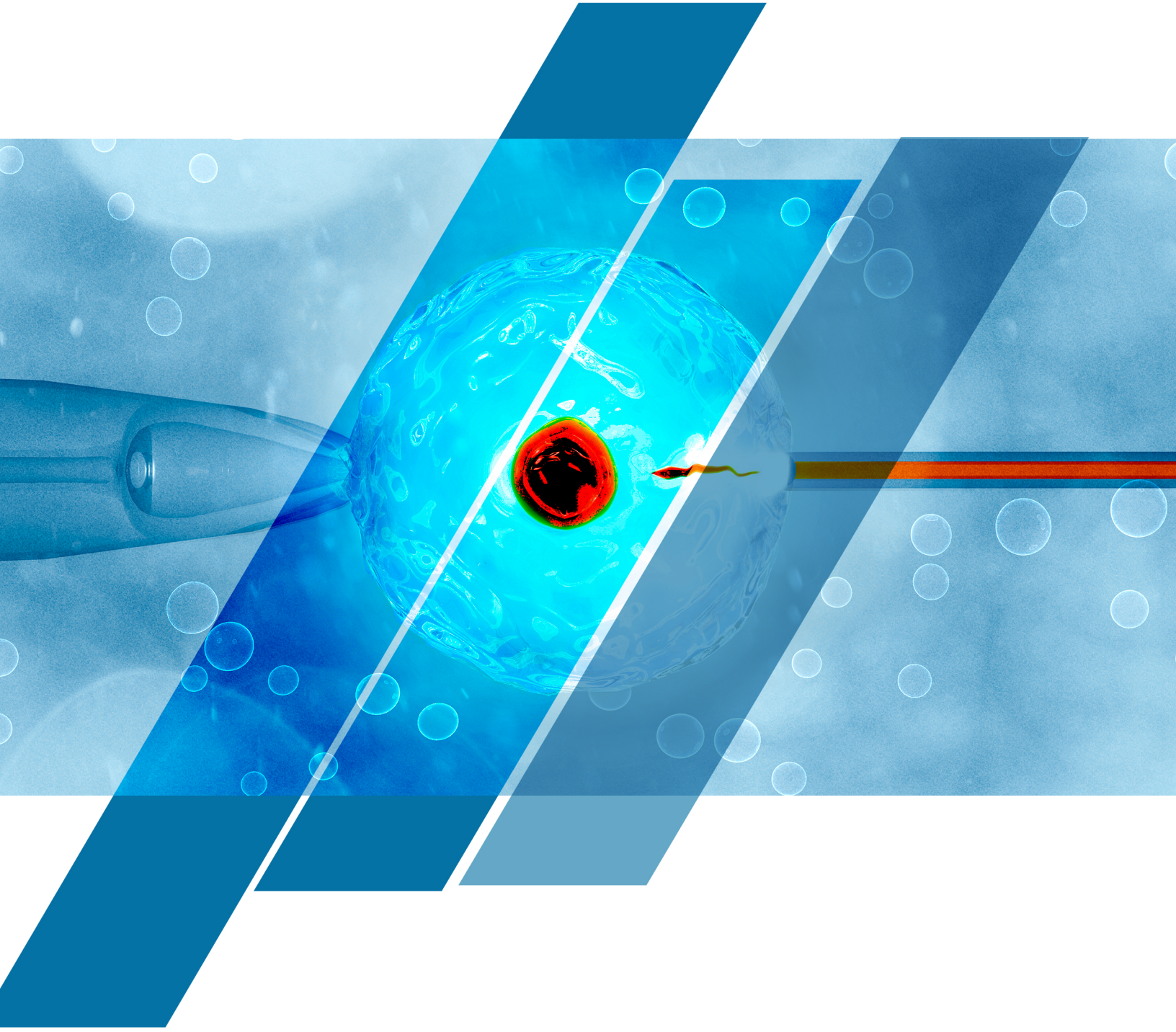
Advances in Assisted Reproduction Technologies



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Advances in Assisted Reproduction Technologies

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PREFACE

More than 4 decades have passed since the birth of the first in vitro fertilized baby in 1978. The use of assisted reproductive technology (ART) to overcome infertility has increased exponentially with the simultaneous increase in the number of fertility centers in every part of the world. The use of ART continues to increase around the world, due to ever-increasing global access to infertility clinics in the treatment of different forms of infertility (like tubal disease, ovarian aging, or ovarian dysfunction).

