum transversum and interrupt the cranial portion of the umbilical and vitelline veins. D: Asymmetric stage with the intrahepatic anastomosis developing between the umbilical-portal and ductus venosus systems. E: Changes in the VVs and changes in the UVs continue. Only the most caudal and the most cranial segments of VVs will persist. F: By the end of the 10th week, small venous branches, named venæ advehentes, convey the blood from the subhepatic anastomosis to the sinusoidal plexus; small venous vessels, named venæ revehentes, drain the blood of the sinusoidal plexus into the subdiaphragmatic anastomosis. SV: sinus venosus, VV: vitelline veins, PL: primordial liver, UV: umbilical veins, PCV: posterior cardinal veins, ACV: anterior cardinal veins, RUV: right umbilical vein, LUV: left umbilical vein, RVV: right vitelline vein, LVV: left vitelline vein, ARCV: anterior right cardinal vein, ALCV: anterior left cardinal vein, DU: duodenum, PS: portal sinus, IDA: intermediate dorsal anastomosis, CVA: caudal ventral anastomosis, PRCV: posterior right cardinal vein; PLCV: posterior left cardinal vein; RHCC: right hepatic common cardinal vein; IVChcs: inferior vena cava hepatocardiac segment; RPV: right portal vein; LPV: right portal vein; UV: umbilical vein; MPV: main portal vein; LP: liver parenchyma; IVC: inferior vena cava; PV: portal vein; MV: mesenteric vein; RA: ramus angularis; RTHV: right terminal hepatic vein; MTHV: median terminal hepatic vein; LTHV: median terminal hepatic vein; HCV: hepatic cardialis venula; OPVR: obliterated portion of venous rings; BD: bile duct; P: pancreas; VA: venae advehentes; VR: venae revehentes; ADPUV: anterior detached portions of umbilical vein. Images and legend from the collection of Dr. Anca-Maria Istrate-Ofițeru, with permission.

The superior portion of the left supracardinal vein obliterates and joins to the right branch, establishing the renal–hepatic segment of the IVC. The inferior portion of the left supracardinal vein obliterates and connects to the right subcardinal vein, forming the sacrorenal segment of the IVC. The superior segment of the supracardinal vein is divided into two branches: the left branch, known as the hemiazygos vein, which forms a cross anastomosis with the right branch, known as the azygos vein, which drains into the superior vena cava.

The proximal left anterior CV recedes and separates from the sinus venosus. During the eighth week, the left brachiocephalic vein develops from the shunt produced to the right anterior CV. Meanwhile, the SVC develops from the confluence of the left and right brachiocephalic veins and the right atrium.

Afferent and efferent venous networks form inside the developing fetal liver. The afferent system consists of the UV, PV, and DV, whereas the efferent system consists of the hepatic veins.

An invagination of the left atrium dorsal wall is visible in the 4-mm embryo, which represents the developing common pulmonary vein. The connection process continues as the atrial cavity grows, and two right and two left branches of the pulmonary stem become connected with the atrial cavity. The pulmonary venous plexus loses its connection to the VVs and CVs throughout time (**Figure 1**) [1–3].

4. Anatomy of the hepatic venous system

Although ultrasound imaging of the fetal venous system has improved, the ability to understand its anatomy and the true anatomical relationships of the portal venous system have not been clearly defined. Within the liver, there are two venous systems: an afferent and an efferent system. The efferent system includes the hepatic veins, which conduct blood from the liver to the heart, and the afferent system includes the portal system, which delivers the blood from the gut and placenta to the liver – through the UV. Thus, the umbilical system that carries blood from the placenta to the liver is part of the hepatic afferent venous system (**Figure 2**).

The extrahepatic portal vein, also known as the main portal vein, results from the confluence of splenic and superior mesenteric veins. It travels behind the duodenum and empties into the portal sinus near the right intrahepatic portal vein's origin. The right portal vein has two branches, anterior and posterior, while the left portal vein has three branches: inferior, middle, and superior. The portal sinus is an L-shaped vascular region that spans from the inferior left portal vein's origin to the right portal vein's origin. As a result, it joins the right and left intrahepatic portal veins, which perfuse the right and left hepatic lobes, respectively (**Figure 3**).

The angle of communication at the confluence of the major portal vein and the portal sinus varies from 900 to a virtually parallel course. There are three types of connections between the main portal vein and the portal sinus: T-shaped, end-to-end anastomosis, X-shaped, side-to-side anastomosis, and H-shaped, parallel anastomosis. The majority of the blood flowing via the main portal vein is delivered to the right hepatic lobe [3].

According to Rudolph et al., portal blood is directed only to the right hepatic lobe, but umbilical venous blood nourishes both hepatic lobes and the ductus venosus. When compared to the left vein, the right portal vein receives less oxygenated blood. As a result, the left lobe is considerably larger than the right lobe throughout fetal life, a circumstance that is reversed after birth when the UV and DV become atrophic. The

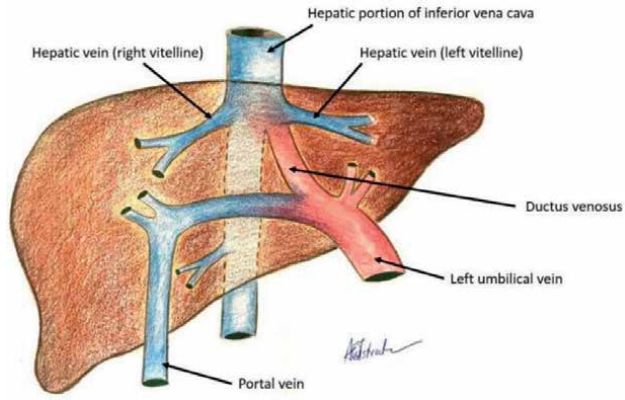

Figure 2.
Schematic representation of the afferent and efferent systems of the fetal liver.

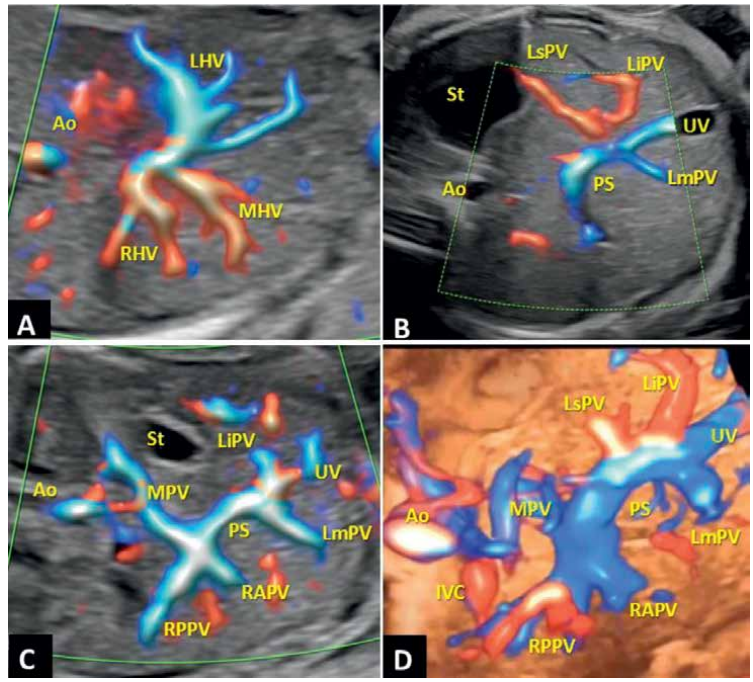

Figure 3.
The normal aspect of the portal venous system (PVS) and hepatic veins. (A) Transverse plane of the fetal abdomen demonstrating normal aspects of the efferent hepatic system: the left, middle, and right hepatic veins. (B) Axial plane of the fetal abdomen with the evaluation of the umbilical vein and left portal vein branches. (C) Transverse plane with the demonstration of the normal right portal vein and its branches. (D) STIC HD-flow evaluation of the afferent system of the liver. UV, umbilical vein; Ao, aorta; IVC, inferior vena cava; St, stomach; MPV, main portal vein; PS, portal sinus; RAPV, anterior branch of the right portal vein; RPPV, the posterior branch of the right portal vein; LiPV, left portal vein inferior branch; LmPV, left portal vein medial branch; RHV, right hepatic vein; LHV, left hepatic vein; MHV, middle hepatic vein.

efferent venous system is made up of three hepatic veins: the right, middle, and left hepatic veins. These veins open to the subdiaphragmatic vestibulum and drain hepatic venous blood into the right atrium [4].

The umbilical vein enters the abdomen within the falciform ligament and joins the liver along its inferior surface, cephalic directed, and opens in the portal sinus, which is aligned with the arising ductus venosus. DV appears as a trumpet-like thin shunt with a diameter that is approximately one-third the diameter of the UV, which determines high blood velocities. Also, the DV direction connects with the right atrium pointing more posterior and cranial toward the foramen ovale (**Figure 4**). These are the reasons why there is a preferential flow of oxygen- and nutrient-rich blood from the placenta to the left atrium via the UV and DV [5].

DV is essential for providing oxygenated blood to the left side of the heart and for the normal development of the intrahepatic portal venous system. The absence of DV causes aberrant hemodynamics because there is a "steal" effect with lower intrahepatic flow, which may result in the vitelline veins failing to transform into the portal system [6]. During pregnancy, umbilical venous blood passes via the portal sinus to nourish the right and left intrahepatic portal veins, as well as the DV. When the placental circulation stops and the ductus venosus closes, blood travels from the extrahepatic portal vein to the left intrahepatic portal veins, perfusing both liver lobes.

Both common iliac veins join together to form the inferior vena cava (IVC), which connects with the two renal veins along its path. The IVC is on the right side of the spine in the lower and mid-abdomen, and then it flows over the posterior liver surface to enter the right atrium.