This book captures the current and recent advances in assisted reproduction technology in humans and livestock in an easy and comprehensive way for the non-expert and the junior embryologists through simplifying each ART tool by providing definition and explanation, and how the methods are practiced and how to overcome troubleshooting, and showing the ARTs uses and significance.

This book will be an ideal reference for junior embryologists as it provides take-home messages for the current and recent ARTs. It contains ten chapters with a detailed explanation of normal reproductive physiology, male ARTs such as sperm retrieval and freezing, female ARTs such as oocyte activation, and cryopreservation, and finally embryo ARTs that include assisted hatching and cloning techniques.

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CHAPTER 1

Physiology of the Reproductive System

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Abstract: The reproductive system of the living organism is the biological system made up of all the anatomical organs involved in sexual reproduction. This system involves the interaction of several fluids and hormones to regulate the functions of the reproductive system. The ultimate goal of the reproductive system is to successfully produce gametes (sperms and oocytes) to attain a combination of genetic material between two individuals, which allows for the possibility of greater genetic fitness of the offspring. In this chapter, we introduce the physiological process of gonadal development, male, and female reproductive system, embryo formation, and development to give the reader the basic concepts for application in the field of assisted reproductive techniques.

Keywords: Oocyte, Ovary, Physiology, Reproduction, Sperm, Testis.

GONADAL DEVELOPMENT AND SEX DETERMINATION

Gonadal Development

The gonads represent a unique embryological situation in that: the rudiments of all body organs except the gonads can normally differentiate into only one type of organ. For example, a lung rudiment can become only a lung, and a liver rudiment can develop only into a liver. On the other hand, the gonadal rudiment has two normal options. When it differentiates, it can develop into either an ovary or a testis. The path of differentiation taken by this rudiment determines the future sexual development of the organism. Before this decision is made, the mammalian gonad first develops through a bi-potential (indifferent) stage, during which time

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it has neither female nor male characteristics (Figs. **1** and **2**). The indifferent gonads consist of several components:

1. Coelomic epithelium, which is the precursor of Sertoli cells in males and granulosa cells in females.

2. Mesenchymal stromal cells, which are the precursor of Leydig cells in males and theca cells in females.

3. Germ cells that have migrated there from the yolk sac endoderm.

This assembly is organized into the indifferent gonads into two layers, cortex and medulla, and proceeds as follows:

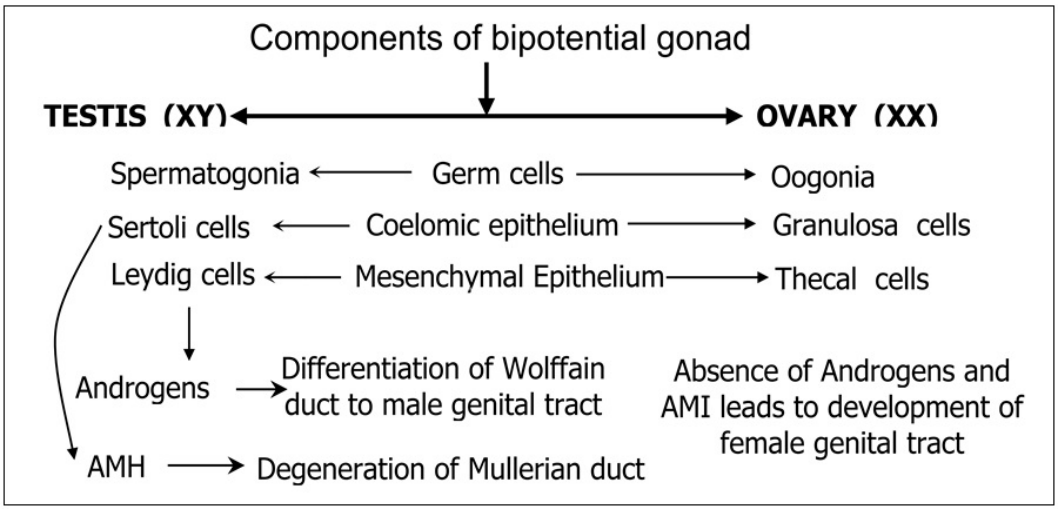

Fig. (1). Differentiation of the indifferent gonad components to their analog in both testes and ovary.

In a Male Fetus

Spermatogenic tubules begin to be formed at 6 weeks. This is followed by differentiation of the Sertoli cells at 7 weeks and Leydig cells at 8 to 9 weeks. At this point, the testes are structurally recognizable, and testosterone secretion begins.