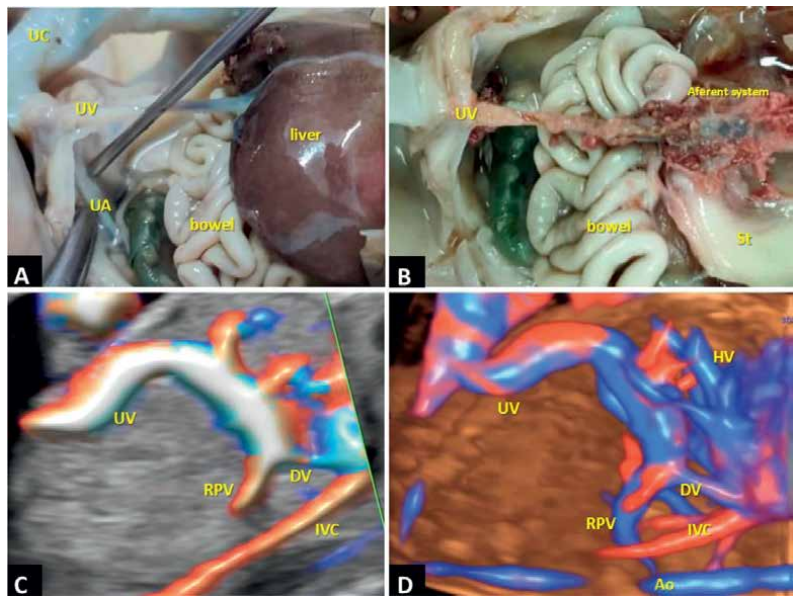

Figure 4.
Macroscopic and ultrasound evaluation of the fetal liver. (A and B) Pathology examination shows the normal extrahepatic (A) and intrahepatic (B, liver tissue removed) pathway of the umbilical vein. (C) Longitudinal plane of the fetal abdomen, Color Doppler evaluation, showing the normal intrahepatic pathway of the umbilical vein and the normal aspect of the ductus venosus. (D) Longitudinal plane of the fetal abdomen, 3D rendering, which demonstrates the normal aspect of the afferent and the efferent hepatic systems. UV, umbilical vein; Ao, aorta; IVC, inferior vena cava; St, stomach; RPV, right portal vein; HV, hepatic vein; UC, umbilical cord; UA, umbilical artery.

5. Ultrasound assessment of the liver vasculature

By the end of the 10th week of gestation, the portal venous system (PVS) is already formed. However, due to the embryo's size, ultrasonographic examination of this system is not possible. At the time of the standard first trimester (FT) anomaly scan (12–13 gestational weeks), the fetal portal venous system (PVS) architecture, as well as the presence of ductus venosus, can be analyzed [7, 8]. It has been also demonstrated that microscopic techniques could serve as an audit to assess the prenatal PVS features determined by first-trimester ultrasound assessment (**Figure 5**).

Ultrasound examination of the embryonic venous system has shown a wide spectrum of abnormalities. This system's aberrant development may be caused by defects in one of the four embryonic systems: the umbilical, vitelline, cardinal, or pulmonary systems. In most cases, venous malformations occur because the primitive veins do not undergo obliteration, or the development of crucial anastomoses does not occur. There has been proposed a novel classification of fetal venous system anomalies that expands on the four major embryonic groups mentioned above, but we will focus on the anomalies of the vitelline and umbilical systems [9].

5.1 Umbilical veins

5.1.1 Primary failure to create critical anastomoses, leading to abnormal UV connection with agenesis of ductus venosus (DV) and intra- or extra-hepatic systemic shunt of the UV

Agenesis of ductus venosus (ADV) occurs secondary to the absence of "critical anastomoses" between the portal and umbilical venous system and the hepatic–systemic venous system. This leads to the shunting of umbilical blood through an aberrant vessel. As a summary of the literature, in isolated ADV with intrahepatic UV drainage, the neonatal outcome is generally good but attention must be given to cases with intrahepatic shunts between the portal and hepatic veins concerning hyperammonemia and elevated liver enzymes that should be monitored after birth until the shunt closes. Also, in cases with extrahepatic UV drainage, it is important to monitor the closure of the shunt. In cases of ADV with extrahepatic shunt, the prognosis is determined by the severity of associated anomalies, the diameter of the shunt, and the development of the portal system [10]. In cases of isolated agenesis of the ductus venosus, the postnatal outcome depends on the persistence of a portosystemic shunt or agenesis of the portal venous system [11]. The association of the ADV with portal venous agenesis affects the long-term outcome due to severe postnatal complications, including congestive heart failure, pulmonary edema, focal nodular hyperplasia, and hepatic tumors [12–14].

5.1.2 Persistent right UV (PRUV) with or without left UV and/or DV

PRUV, an anomaly that results from the failure of the right umbilical vein atrophy, represents the most frequently detected fetal venous system anomaly. The left umbilical vein may be replaced or the RUV may be found as an intrahepatic vein, connecting to the right portal vein. Also, it may determine anomalous drainage of blood into the IVC or right atrium, bypassing the liver [15–17]. It has been suggested that primary or

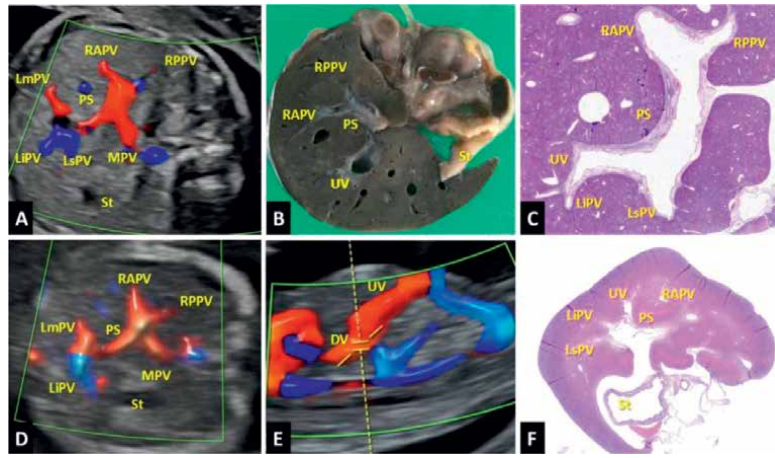

Figure 5.
The fetal liver's macroscopic, microscopic, and ultrasonographic features. (A, B, C): Transverse planes at the level of PVS in the second trimester of pregnancy. (A) Color Doppler evaluation, (B) macroscopic evaluation, and (C) microscopic evaluation of the fetal liver, showing the hepatic course of the umbilical vein (UV), the L-shaped portal sinus (PS), the junction of the PS with the main portal vein (MPV), the left portal vein, the right portal vein, and their branches. (D, E, F): First-trimester evaluation of the hepatic venous system. (D) Color Doppler evaluation of the portal venous system and (E) the normal aspect of the ductus venosus. (F) Microscopic demonstration of the portal venous system features. St: stomach, PS: portal sinus, MPV: main portal vein, UV: umbilical vein, LsPV: superior branch of the left portal vein; LiPV: inferior branch of the left portal vein; LmPV: middle branch of the left portal vein; RAPV: anterior branch of the right portal vein, RPPV: posterior branch of the right portal vein. Adapted from [7], Courtesy of Nagy et al., with permission.

secondary occlusion by thromboembolic events arising from the placenta may lead to early streaming of blood through the right UV to cause this anomaly [18]. Echogenic foci situated within the fetal liver suggest this etiology. Ultrasound visualization of an aberrant vein passing laterally to the right of the gall bladder, in the plane of measurement of the abdominal circumference, is suggestive of PRUV. According to the reported data from the literature, in the absence of other anomalies, PRUV with normal DV connection usually represents a normal anatomical variant with no clinical significance [15, 19, 20].

5.1.3 UV varix

Umbilical vein varix is a focal dilatation of the vein, more often at the level of intra-abdominal UV, being diagnosed when a sonographically anechoic cystic mass is seen between the abdominal wall and lower liver edge, continuing the UV and with venous Doppler signal. The intra-abdominal UV varix has been defined as an intra-abdominal UV diameter at least 1.5 times greater than the diameter of the intrahepatic UV, or as an intra-abdominal UV diameter exceeding 9 mm [21]. Diagnosis of UV varix requests a detailed anatomical examination, karyotyping, echocardiography, and close monitoring of the fetus for sonographic signs of hemodynamic disturbance because of the high risk for unfavorable outcome [22].

The umbilical-portal-DV complex is a vascular unit, and each component can be affected. Abnormal communications of the portal, umbilical venous system, and hepatic-systemic venous system have been classified under the term "umbilical–portal-systemic venous shunts" (UPSVS). Three types of shunts have been previously reported: type I, umbilical-systemic shunt (USS), type II, ductus

venosus–systemic shunt (DVSS), and type III, portal-systemic shunt, divided into type IIIa, intrahepatic portal-systemic shunt (IHPSS) and type IIIb, extrahepatic portal-systemic shunt (EHPSS). More recently, a new type of shunt has been reported, type IV, which includes multiple shunts of different types [23]. The outcome in UPSVS and ADV cases depends on the presence of concomitant structural or genetic abnormalities. Venous system anomalies can be accompanied by heart, digestive, and body symmetry abnormalities, known as left and right atrial isomerism.