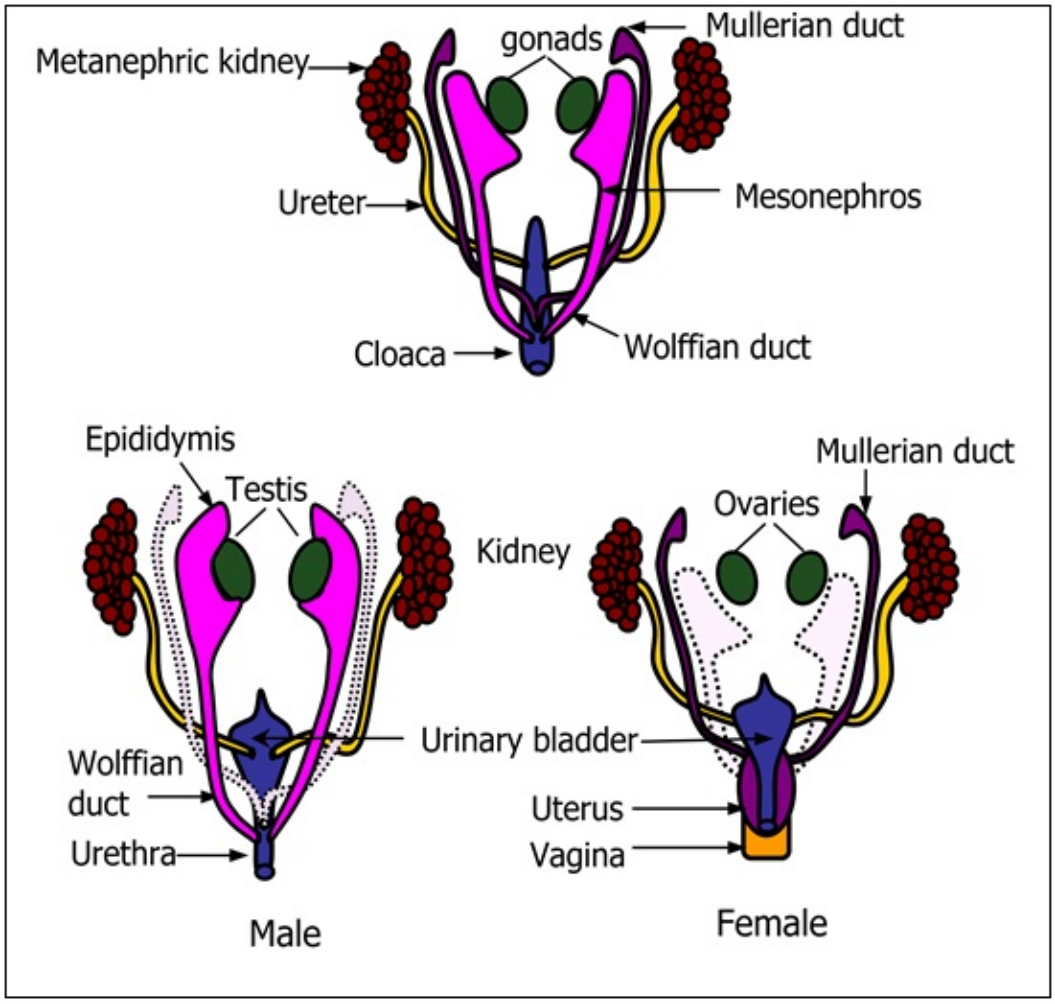

Fig. (2). Development of the gonads and their ducts in mammals. The upper figure represents the undifferentiated gonads and the presence of both male and female ducts. In the lower figures the male and female development is due to gonadal differentiation.

The germ cells become enclosed within the medulla, whereas the cortex is regressed. No known hormonal influences are required for the differentiation of the indifferent gonad into a testis till that stage. The urogenital groove (sinus) is the progenitor of the external genitalia. The Wolffian duct differentiates into the epididymis and the vas deferens.

In a Female Fetus

Differentiation of the indifferent gonad into an ovary does not start until 9 weeks

CHAPTER 2

Sperm Assessment and Processing

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Abstract: Semen analysis has become a definitive tool for understanding the male fertility factor. The volume of the ejaculation indicates the functioning and patency of testicular organs, whereas the quantity and quality of sperm define one's fertility profile. Thus, semen analysis is the cornerstone for diagnosis as well as for medical management. Semen comes with both cellular and non-cellular components. For the management of male infertility, it is necessary to have better quality sperm. As a result, semen processing has become an integral part of any medically assisted reproduction. There are different standardized preparation methods available that include sperm wash, swim-up technique, pelleting method, density gradient, *etc*. The aim of this chapter is to discuss semen analysis, its relevance in understanding male fertility factors, and the available sperm preparation methods. Based on the literature, it can be concluded that a proper semen assessment, according to the latest WHO guidelines, is mandatory for diagnosing male fertility issues. The appropriate sperm preparation method and good quality of sperms combined with the skill set of embryologists and environmental conditions will be the decisive factor in any IUI/IVF/ART success.

Keywords: Density gradient, Pelleting method, Semen analysis, Semen processing, Sperm wash, Swim-up technique.

INTRODUCTION

The creation begins with conception. Conception is achieved by two germ cells called sperms and oocytes. All conceptions are not achieved naturally, thus resulting in infertility. It turns out to be a global problem since it affects about 20% of couples worldwide. Male and female factors equally contribute to this condition. For the male fertility evaluation, a semen analysis (SA) is the primary screening procedure. It is advised to perform a minimum of two standardized

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semen analyses at an interval of 4 weeks apart. SA is repeated since semen parameters vary from time to time in one person as like other fluid parameters. It is also done to neglect technical or manual errors. World health organization (WHO, 2021) instructed to perform at least one diagnostic semen analysis before initiation of a treatment cycle. It has provided guidelines, reference values, and protocols to perform a semen analysis (Table **1**) [1].

Table 1. WHO 2021 guidelines for seminal parameters and reference values [1].

Parameter	Lower reference value
Semen volume	1.4 ml
Total sperm number (10^6 per ejaculate)	39
Sperm concentration	16 M/mL
Total motility (PR + NP, %)	42%
Progressive motility (PR, %)	30%
Vitality (live spermatozoa, %)	54%
Sperm morphology (normal forms, %)	4%
pH	≥7.2
Peroxidase-positive leukocytes (10^6 per ml)	<1.0
Immunobead test (motile spermatozoa with bound beads, %)	≥50
Seminal zinc (mol/ejaculate)	≥2.4
Seminal fructose (mol/ejaculate)	≥13
Seminal neutral glucosidase (mU/ejaculate)	≥20

Semen processing is a vital part of any IVF (*in vitro* Fertilization) treatment. It can be done for ongoing procedures as well as for cryopreservation for future use. It is directly related to the success of the procedure. A variety of semen preparation methods are followed in IVF laboratories. In this chapter, we are going to discuss various insights into sperm assessment and processing in detail.

WHAT IS SPERM?

Sperm is the fundamental unit of the male reproductive system. Sperm, illustrated in Fig. (**1**) is a haploid microscopic male reproductive cell. It is produced in the seminiferous tubules of testes, matures and stored in the epididymis, and transported through the ejaculatory duct along with the fluids secreted from the accessory glands [2].

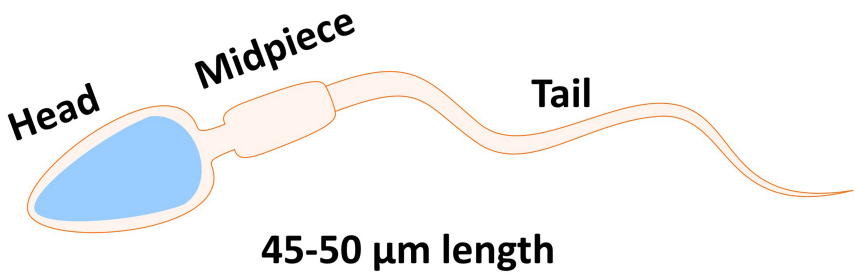

Fig. (1). Normal human sperm.

The head of mature sperm is made up of a well-defined nucleus and acrosome region. The nucleus contains the 23 chromosomes of parental DNA ranging from 30-35% while the acrosome contains proteolytic enzymes that constitute around 65-70%. Unlike most somatic cells, it lacks a large cytoplasm. The head is followed by a mid-piece that contains mitochondria, and a tail facilitates its mobility [3].

WHAT IS THE DIFFERENCE BETWEEN SPERM AND SEMEN?

Semen is a term that is often used interchangeably with sperm, but both are different, where semen refers to the seminal fluid that contains millions of sperm cells along with other plasma fluid. It can look white, yellow, or slightly greyish in color and viscous. The composition of the semen is of mature and immature sperm cells, white blood cells, and other non-cellular components like spermine, prostaglandins, electrolytes, *etc* [4].

Semen Collection

Semen collection is a vital step. Clear instructions should be given to the patients for collecting the entire sample in a wide-mouth non-toxic sterile container (Fig. **2**). The patient can be asked to collect the sample through masturbation in a room near the andrology lab. If the patient faces difficulties in collecting samples, it may be advisable for taking phosphodiesterase (PDE) inhibitors (prescribed based on clinician's advice), home collection in a sterile container or a non-spermicidal condom, or through electroejaculation. After home collection, the sample should be transported to the examining laboratory within 45 minutes (avoid extreme temperature), since the semen analysis should be completed within 1-hour post ejaculation. The semen container can be kept at 37°C or room temperature (RT). The detailed reports should be maintained for every sample (Fig. **3**). All semenology procedures should be carried out in an aseptic condition (Fig. **4**) [1].

CHAPTER 3

Sperm Freezing

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Abstract: Sperm freezing is one of the reproductive technologies responsible for increasing domestic animals' fertility and preserving wild animal fertility over the last 100 years. This chapter will shed light on the current understanding of the effect of cryopreservation on sperm cells, advances in cryopreservation in terms of cryoprotectants and freezing protocols for bovines, caprines, ovines, equines, and camelids. Moreover, this chapter will shed light on other technologies needed to be adopted for different animal species.

Keywords: Cryopreservation, Cryoprotectants, Fertility, Sperm.

INTRODUCTION

Sperm freezing is essential for the distribution of superior genetics among domestic animal species and the preservation of wild animal species. Sperm freezing enables increasing sperm longevity and availability to inseminate animals in another part of the world or even in the future when the original donor is already dead. However, this at the expense that sperm cells thawed after freezing undergo alternation in their functionality, and intracellular and plasma membrane structures, which leads to compromised fertilizing ability.