5.2 Vitelline veins

Complete agenesis of the portal system (portosystemic shunt) or *partial agenesis of right, left, or both portal branches* (porto-hepato-systemic shunt)

Few cases of total or partial agenesis of the portal venous system have been reported during fetal life and apparently this anomaly is most likely underdiagnosed, but detection increases significantly with a systematic examination and the routine use of color Doppler is implemented. Lately, a classification of this condition has been proposed; there are two types of portal agenesis: type I (complete absence of portal venous system) and type II partial agenesis, which is further classified into IIa, IIb, and IIc, according to the shunting site [9, 24].

a. ***Complete absence of the portal venous system*** (CAPVS) occurs due to the failure of the vitelline veins to transform into the portal system, which also leads to a failure to form the critical anastomosis with the hepatic sinusoids or UVs. The splenic and superior mesenteric veins do not joint to form the portal vein or, if they form it, the drainage is done into the systemic circulation (IVC, renal vein, azygos vein, etc.). As a result, liver development is supplied by a compensatory increase in the hepatic arterial perfusion. The failure to form the critical anastomosis between the UV and the vitelline venous system seems to be the reason and the link between the agenesis of the DV and the agenesis of the portal venous system. Achiron et al. suggested that when ADV is detected, caregivers should rule out the presence of the total or partial agenesis of the portal venous system, and the splenic or superior mesenteric vein should be investigated for the presence of a triphasic pulsatile flow, which raises the suspicion of a portosystemic shunt [9]. Mesenteric and splenic venous blood may drain directly into the IVC, renal veins, or hepatic veins, or via the caput medusa to the heart. Isolated CAPVS is rare, as the frequency of associated major anomalies is high [12]. There have been reported heterotaxy–polysplenia cases, congenital heart disease (CHD) – atrial septal defect and/or ventricular septal defect, Goldenhar syndrome, and chromosomal anomalies.

b. ***Incomplete absence of the portal venous system*** (IAPVS) occurs due to a partial failure to form critical anastomoses. This is a more benign presentation of vitelline vein abnormality and with a better outcome, due to the rare association of other malformations and with a high chance of neonatal spontaneous resolution. The consequence of partial maldevelopment of critical anastomosis is agenesis of the right portal system, with a persistent left vitelline vein connected to the hepatic vein (portohepatic shunt) with or without the development of the DV. However, the intrahepatic shunt ratio may influence the hemodynamics with repercussions on the fetal growth – intrauterine growth restriction [25].

The decreased portal blood flow into the liver due to the hepatic bypass of the portal circulation increases blood flow in the hepatic artery – the so-called hepatic artery buffer effect.

Long-term metabolic sequelae of portosystemic shunting are often identified in retrospection and include hypergalactosemia, hyperbilirubinemia, hyperammonemia, and liver masses, including focal nodular hyperplasia, adenoma, hepatoblastoma, hepatocellular carcinoma, and rarely encephalopathy [10].

6. Conclusions

Abnormalities in the PVS are more likely to occur along with abnormalities of the ductus venosus due to the connections established during embryology. An important finding from previous research is that the integrity of the PVS during the FT anomaly scan can be achieved. The audit of the ultrasound examination of the PVS may be performed macroscopically in the second trimester of pregnancy and microscopically in the FT, with very few resources and good results. The incidence of UPSVS in a tertiary unit is higher than previously reported but the early detection is feasible, which is important for proper management and prenatal counseling. To understand the abnormalities of the hepatic fetal venous system, it is important to understand its normal embryologic development.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 9

Historic Background and Current Perspectives in Dental Crown Formation

David F. Gómez-Gil, María Camila Orjuela-Vásquez, Manuela Pino-Duque, Angie Pino-Araujo and Juliana Sánchez-Garzón

Abstract

Understanding the cellular principles of odontogenesis requires an incremental and up-to-date understanding of the sequential molecular embryological processes leading to a complete normal dental formation. This topic review provides a state-of-the-art explanation of these dental morphogenetic processes and the subsequent crown development in normal deciduous and permanent teeth, based on an upgraded version of the "odontogenic homeobox code". The description of these processes is shown from the differential epithelium-ectomesenchyme and epithelium-mesenchyme interaction stand-points, necessary to produce cell-cell and extracellular matrix-cell transformations. These cellular processes lead to the sequential stages of classic histological dental formation, which progressively correspond to the development of dental regions, identities, and forms, to obtain complete deciduous and permanent human dentitions.

Keywords: Odontogenesis, Amelogenesis, Dentinogenesis, tooth root/embryology, dental crown formation

1. Introduction

Odontogenesis (dental -or tooth- formation, development, organogenesis or morphogenesis) is the intraoral molecular embryological process in which teeth are formed from specialized embryonic cells until completion of their root formation and eruption [1, 2]. Such formation is a mix of genetically complex processes determined by a series of non-*HOX* homeobox genes, which regulates initial dental histological organization, histodifferentiation, morphodifferentiation, spatial tooth arrangement, amelogenesis, dentinogenesis, periodontal ligament formation and tooth eruption [3]. In this spatial-temporal sequence, the ectodermal cells of the stomodeum are surrounded by the underlying ectomesenchyme -derived from ectoderm-originated cranial neural crest cells- to collaboratively form dental structures, give shape to future dental crowns, and create supporting tooth structures [4]. This sequential process facilitates the formation of specific dental patterns (dental location, structure,

size and shape [type]) and extends until the completion of tooth eruption [1–8]. More than 300 genes are involved in these interactions [8]. The present literature review considers all topics related with the current molecular embryology state-of-the-art on crown formation and gives a baseline to accurately point out critical aspects of dental development that are important in clinical practice.

2. Normal molecular embryology in odontogenesis

As eutherian mammals, humans are heterodonts and diphyodonts [1, 9]. To explain the heterodont origin of teeth, Sharpe (1995) proposed the "odontogenic homeobox code" [10], as a different approach from both Butler's morphogenetic fields (gradient) model (1939–1956) [11–13], and Osborne's clonal model (1978) [14]. Histologically, tooth formation requires simultaneous formation of multiple tissues closely interrelated. Dental patterning is a 3D gradual expression of several and restricted non-*HOX* homeobox genes in specific craniofacial (mandibular and maxillary) ectomesenchymal cell clusters controlled by the same genes expressed inside dental mesenchyme, to develop either uni-, bi- or multicuspid dental patterns [10, 15–17]. This divided tooth formation origin is genetically orchestrated according to the "clock and wavefront" model of vertebrate segmentation [18, 19], currently understood as the "spatio-temporal dynamics of gene expression patterns" [20–22]. Tooth identity interacting genes follow Sharpe's "code", similar to homeobox codes in other developmental systems [10, 15, 16, 23, 24].

The presence of epithelial-ectomesenchymal (EEI) and epithelial-mesenchymal interactions (EMI) through cell-cell and extracellular matrix-cell transformations is essential. Cross-talk between these tissue layers is mediated by diffusible signaling molecules (growth factors), required for normal cell survival and differentiation [25]. These interactions use similar genetic expression cascades in a repetitive manner that depends on several non-*HOX* homeobox genes [10]: *LHX6* and *8, MSX1* and *2, PAX9, RUNX2, DLX1* and *2, BARX1* and *2,* and *ALX3* [3, 24, 26–28]. These genes control fundamental processes of organogenesis by coding transcription factors needed for the formation in dental crown and root of regulatory proteins, cell signaling molecules, and structural proteins essential for multiple cellular processes -proliferation, condensation, adhesion, migration, differentiation and secretion- in the establishment of a totally differentiated tooth [29]. These series of interactions requires the completion of sequential embryological phases, which almost match the histological phases and stages of tooth development: proliferation and cell migration (dental initiation phase: dental lamina-bud stages); cellular differentiation (dental histo -or cyto- differentiation phase: cap-initial bell stages); and formation of specialized dental tissues -enamel and dentin structural protein secretion- (dental morphodifferentiation phase: late bell stage) [26, 30].

A synthesis of the molecular embryological process of tooth crown formation and development appears below, with emphasis on the gene-protein interactions present in each step.

3. Crown formation

3.1 Primary epithelial band (PEB)

This stage, called Dental Placode (DPL) later on, starts when a portion of the ectoderm (future PEB) begins to proliferate in the surroundings of the stomodeum

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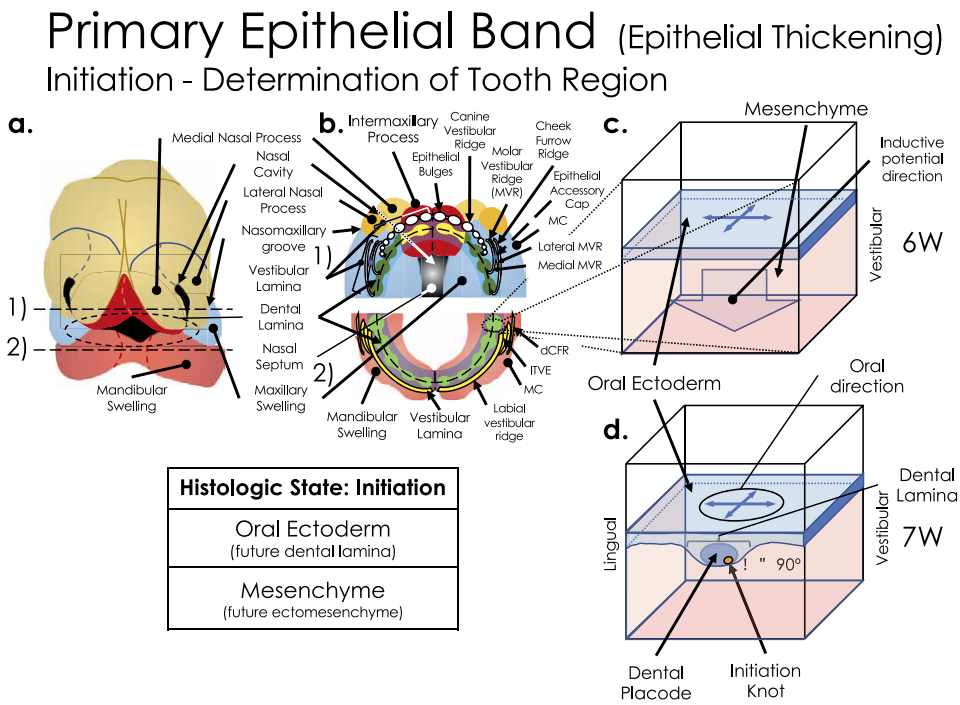