Advances in sperm cryopreservation go around improving post-thawing sperm parameters to be as close to pre-freezing sperm parameters. In this context, a substantial amount of work has been dedicated to testing different cryoprotectants, additives, cooling rates, and freezing dose. It is worth noting that a freezing protocol that is suitable for one animal species does not essentially work for another. This is could be attributed to many reasons but primarily due to the different composition of sperm cell plasma membrane of different animal species. The cryotolerance of the sperm plasma membrane is determined according to membrane cholesterol to phospholipid ratios. In this context, species can be

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ordered, according to their plasma membrane cholesterol to phospholipid ratio, hence sperm cryotolerance is as following: human (0.99-0.83), rabbit (0.88), bull (0.40-0.45), ram (0.38), stallion (0.36), rooster (0.30), and boar (0.20-0.26) [1 - 5].

EFFECT OF CRYOPRESERVATION ON SPERM CELLS

As with any plasma membrane, the spermatozoa plasma membrane is built up of lipids and proteins. Lipids are in the form of phospholipids and cholesterol. The phospholipids are arranged as a dynamic bilayer. Each phospholipid is composed of a phospho-head group and two fatty acyl chains. The fatty acyl chains are varying in length and structure ranging from 14 to 22 carbons and contain 0–6 unsaturated bonds. Cholesterol is composed of four lipophilic carbon rings (associated with the fatty acyl chains) and a carbon side chain (associated with the head groups of the phospholipid). Therefore, cholesterol can fill in any gaps created in the membrane's core because fatty acyl chains have different carbon lengths or different degrees of unsaturation. Cholesterol, therefore, helps to stabilize the membrane at body temperature. When the spermatozoa undergo cooling, the lipid parts undergo a transition state called a phase transition, where they transform from being in a fluid state into a solid (gel) state. Cellular plasma membranes do not undergo the phase transition at a single temperature but over a range of temperatures. This is because the phase transition of each fatty acyl chain occurs at a different temperature. As lipids undergo the phase transition coalesces into ‘icebergs’, they leave their protein partners, which in turn coalesce to form protein clumps within the membrane, which significantly alters their functional capacities. As the temperature is reduced further, more and more lipid species undergo the phase transition and coalesce in these ‘icebergs’ of solid lipid, leaving a constantly smaller portion of the fluid membrane. Finally, upon reaching the lowest temperature of the membrane range of transition, the entire membrane is in the ‘gel’ state. At −5 °C, the extracellular solute, the cells, and intracellular water are still not frozen but in a supercooled state. Between −5 to −15°C, the intracellular water is still in a supercooled state, but the extracellular medium starts to form ice crystals [6]. Due to concentration differences, the intracellular water will diffuse to the extracellular medium and freeze. If the cooling speed beyond -15°C is slow, almost all intracellular water will diffuse out to an external medium leading to dehydration and shrinkage of the cell, and ultimately a hyperosmotic shock. However, if the cooling speed is fast, ice crystal formation will start due to the presence of intracellular water that did not get out [7]. Therefore, sperm cooling and freezing rate should be fast sufficiently to circumvent spermatozoa dehydration and shrinkage but slow adequately to dodge intracellular ice crystals formation [7]. Moreover, cooling, and freezing temperatures lead to the compromise of sperm ATP-dependent ion channels of potassium, sodium, magnesium, and calcium leading to depolarization and an

increase in permeability of plasma membrane and mitochondria, leading to premature capacitation, cell death, peroxidation, loss of lipids from the plasma membrane, and release of reactive oxygen species (ROS) [8].

The objective of cryopreservation protocol is the survival of sperm cells during freezing and after thawing. During thawing, sperm cells undergo rehydration, osmotic stress, and ultimately plasma membrane disruption. Therefore, optimizing sperm cryopreservation consists of optimizing three main components: sperm cryoprotectant, cooling/freezing rate, and thawing rate.