Figure 1.
Primary epithelial band (6-7th IU week) – Deciduous dentition. Embryo head: a. frontal view and b. coronal view: 1) A horizontal maxillary section (formed by maxillary swellings and the intermaxillary process of the medial nasal processes) and 2) a horizontal mandibular section can be identified. Note the different components of vestibular lamina as well as the combined formation of maxillary dental lamina at lateral incisor level (from intermaxillary process and maxillary swellings, respectively); tooth forming site: c. At 6th IU week, the oral ectoderm and mesenchyme starts to differentiate, guided from an inductive potential coming from the ectoderm; d. At 7th IU week, the epithelial band thickens to form the dental placode, containing a transient initiation knot. ITVE: Irregularly thickened vestibular epithelium; dCFR: Differentiated mandibular cheek furrow ridge; MC: Mouth corner.

during the sixth intrauterine (IU) week (**Figure 1**), under FGF8 influence -component of the "clock and wavefront" model that brings periodic pulses of Notch, FGF and WNT signaling [19, 31]. FGF8 acts as a strong inducer of *LHX6* and *8* (formerly *LHX7* or *L3*) gene expression in the embryonic molar region [32]. *LHX* genes encode transcription factors that determine cell fate specification and differentiation in maxillary and mandibular processes' oral ectomesenchyme, palatal shelves and forebrain [32–35].

The PEB invades the underlying mesenchyme, dividing it in Vestibular Lamina (VL) and Dental Lamina (DL). VL generation and cell proliferation is induced by the EGF family (with EGFR internalization) and FGF2 [36]. FGFR1 expressed strongly in both VL and DL/enamel organ [36]. Expression of PCNA (a DNA scaffold for DNA replication proteins) and pRb (an initiator of cell cycle) -intensely localized in VL stratum basale- were found [36]. Both proteins are related with temporal and spatial expression of cytokines and receptors in the PEB, VL, DL/enamel organ, and ectomesenchyme [36]. pRb expression corresponded to CK10 expression in the keratinizing VL [36].

The primary dentition begins to form from the DL inside the embryonic mandibular -first- and maxillary processes in the seventh IU week [30]. At 45–48 IU days, FGFR1 up-regulates pRb to induce cell proliferation, keratocyte differentiation,

and exfoliation of keratinizing VL cells to form the oral vestibule space [36]. Simultaneously, DL -under FGFR1 regulation- forms several partially discontinuous thickenings of oral ectoderm inside the different dental fields (in a dorsal-ventral [molar-first] pattern) [36–38]. At these locations, LHX6 and 8 transcription factors [39, 40] restrict the expression of *GSC* gene to the caudal mesenchyme, establishing the oral–aboral polarity in dental arches [41–43]. These thickenings grow inward in the future dental arch mesenchyme [37], to give origin to the cellular component of deciduous dental germs formation [44]. At this point, a shift of inductive potential from the oral ectoderm to the underlying ectomesenchyme occurs [37].

In the ectomesenchyme, *BARX1* gene is expressed in restricted areas of head and neck mesenchyme [45, 46]. Molar tooth papillae -derived from ectomesenchyme- form under its expression [45]. *GLI1* gene (from the SHH signaling pathway) is also expressed [47–49]. The mesenchymal induction of two members of the TGFβ family -BMP4 and Activin βA- induce overlying epithelial cells to form the DPL [49].

Several subsets of cells inside the DPL become quiescent and form a series of early signaling centers -Initiation Knots (IK's)-. These centers govern epithelial budding and folding, and adjacent ectomesenchyme condensation [9, 50]. These cells start to migrate centripetally and condense to form a non-proliferative mature signaling center. Soon after, bud formation begins with a burst of epithelial cell proliferation in the surroundings of, and oriented away, from the IK's [50], followed by centripetal cell movement and cell intercalation that possibilitate cell rearrangement inside the bud [51]. Tooth bud size and the number of IK cells are regulated by the EDA/EDAR/EDARADD pathway [50, 52]. Among the genes expressing inductive signals from these DPL non-proliferative cells clusters are *p21*, *SHH, EDAR, DKK4, FGF4*, and *FGF20* [53–60]. Cell cycle regulatory protein p21 mediates growth arrest at specific stages in the cell cycle by binding to and inhibiting cell division control proteins CDK1 [61, 62], and CDK2 [63]. The levels of EDA (mainly) and FGF signaling -present both in DPL (FGF4 and 9) and newly formed ectomesenchyme (FGF3)- help defining tooth number [32, 64–68]. Epithelial expression of FGFR2c, FGF4 and 9 regulates mesenchymal proliferation, while epithelial proliferation is promoted by mesenchymal expression of FGF3, 10 (both important in dental morphogenesis [tooth number] -mostly FGF3- and tooth size) and FGFR2b. In addition to FGF signaling, TWIST1 modulates FGF signaling within DPL and ectomesenchyme, defining tooth germ size and cusp formation [69].

3.2 Dental germ (bud) (DG)

By the eighth IU week (**Figure 2**), the odontogenic epithelium located in the innermost region of the DL enters a mitosis phase in aboral direction (away from the oral cavity and inside future mandibular and maxillary bones) and begins to invade the subjacent mesenchyme, forming outbreaks of identical cells (epithelial or dental germs -DGs-) that will shape all enamel organs [39]. Each DG is connected to the DL through a pedicle -gubernaculum dentis or gubernacular canal- [70]. The DG's and ectomesenchyme are separated at this stage by a basement membrane [71]. Cell cycle regulatory proteins CCNA2 -from *CCNA2* gene, which form a complex with CDK2-, Cyclin D1 -encoded by *CCND1* gene-, and Ki-67 -encoded by *MKI67* gene- in molar dental epithelial and mesenchymal cells, and Ki-67 and Cyclin A2 (in lesser amount) in both incisors' EO and dental mesenchyme (with some Ki-67 negative cells in the EO bulbous protrusion) start to be expressed at this time [72]. A Cyclin D1-CDK complex formation (with CDK2, which compete with canonical CDK4 or 6 [73])

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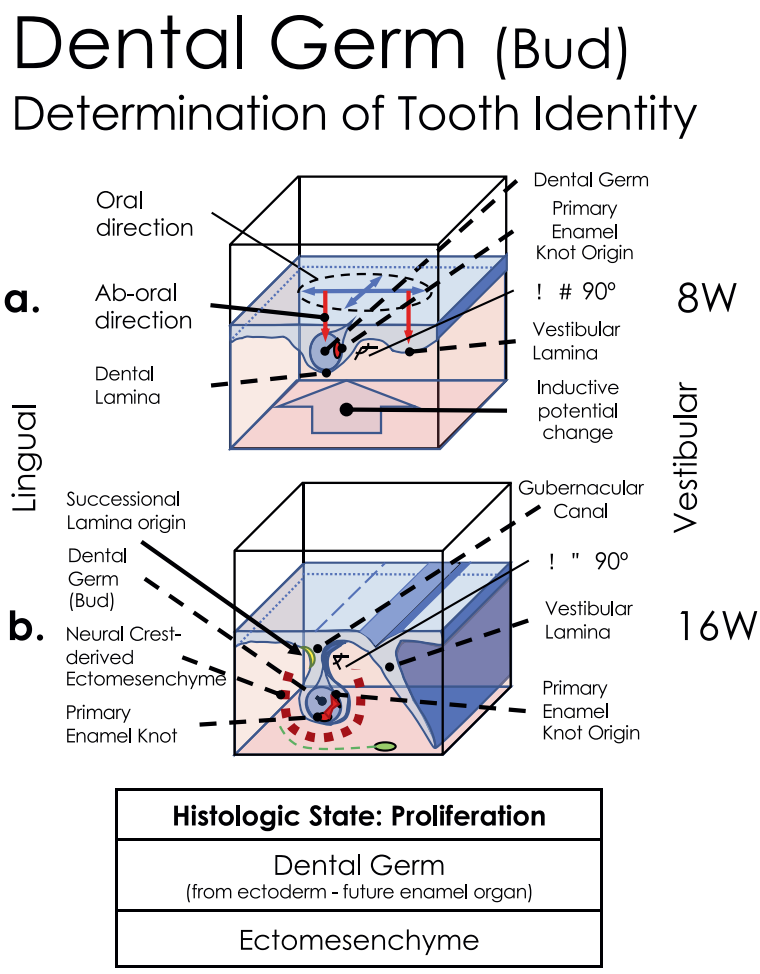


Figure 2.
Dental germ (8-16th IU week) – Deciduous dentition. Tooth forming site: a. At 8th IU week, the dental placode starts to grow in specific points in an aboral direction to form dental germs, due to local changes in the expression of PAX9, MSX1–2, DLX2–5, SHH, and cell cycle regulatory proteins. The forming ectomesenchyme also expresses MSX1–2 (regulated by PAX9), DLX1–2 (molar region), BARX1 (molars and bicuspids), and GLI2 and ALX3 (incisor region). At this point, a change in the direction of the inductive potential takes place; b. At 16th IU week, the dental germ is fully formed, containing a definitive enamel knot. Ectomesenchymal cells surrounding the dental germ start to condense to form the future dental papilla. A successional lamina appears lingual to the dental germ to give rise to the permanent tooth replacement.

is needed to regulate transition from G1 to S phase [72]. However, upregulation of p21 slightly precedes the cessation of cell proliferation of the forming Enamel Knot (EK -cell cluster located in the center of each internal enamel epithelium-) by CDK2 inhibition [55].