Cryoprotectants are used to decrease cryoinjury during freezing and thawing. Cryoprotectants are classified according to their ability to penetrate the sperm cell into permeating or non-permeating cryoprotectants. *Permeating cryoprotectant* is a type of cryoprotectant able to penetrate the sperm cell membrane, consequently, they change the viscosity of the cytoplasm and decrease the concertation of intracellular electrolytes. Therefore, upon subjecting sperm cells to freezing temperatures, they will cause dehydration of the sperm cell and reduce the formation of intracellular ice and minimize the extent of osmotic shrinkage of sperm cells [9, 10]. Glycerol is generally the most used permeating cryoprotectant in mammalian spermatozoa cryopreservation. Also, ethylene glycol and dimethylacetamide have been used as penetrating cryoprotectants [11]. Glycerol and other permeating cryoprotectants can penetrate the sperm cells, therefore it is relatively toxic to the sperm cells. Since cytoplasm viscosity of sperm cell differs between animal species [12], therefore, glycerol concentrations tolerability depend on animal species. For example, bull and boar semen is relatively tolerate higher concentration of glycerol compared with stallions [13]. *Non-permeating cryoprotectants are a* type of cryoprotectants that do not penetrate the sperm cell membrane. It acts extracellularly by stabilizing the plasma membrane, decreasing the freezing point of the extracellular compartment, therefore, lessening formations of ice crystals around the sperm cells [14]. Non-permeating cryoprotectants include sugars such as lactose or trehalose or dextran, and proteins [15]. The protein of animal sources has been used as a cryoprotectant, as chicken egg yolk and fat-free skimmed milk are widely utilized for different animals' sperm cryopreservation. However, there is an increasing interest to use animal-free cryoprotectants to avoid risks for viral or other disease transmissions.

As previously mentioned, since sperm cell wall composition is different between species, hence does each animal spices sperm prefer a particular freezing medium? what is the ideal concentration of the ingredients? The next sections will discuss the current understanding of cryopreservation protocols for different animal species.

CHAPTER 4

Controlled Ovarian Stimulation

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Abstract: For conception to occur, whether naturally or via assisted reproductive technology (ART), three things are required; a healthy sperm for fertilization, an ovum released from both ovaries (in the fallopian tubes for natural pregnancy or in the laboratory in IVF/ICSI), and a healthy place for the embryo to implant (a healthy receptive endometrial cavity), so any defect in any of the aforementioned pregnancy requirements will affect the achievement of pregnancy in both types; the natural one or that resulting from ART [1]. Ovarian stimulation is used to overcome any ovulatory disorder or in ART to collect as many eggs as possible (10-15 eggs at least) [2] to maximize the chances of pregnancy. Assessment of ovarian reserve is the first and most important step in ovarian stimulation, as this will indicate the proper method and dose needed for ovulation induction. We test for ovarian reserve using Biomarkers (FSH and AMH) and physical markers (antral follicles count(AFC)) by transvaginal ultrasound(TVS). Both AMH and AFC have similar accuracy in assessing ovarian reserve and are both superior to FSH as they have no cyclic variations [3]. This chapter will discuss all aspects of the physiology of the ovulation process and ovarian stimulation.

Keywords: Anovulation, ART, Ovarian stimulation.

THE OVARY

The human ovary is composed of the following; a) the hilum; b) the outer cortex; c) the central medulla. The hilum comprises the blood supply, nerves, and hilus cells. At the embryonic stage, the ovary is part of the mesovarium. The ovarian cortex consists of two parts, the outer layer (the tunica albuginea) and the inner one (ovarian follicles are implanted in stromal tissue). Further, mesonephric is the origin of the central medulla.

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Embryology and Ovarian Reserve

It is well known that in average cases the primitive ectoderm is the origin site for human germ cells after they develop and migrate to the gonadal ridges (the migration takes place within 5 weeks of gestation). At this time window, the gonadal ridges and mesonephric ducts formulate. The process of migration entails the proliferation of germ cells followed by mitosis. Importantly, the variation of different gonads into either ovary or testis is controlled by genes-related sex chromosomes. However, at this early stage, rapid mitotic germ cell multiplication takes place in which 6-7 million oogonia develop(in the ovarian cortex), which is the highest number or the peak number, keeping in mind that this takes place by 20 weeks gestation. After delivery, the number of eggs decreases with age reaching puberty with less than one million and menopause with around 1000 eggs [4].