Several key genes also initiate expression. *PAX9* gene (encoding PAX9 transcription factor) is a key control element during odontogenesis initiation. It is specifically expressed in all prospective tooth sites before any morphological odontogenetical signs [2, 5–8, 26, 29, 30, 74–79]. *MSX1* and *2* genes encode regulatory proteins that act as transcriptional repressors inside the cell nucleus [80]. *MSX1* gene is related with the development of ectodermal derivatives, with a strong expression in dental mesenchyme [1, 2, 4–8, 10, 26, 29, 30, 74–79]. *MSX2* gene also participates from the

onset of odontogenesis in both oral epithelium and ectomesenchyme [81]. In order to regulate their effects, MSX homeoproteins form inactive homodimers or heterodimers with DLX homeoproteins (2 and 5) [82–84]. PAX9 homeoprotein has a genetic epistasis with MSX1 protein, being able to directly regulate *MSX1* gene expression and interact with MSX1 protein to enhance its ability to transactivate *MSX1* and mesenchymal *BMP4* gene expression during tooth development [83, 84]. Both genes are regulated by OSR2 transcription factor, responsible of downregulating tooth number [85]. *SHH* gene also participates in epithelial cell proliferation involved in tooth bud formation [86]. In addition, *AXIN2* gene expression was observed in epithelium and mesenchyme of mandible, maxilla and lateral nasal processes. *AXIN2* expression in the incisors and molar buds is located in spherical cell balls at the anterior part of the tooth bud where the EK's form, as well as in the surrounding mesenchyme [87].

Simultaneously, ectomesenchyme condensation surrounding these ectodermal cell bursts express several transcription factors, which differentiate the forming individual condensations from the rest of mesenchyme [88]. *DLX1* and *2* gene expression is found in proximal ectomesenchymal regions where molar teeth are located [15]. *GLI2* gene is responsible of maxillary incisors normal development, while *GLI3* gene is responsible of molars and mandibular incisors size, and maxillary incisors development at the epithelial thickening stage [86]. *PITX2* gene acts as an exclusive marker for developing tooth epithelium and is involved in tooth orientation regulation [25, 89–92], BMP4 restrict both *PAX9* expression to the dental mesenchyme and *PITX2* expression to the dental epithelium [93].

The DG cells also produce glycosaminglycans [94]. Versican (coded by *VCAN* gene) V0/V1 is involved in cell proliferation, while V2 isoform is associated with histodifferentiation phase [94]. These two molecules act as scaffolds for extracellular matrix formation [95], and in EEI in early dentinogenesis [96]. Several glycoproteins such as TN-C -that modulate with anti-adherent effects the cell-matrix interactions [97, 98]- and TN-X -a TN-C complementary protein [99, 100]- are produced also. TN-C is found widely in loose connective tissue, with a regulating role in collagen deposition and indirect binding and bridging of these fibrils [97, 99, 101]. FN, collagen type III, and other chondroitin sulfates are also found [102]. CPNE7 expression -important in EMI- is also seen in dental epithelium [103]. This variation in intracellular molecular contents causes cell turgor and marks the initiation of DG histodifferentiation phase [104]. Trigeminal nerve fibers first appear in the DF at late DG stage. Some nerve fibers from PRPH-reactive neurons are located within tooth germ ectomesenchymal cells [105].

When fully formed around the 16th IU week, deciduous DG's are composed by ectodermal cells groups that will differentiate into future Enamel Organs (EO's), surrounding condensed ectomesenchyme cells that give shape to Dental Papillae (DPa's). These structures will form enamel (from EO), dentin and pulp (from DPa). The mesenchyme surrounding both cell groups is called Dental Follicle (DF) or Sac. This cell group further differentiates in alveolar bone, cementum and periodontal ligament [106].

PAX9 and *MSX1-2* are among several dental patterning genes found in dental mesenchyme [6, 107–109]. Other genes are *DLX1-2* -particularly expressed in maxillary primary molar mesenchyme where they play an important role in maxillary primary molar tooth patterning [15, 23], *BARX1* -involved in multicuspal mesenchymal morphogenesis (molars and bicuspids) [46, 110], and *ALX3* –coding a transcriptional regulator involved in cell-type differentiation and development [24]. Using a different animal model from McCollum and Sharpe [27, 28], Wakamatsu et al. [24] used

4-tooth-classes animal species (opossum and ferret) to affirm that dental formula differences in humans (that follow the "4-tooth-classes" pattern) were due to a large overlap of the proximal part of the *MSX1* expression and most of the *BARX1* domain in the middle of the mandible primordium [24]. According with their view, *MSX1/BARX1* positive domains correspond to an FGF-dependent activation of *MSX1* overlapping with *BARX1*, which partially produces bicuspid differentiation. Maxillary and mandibular canine correspond to *MSX1*-positive region, and incisors correspond with *MSX1*/*ALX3* positive region, respectively (**Figure 3**) [24].

Formation of permanent dentition up to second bicuspids started with the derivation of lingual groups of cells from the deciduous enamel organs at the time of deciduous morphodifferentiation (between the 10th and 13th IU week), forming a successional DL under the influence of *SOX2* gene [9, 75]. These lingual cell clusters at each deciduous tooth constitute the successional permanent teeth [76]. The formation of the permanent molars, or additional permanent teeth, originates from the expansion of a subepithelial continual (or additional) DL that comes off the last deciduous DG [14, 76]. Permanent molars represents members of the primary dentition without successional replacement that show later development and eruption [111]. From these two origins, the permanent DG's start to form around the 3–4 months of age and extend their initial formation until the early teen years, when the 3rd molars begin to form (**Figure 3**) [30].

3.3 Dental cap (DC)

The DG invaginates towards the aboral direction (**Figure 4**), forming a cap-like structure -the EO- that begins to encapsulate the subjacent mesenchyme [71]. This bud-to-cap transition -regulated by FGF3 and RUNX2 [112]- constitutes an epithelial folding deformation phenomenon, fundamental for the differentiation and specialization of dental cell types and the definition of final tooth size and shape, as the dental epithelium elongates and the underlying mesenchyme grows [71].

The epithelial cells of the deepest part in the internal portion of the EO -preameloblasts- begin their histodifferentiation with the formation of the EK's [113–115] -significant non-proliferating tooth signaling centers [50, 116], under the influence of mesenchymal BMP4 [112]. EK's are not derived from the early DPL signaling center, but rather they form de novo at the inner tip of the cap [50]. They are divided into primary EK's -appearing at this stage- and secondary EK's -developing during bell stage- [112, 116]. The number of EK's determine the number of cusps [1, 24, 117, 118] (**Figure 4a** and **b**). EK signaling controls reciprocal EEI -responsible for EK maintenance and morphogenesis-, with proliferation and movement of surrounding EO cells and ectomesenchyme during folding and patterning of dental epithelium and cusps [2, 24, 49, 56, 112]. p21 (protein associated with cell exit from the cell cycle) and Cyclin D1 (a cell cycle enzyme coded by the *CCND1* gene, regulated positively by Rb) -both from cell proliferation-regulating genes-, elongate the cell cycle at G0 phase in the EK [72, 116]. Molar EK cells only express Cyclin D1, while stronger localization of this enzyme and other DNA regulatory genes are found in incisors' dental epithelium compared with dental mesenchyme at this stage [72]. EK's also express in an encapsulated pattern *MSX2* –coding a transcription factor that organize normal cusp generation- plus several extracellular signal molecules belonging to four protein families (BMP, HH, FGF, and WNT), including BMP2, −4 and − 7, SHH, FGF3, −4, −9 and − 20, and WNT3, −10a and -10b [5, 49, 53, 56, 112]. These molecules (mainly FGF4 and WNT10a) and other signal molecules (like CPNE7) are the first inductive

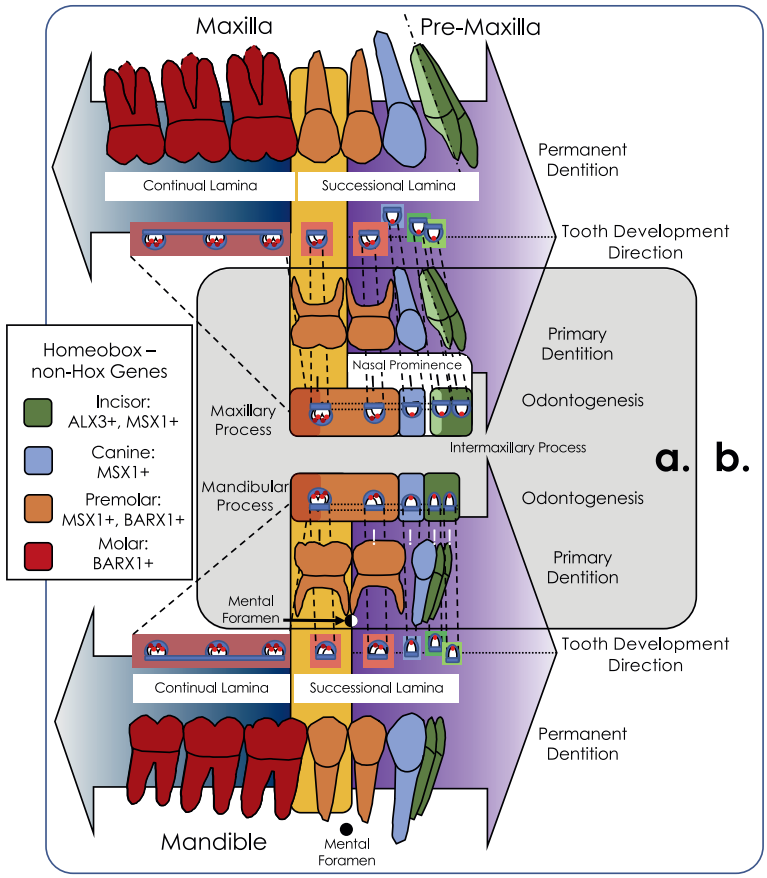


Figure 3.
Homeobox non-Hox genes involved in the development of primary, continual and successional dental laminae. a. Starting at second deciduous molar dental germ level, primary dentition develops from the dental lamina in a distal-to-mesial pattern; b. permanent dentition develops from two different origins: The successional lamina -lingual extension of each deciduous dental germ for incisors, canine and bicuspids-, and the continual lamina -a distal extension of the deciduous second molar germ that gives origin to permanent molars-. The genes involved vary according with the level of dentition they belong to (incisor, canine, bicuspid [primary molar], molar), respectively. (modified from Juuri and Balic [77], Wakamatsu et al., [24]).

signals for pre-odontoblasts differentiation and primordia of other dental pulp cells, normal cusp growth, and patterns of cusp formation that give rise to different dental morphologies in the initial bell stage [1, 30, 56, 103]. Particularly, CPNE7 induces the differentiation of mesenchymal cells (of dental or non-dental origin) into pre-odontoblasts and odontoblasts through EMI [103].

Expression of *LEF1* was also shown in EK's and dental ectomesenchyme at DC and DB stages [116]. This pleiotropic gene codes a member of the LEF/TCF family of transcription factors. It forms a transcriptional regulatory complex with β-catenin to control WNT signaling-mediated cell survival, intercellular adhesion, cell proliferation, pattern formation, axis specification, and cell fate determination -by regulating the cell cycle progression in progenitor cells- [119–127]. In tooth EEI, LEF1 -under direct mediation by BMP4 and WNT10a and b- induce epithelial FGF4 expression, which regulates the induction of mesenchymal FGF3. Reciprocally, FGF3 -and possibly BMP4- induce expression of SHH in the epithelium [119, 128, 129].

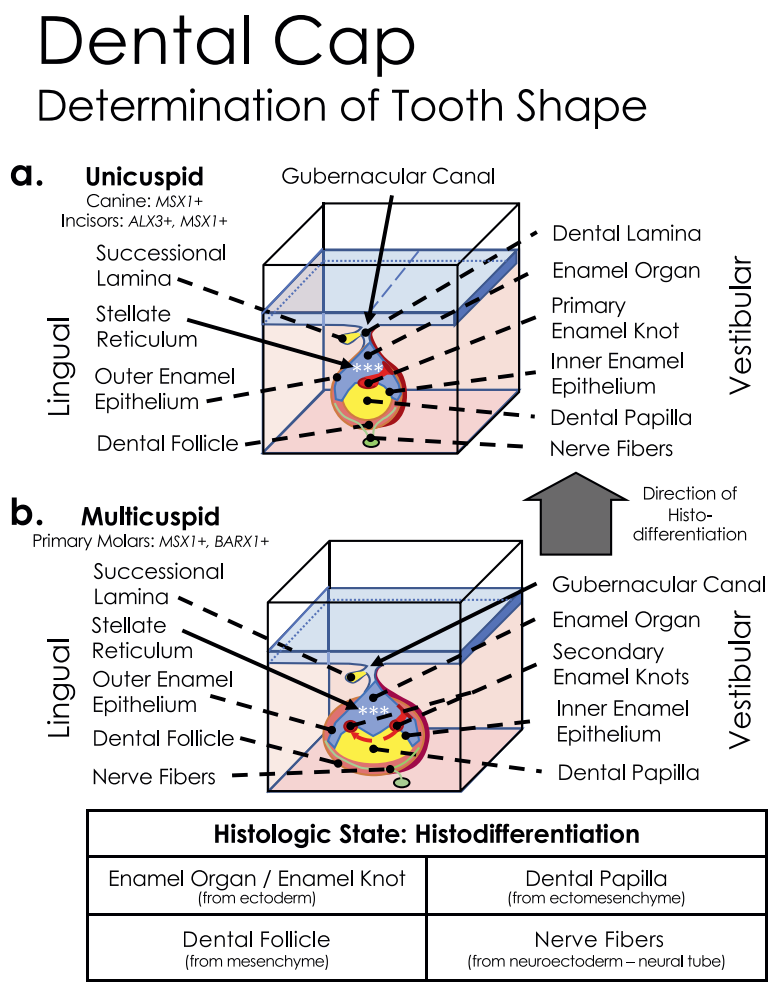


Figure 4.
Dental cap – Deciduous dentition. Tooth forming site: a. Unicuspid tooth formation: After being submitted to an epithelial folding deformation phenomenon -under epithelial FGF3/RUNX2 control-, the dental germ invaginates in an aboral direction and forms the enamel organ and the enamel knot -under epithelial SHH and mesenchymal BMP4-. The ectomesenchyme is partially surrounded by it, assuming the role of dental papilla. Pre-odontoblasts start expression of type I collagen and NCP's genes; b. Multicuspid tooth formation: The primary enamel knot further divides in secondary enamel knots at early bell stage to define the number of cusps.

In addition, it induces the ectomesenchyme competency to form the DPa -a hallmark of the DC stage- [128–130]. It is expressed in crest cells and ectomesenchyme-derived structures during embryogenesis and is critical for EK cell survival [119]. SHH signaling from EK is needed for cervical loop growth and tooth crown patterning regulation during early DB stage, by initiating secondary EK's -expressing almost the same signal molecules as the primary EK's- [112].

The pre-odontoblasts subsequently express *COL1A1* and *COL1A2* genes (which encode the amino-acid chains of type I collagen), whose future constituent protein forms almost 90% of the organic matrix of dentin. In addition, they also express non-collagenous proteins (NCP's) genes such as *DSPP* (which encodes dentin sialophosphoprotein -DSPP-, belonging to the Small Integrin-Binding LIgand Nitrogenous Glycoproteins [SIBLING] family [131]). DSPP pre-protein is secreted

and proteolytically processed in several functional domains to generate DSP, DPP, DGP, and BSP. These proteins perform different functions in dentinal mineralization [106, 131]. Other NCP's include DMP1, SPARC, OPN (from *SPP1* -Secreted Phospho-Protein 1- gene), MEPE (from *MEPE* gene), decorin (encoded by *DCN* gene), biglycan (from *BGN* gene) and two vitamin K-dependent calcium binding proteins of the extracellular bone matrix [132]: MGP (from *MGP* gene) [133, 134], and OSTCN (from *BGLAP* gene). EK apoptosis is the final stage of an EK cell, where induction of p21 and EDAR by BMP4 inhibits EK cell proliferation between G1 and S phase and makes cells responsive to EDA, expressed in the flanking epithelium of the tooth bud [55, 112, 135]. EDA/EDAR/EDARADD signaling regulates the formation and the signaling activity of the EK [64, 112].

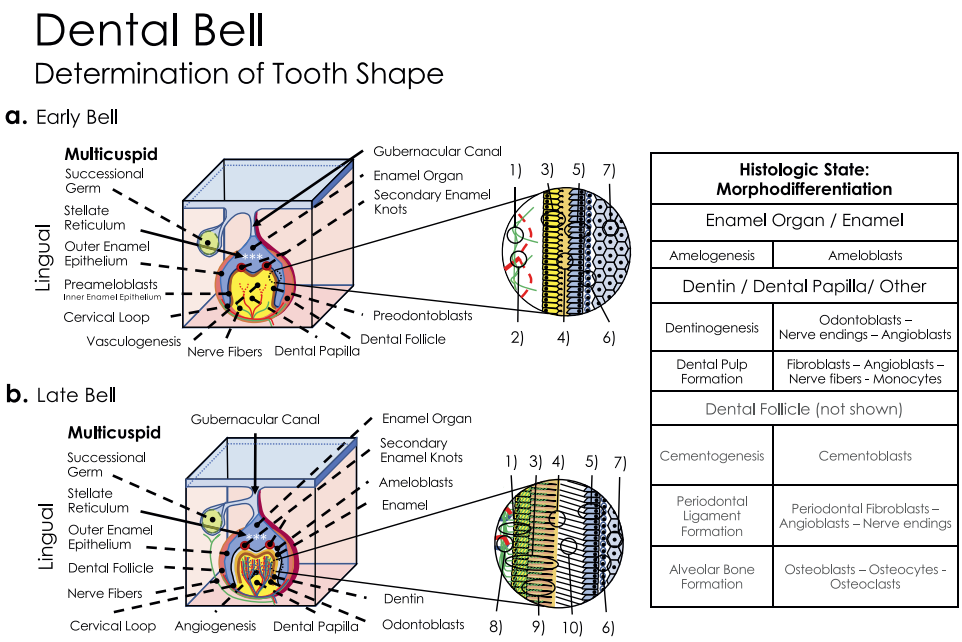
Two theories (differentiation and concrescence) explain how these centers can form multicuspid coronal patterns [30]. The theory of differentiation (Cope-Osborn or trituberculate theory) is based on 19th century paleontological and anatomical comparative findings [136, 137]. Phylogenetically, complex multicuspid teeth (initially tricuspid) evolved by differentiation of a unicuspid tooth [30]. The phylogenetic decrease in the number of teeth involved the deletion of the simplest distal teeth, while the remaining teeth underwent further differentiation to produce multicuspid crowns [132, 136, 137]. Osborn et al. (1972-1975), [138] (a different first author, who also proposed the Clone theory of dental formation [14]) stated that this decrease in number involved the deletion of the simplest distal teeth, while the remaining teeth underwent further differentiation to produce multicuspid crowns [138–143]. Also originated in the 19th century, the theory of concrescence (theory of the dimer [Bolk] or of the integrated development) is supported by the dental embryological development that occurs during ontogenesis in different species [30, 144, 145]. According to this theory (partially described by Kükenthal, 1892 and summarized previously to Bolk's work by Adloff, 1916), multicuspid teeth in mammals evolved through the integration of several individual dental primordia (similar to that of reptiles) [30]. This process would integrate two lines of dental primordia in the longitudinal (buccal) and medial (lingual) directions, to form more complex multiple molars. This concrescence of the dental primordia may be accompanied by a shortening of the jaws [137]. It is different from the better-known definition of dental concrescence ("fusion" of adult tooth roots by cementum) [146]. While the concrescence theory explains the multicuspid dental development process, the differentiation theory describes its outcome in adult dental morphology [30].

During the bud-to-cap formation process, the EK's create a spatial deformational anisotropy along the buccal-lingual axis with minimal volume growth, while the remaining epithelial EO cells grow at a higher rate. This epithelial growth increases the internal pressure around the EK's, in addition to the mechanical restrictions occurring at the epithelium-mesenchyme interface. This change might induce the buccal-lingual flattening of tooth germs during cap stage [71].

3.4 Dental bell (DB)

At this stage the future shape of the crown is determined (**Figure 5**). The DC begins to develop a bone crypt within the ossification zone. The EO acquires the shape of a bell due to the folding and complete cellular differentiation of the internal epithelium of this structure. The DF delimits the external portion of the bell, while the DPa is located towards its internal portion [147].

The DB presents two phases from the histological point of view:



Histologic State: Morphodifferentiation	
Enamel Organ / Enamel	
Amelogenesis	Ameloblasts
Dentin / Dental Papilla/ Other	
Dentinogenesis	Odontoblasts – Nerve endings – Angioblasts
Dental Pulp Formation	Fibroblasts – Angioblasts – Nerve fibers - Monocytes
Dental Follicle (not shown)	
Cementogenesis	Cementoblasts
Periodontal Ligament Formation	Periodontal Fibroblasts – Angioblasts – Nerve endings
Alveolar Bone Formation	Osteoblasts – Osteocytes - Osteoclasts

Figure 5.
Dental bell – Deciduous dentition. Tooth forming site - Multicuspid tooth formation: a. early bell stage: Starting at the enamel knots level, the newly formed odontoblasts begin to delay mantle dentin. Vasculogenesis is active as well as the growth of nerve fibers. A highly stratified enamel organ can be seen, but no morphodifferentiation changes are visible yet; b. late bell stage: As the delay of dentin and enamel is in process, nerve fibers start to get in contact with odontoblasts to establish the dual role of odontoblasts in tooth formation as both mineralized tissue forming cell and tissue specific neuronal ending. Vascular supply is exquisite for the development of mineralized dentin at this point. 1) nerve fibers; 2) Vasculogenesis; 3) odontoblasts; 4) mantle dentin; 5) Ameloblasts; 6) SI cell; 7) SR cell; 8) angiogenesis; 9) dentin; 10) enamel.

3.4.1 Initial or early DB

This phase continues from the histodifferentiation in the DC stage towards enamel and dentin formation in the DB stage (**Figure 5a**). Histologically, the EO is stratified in four groups [148]:

1. Outer Enamel Epithelium (OEE): cube-shaped epithelial cells located on the periphery of the enamel organ. It is separated from the ectomesenchyme by a basement membrane. These cells express *SLCO4a1* (coding an anion membrane transporter in cell junctions, involved in the transport of sugars and organic acids, metal ions, amine compounds, and estrogen), *TH* (coding the enzyme tyrosine hydroxylase, which takes part of the pathway that produces catechol-amines -group of hormones including dopamine, norepinephrine and epineph-rine-) and *AMER1* (coding a regulator protein of the WNT signaling pathway -Adenomatous Polyposis Coli [APC] protein- during cell division and move-ment) genes, with OEE progenitor gene expression of *FOS* (Fos proto-oncogene or AP-1 Transcription Factor Subunit - coding a leucine zipper protein that dimerize with JUN proteins to form the transcription factor AP-1, a regulator of cell proliferation, differentiation, transformation and cell death), *EGR1* (Early Growth Response 1 – also a regulator of cell survival, proliferation and death), and *VRTN* (coding vertebrae development associated protein -or vertnin-, which

act as a transcriptional repressor functioning independently but in coordination with canonic WNT signaling to regulate dorsal-ventral patterning) [149, 150].

2. Stellate Reticulum (SR): central portion of the enamel organ with turgor cells joined by desmosomes. These cells are marked by *VAT1L, FAM19A4*, and *HEY2* [149].

3. Stratum Intermedium (SI): Two or three layers of flat alkaline-phosphatase-producing cells that are needed for enamel mineralization, marked either by *RAB3IL1, PMCH*, and *CYP2S1* or *PSMB10, C1QB*, and *IBSP* [149]. There is a third layer of cuboidal SI cell that contains *THBD, GNRH1*, and *JPH4* gene expression [149]. The SI progenitors are marked by *CDH6, LRP11* and *CPNE5* genes [149].

4. Internal Enamel Epithelium (IEE): cylindrical cells that differentiate into ameloblasts -enamel-producing cells-. This epithelium has an internal basal portion of undifferentiated mesenchymal cells, followed by another cell layer closer to the epithelial surface composed of pre-ameloblasts -elongated cells in ameloblastic differentiation, marked by *COL22A1, VWDE, KIF5C* gene expression-.

An additional cellular region is identifiable in the apical side of the EO at this stage: the Cervical Loop (CL) (after Diamond and Weinmann, [unpublished data]) [151] (precursor of the Hertwig's Epithelial Root Sheath - HERS). This transient structure is formed at the union of both IEE and OEE, and is composed on its central portion of loosely aggregated SR surrounded by SI [152]. Currently, there has been an agreement on the stages of development inside this epithelial zone. While CL refers to an initial enamel-forming phase inside this ectodermal structure, HERS is established when an EMI occurs in the OEE cellular component during dental root formation to give rise to cementoblasts [152]. HERS and its remnants (Malassez' epithelial rests) are involved in the root formation process. HERS apical growth and subsequent cementoblast transformation splits root dentine from the incipient periodontal ligament [152].

Regulation of the programmed EK and IEE cell-cycle phase-timing controls tooth morphology [116]. Secondary EK's follow a precise sequence to determine sites where the epithelial sheet folds and cusp' development starts, with mechanisms of lateral inhibition and central on-off signaling [112]. Their development is regulated by reiterative signals from primary EK's, previously formed secondary EK's, and mesenchymal signals that code different tooth morphologies through an intricated gene expression network [112]. These histodifferentiation changes in crown formation are mediated by various transcription factors (regulatory proteins such as PITX2, LEF1, MSX1, PAX9 and with less effect DLX1-2 and GLI2-3) and extracellular signal molecules/receptors (AXIN1–2; WNT3, –7b, and 10a-b; EDA-EDAR-EDARADD; BMP2,4, and 7; FGF1,2,4, and 8; SHH; HGF; PTC1; SLIT2; and SMO) [2, 4, 8].

Ectomesenchymal BMP4 signaling -expressed after DPa WNT/ß-catenin signaling [92, 153]- is fundamental for ameloblast differentiation [154]. EPFR -a Krüppel-like family (KLF) transcription factor expressed in ameloblast- and odontoblast-lineage cells [155]-, also produces enhanced ameloblast WNT/ß-catenin and BMP4 expression and induces intranuclear ß-catenin accumulation and formation of cell junctions during predifferentiation bell stages [156]. CPNE7 protein expression also raises in DPa ectomesenchymal cells at this stage [103].

3.4.2 Late or advanced DB

In this phase, changes occur in morphodifferentiation for the formation of enamel, dentin, cementum, periodontal ligament, and alveolar bone (**Figure 5b**). At crown formation stage, CPNE7 is localized in differentiating odontoblasts and odontoblast processes during dentinogenesis [157]. It initiates dentinal hydroxyapatite nucleation, promotes the formation of dentinal tubule-like structures, and stimulates obliteration of dentinal tubules due to its calcium-binding properties [157, 158]. CPNE7 expression is lost when odontoblasts reach full differentiation [103].

Afterwards, odontoblasts produce a new cascade of transcription factors associated with the production of structural dentin proteins (DSPP, DMP1, BSP, OPN, collagen I, III, and V) [2, 4, 8], initially creating a relatively atubular and hypomineralized outer mantle dentin (15– 30 μm thick, without or with a very low content of dentinal tubules) at the dentin-enamel junction (DEJ) [159]. This layer serves to dissipate pressures and forces concentrated in the DEJ [159]. Crown mantle dentin contains phosphorylated proteins in underphosphorylated or non-phosphorylated forms, with particular elastic properties and resilience [159, 160]. This characteristic is due to different NCP's found in this layer compared with circumpulpal dentin [159]. The deposition of mantle dentin acts as a stimulus for IEE cells of the EK's to differentiate into ameloblasts and start producing structural EMP's (Enamel Matrix Proteins) organic matrix. EMP's are divided in amelogenins (90%) and non-amelogenin proteins (remaining 10%). AMEL is formed from two gene isoforms: *AMELX* (90% of the amelogenin) and *AMELY* (remaining 10%). The remaining EMP's are: AMBN (secreted by odontoblasts and HERS cells) involved in regulation of ameloblast adhesion, proliferation, differentiation, enamel mineralization and structural organization [161]; ENAM, necessary for ameloblast adhesion to enamel, crystal elongation and regulation of mineral formation; AMTN, involved in the maturation and hardness of enamel; and TUFT1, implicated in enamel mineralization [2, 4, 8, 162–164]. EMP's will shape the nanocrystalline hydroxyapatite that make up the enamel crystals. EPFN and MSX2 participate conjointly in a network of transcription factors against FST -inhibitor of TGFβ that regulate ameloblast differentiation-, controlling ameloblast life cycle and amelogenesis at later stages [115].

Then, the odontoblasts' dentinal extensions initiate dentin secretion. Only at these locations inside forming teeth, polarized odontoblasts start to produce membrane protrusions that detach and form matrix vesicles, which contribute to initial dentin mineralization [165, 166]. Dentin is deposited concentrically around odontoblastic extensions -dentinal tubules- to form peritubular dentin. Intertubular dentin is deposited between the tubules. After this step, the rest of dentin and enamel layers begin to be deposited by apposition [2, 147].

Enamel formation is divided in secretory and maturational phases. In the first phase, ameloblasts secrete EMP's and favors the organized formation of very long apatite crystals, forming the full thickness of a partially mineralized enamel [163, 167]. To reach its final hardness during the maturation stage, most of the EMP's are disassembled by three enzymes secreted by ameloblasts as the crystal growth continues: MMP20, KLK4 and AMTN. While MMP20 inactivates EMP's during the secretory stage, KLK4 totally breaks down the remaining EMP's during maturation phase [163, 167]. The possible AMTN role is the engagement in proteolytic processing of the enamel matrix, similar to KLK4 [164]. After being removed during dental differentiation, a thin basal-like layer at the interface between the apical cell membranes of ameloblasts and the surface of maturing enamel is reformed (and named enamel

matrix). It is composed mainly from LN5 [168, 169], together with glycoconjugates and highly glycosylated components that constitute a differential hallmark from typical basal laminae [170, 171]. LN5 is encoded by *LAMA5* (or *3*) (found in dental epithelial basal membrane [172]), *LAMB3* (found at basal and apical sides of enamel-secreting ameloblasts of the first molar [173]), and *LAMC2* genes [172, 174, 175]. LN5 forms a protein complex localized in the anchoring filaments underneath the hemidesmosomes [176, 177], which participate in cell-basement membrane adhesion [178, 179]. LN5 is normally required for cell migration and differentiation, and is essential for epithelial morphogenesis, hemidesmosome assembly and stability [174, 180, 181]. In developing teeth, LN5 is expressed by functional ameloblasts, before the initiation of final enamel mineralization and hardening (maturation stage) by cyclic modulations [173].

The DPa at this point is starting to convert in a loose connective tissue -containing cells, blood vessels, lymphatics, and nerve fibers-. There is a common and abundant vascular supply that irrigates in a loop-form not only the DPa (future dental pulp), but also the DF (future periodontium and alveolar bone marrow) [182]. A normal blood supply system in the developing tooth guarantees adequate gas exchange, nutrient supply, and waste removal [183]. The development of a mature vascular system -capillaries, arterioles, arteries, and veins- requires fine regulation among hemangioblasts (a mesenchymal stem cell type) and their successors -angioblasts and vascular endothelial cells (EC's), and mural (supporting) cells such as pericytes and vascular smooth muscle cells (VSMC's)- [184]. For this purpose, an EMI is needed to initiate dental germ vasculogenesis. Developmental vasculogenesis is the formation of new blood vessels during embryogenesis from angioblasts (endothelial precursors cells or hemangioblast-derived progenitor cells, a division of splanchnic -visceral- or splanchnopleuric mesoderm, part of lateral mesoderm) at the third IU week [183–185].

Early in embryogenesis, hemangioblasts (without a lumen) are induced by FGF1 and 2 -on a VEGF-dependent manner through stimulation of VEGF expression and upregulation of VEGFR2 signaling [186]- to migrate from the splanchnopleuric mesoderm into the head region, differentiate in angioblasts and organize to form a primitive capillary vascular network to supply the developing brain [183, 184]. Angioblast differentiation depends on paracrine signaling in part due to VEGF and VEGFR1 [183]. Simultaneously, FGF2 and PDGF-BB act synergistically to form stable vessels even after the decrease of angiogenic factors. Mural cells (particularly VSMC) around newformed microvessels have abundant PDGF receptor (PDGFR) protein expression and demonstrate potent PDGF signaling response (to PDGF-BB) to promote mural cell recruitment and maturation [186].

Angioblasts are the first mesenchymal (mesodermally-derived) cells to reach the developing DPa at late DC stage [184, 187]. Vasculogenesis of the dental germ starts at early DB [183]. Migrating angioblasts inside DPa mature to create a network of EC's, aggregate and invade it to form new dental blood vessels with a basement membrane, which provide DPa blood supply [183, 187]. Thin terminal arterioles -and venules, compared with their lumen- enter the pulp, with few collateral circulation [182]. Terminal pulpal capillaries (diameter up to 30 μm), forming a rough and thin vascular network, were observed in the inner region of the DPa of the upper first molar germ at the early DB stage before ectomesenchymal histodifferentiation [188].

Oxygen diffusion to support good oxygen interchange with DPa cells depends on the presence of a vascular network at 200 μm maximum [189]. Oxygen delivery to tissues depends on three conditions: O_2 availability, O_2 arterial blood transportation (O_2% -ca [capacity]-O_2- or O_2 content), and tissue perfusion [190]. In addition, ca-O_2 blood characteristics are determined by the pressure of inspired O_2, ventilation

and gas exchange, the Hb concentration and its affinity for O_2. The O_2 blood carrying capacity is 20 ml of O_2/100 ml of blood [190]. The O_2 saturation (sa-O_2) -% of Hb binding sites filled by O_2 and related with O_2 partial arterial pressure (p-O_2), or O_2 tension- is between 96 and 98% in blood but can vary among tissues [190]. Hypoxia -deprivation of oxygen (and nutrients) by inadequate diffusion at greater distances- is the driving force of vasculogenesis [183]. This event improves VEGF (A -mainly- and B) and HIF1*α* (proangiogenic factors) expression in hypoxic DPSC's and dental pulp fibroblasts (DPF's), which act on adjacent EC's to promote endothelial precursor invasion on DPa [191]. VEGFA stimulates endothelial cell proliferation by binding to VEGFR2. VEGFA increases pulpal blood flow, capillary hyperpermeability [192], and releases ANG's and other angiogenic factors [193, 194]. Either ANG1 or 2 bind competitively to TIE2 receptor to exert their effects [185]. TIE2 activation of EC's requires ANG1 binding [195], while ANG2 (mostly expressed on EC's) acts as their inhibitor [196].

After odontoblast histodifferentiation but before dentin deposition, the terminal pulpal capillaries gradually approach odontoblasts. The terminal capillary network increased in extent, cover all pulpal horns, their density increase and capillary diameter decrease up to 20 μm [188]. At later developmental stages, new blood vessels are mainly formed by angiogenesis -sprouting of new blood vessels from existing vasculature [185]. At late DB, 10-μm terminal pulpal capillaries invade the odontoblastic layer when DB morphodifferentiation starts in the cuspal region. The capillaries create an irregular vascular network surface with many low-capillary loops facing the predentin, until around 5 μm from it [188]. At this point, the dense network decreases the capillaries diameter to 5 μm [188].

Neuronal cells play an organogenetic regulatory role in addition to their tooth innervation function during embryonic development [105]. Dental pulpal afferent fibers from trigeminal (semilunar or Gasser) ganglion provide mainly dentition nociception in injury or infection [197], and mechanoception [198–200]. Axon guidance and pulpal innervation patterning parallel tooth formation process, and depends on a tight spatiotemporal regulation guided by chemoattraction and chemorepulsion [197].

The first axons reach the deciduous mandibular first molar germ at DC stage [201]. Chemorepulsive signals such as SEMA3A (and its NRP1-PLXNA class receptor complex) inhibit the entrance of axons by its axon repulsion and growth cone collapse properties [202]. Ramification of nerve fibers in the DF base and presence of some peripherin-reactive neuronal cells next to DL and oral epithelium at the buccal side of the tooth germs are recognized [105]. Axons do not enter DPa until final crown shape is completed at the end of early DB stage, just before the beginning of mineralization [197].

Regarding chemoattractant signaling, there are three groups of neurotrophic factors involved [203]: neurotrophins (or neurotrophic growth factors) such as NGF, BDNF, NT3, NT4/5 and their receptors LANR (for all neurotrophins), TRKA (for NGF in nociceptive neurons [204–206], TRKB (for BDNF, NT3 and 4) and TRKC (for NT3 -which mediate neurotrophin signals inside cells [207]); GFL's like GDNF, NRTN, ARTN, PSPN and their receptors GFRα1 (for GNF), GFRα2 (for NRTN), GFRα3 (for ARTN) and GFRα4 (for PSPN); and neuropoietic cytokines such as CNTF and LIF [105, 203, 208].

It has been suggested a role of GFL(GDNF)/RET signaling in promoting DPa/dental pulp innervation. GDNF/GFRα1 complex mediates RET receptor activation [197, 209] and triggers PI3K/AKT, MEK/ERK, SRC and WNT signaling pathways to

regulate neuron cell function [203]. GFL-RET signaling might mediate dental pulpal afferent chemoattraction [197] by upregulation of GDNF and NRTN during the period of tooth innervation [210]. The differential expression of receptors GFRα2 and GFRα4, co-receptor RET, and ETV5 in tooth organogenesis (mainly in SHED) could indicate a change in regulation compared with adult DPSC [203]. After these changes, peripherin positive nerve fibers can be distinguished in the deciduous first molar tooth germ. At late DB stage, neuronal cells are detected within the mesenchymal cells beneath pre-odontoblasts and in the surrounding mesenchyme [105]. This dental primordium, with all its differentiated elements, gives rise to the coronal portion of the tooth.

4. Conclusion

Understanding the dental development process from the molecular embryological point of view -as described in the present synopsis of dental crown formation- allows the clinical dentist to evaluate the presence or absence of these events from the clinical evaluation. This is important to detect and accurately describe events that does not follow the normal pathway of odontogenesis.

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Embryology is the study of the prenatal development of gametes (sex cells), fertilization, and development of embryos and fetuses. There have been many technological innovations and advancements in this field over the last decade, especially in in vitro fertilization (IVF). This book discusses these innovations and presents recent and relevant research. The chapters discuss gamete collection and freezing, how to improve IVF outcomes, embryo development, organogenesis of the early embryo, and much more.

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