Ovarian Physiology

The cyclic activity in women is their reproductive capacity; a characteristic that mirrors the development of dominant follicles. In normal conditions, in each menstrual cycle, a single dominant follicle is produced by the ovaries, in which, this follicle plays a major role during the follicular phase; the production of estradiol. Afterward, ovulation takes place. The dominant follicle transforms into the corpus luteum, through which progesterone production takes place (luteal phase). Together, estradiol and progesterone are needed to facilitate the implantation of the human embryo. Hence, a thorough understanding of the life cycle of dominant follicles will facilitate an understanding of female fertility. This section will briefly discuss the following aspects: Follicular structure and function, the process of folliculogenesis, ovulation, and luteogenesis.

FOLLICULAR STRUCTURE AND FUNCTION

Folliculogenesis resembles the maturation of the ovarian follicle. This follicle is comprised of a shell composed of somatic cells consisting of the immature oocyte and represents the transformation of small primordial follicles into large preovulatory follicles and atresia of the remaining ones. This takes place monthly during the menstrual cycle. Folliculogenesis is divided into two stages; the first stage is gonadotropin-independent while the second stage is gonadotropin-dependent. The first stage represents the development and differentiation of the oocyte and it is organized by the production of growth factors through paracrine mechanisms. The second stage is follicular size increment until it reaches around 25-30 mm, and is controlled by FSH, LH, along with growth factors.

The Process of Folliculogenesis, Ovulation, and Luteogenesis

This process entails the maturation of the follicle from primordial through the secondary follicle.

Primordial

The primordial phase takes place at 18–22 weeks intrauterine, at which the ovarian cortex has its peak number of follicles (4 to 5 million in average case), however, the highest number of follicles could reach 6 to 7 million. These primordial follicles consist of immature oocytes separated from ovarian stroma by the basal lamina and supported by granulosa cells. These cells are dormant, and they can stay at the dormant stage for up to 50 years. Thus, it explains the fact that ovarian cycle length precludes this period. Hence, before birth, the follicles are reduced to around 180,000 by puberty. However, by quality, only 400 follicles reach the preovulatory phase, and by menopause, around 1,000 follicles remain. Thus, it is plausible that early and late menopause are bound by the number of populations at birth, however, no definite evidence is reported. Of note, when primordial cells 'wake up', the initial recruitment phase would take place mediated by hormones and growth factors.

The Primary Phase

In this phase, the activation of the ovarian follicle takes place. The granulosa cells that are around the primordial follicles change their morphological flat features to a cuboidal structure, signposting the launching of the primary follicle, and genes are then transcribed. Besides, paracrine mechanisms begin to initiate the signaling between the follicle and the oocyte. The oocyte and the follicle develop and grow to nearly 0.1 mm in diameter.

The primary follicle during this stage creates and develops follicle-related stimulating hormone (FSH) receptors, in addition, the oocyte will be surrounded by the zona pellucida (A glycoprotein polymer), to form a buffer zone between the oocytes and granulosa cells. The zona pellucida will be kept with the oocyte when the ovulation process is completed as it releases enzymes that enhance the ability of the sperm to penetrate the ovum.

The Secondary Follicle

At this stage, the oocyte-secreted signals will gather around the theca cells; the outermost layer of the follicle, the basal lamina, and undergo cytodifferentiation and will be surrounded by these cells to emerge as the theca externa and theca

CHAPTER 5

Recent Approaches in Intrauterine Insemination in Livestock

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Abstract: In the present chapter, the recent studies on artificial insemination (AI) of livestock (large and small) animals via the intrauterine route are summarized. For this, intrauterine inseminations especially in cattle, horse, camel, buffalo, sheep, goat, dog, and cat species will be considered in detail. Brief data on inseminations in other species (pig, turkey, hen, honeybees, silkworm) is also given.

Once semen sample is collected from sexually mature and clinically healthy breeding male animals, it has to be kept alive (mainly by dilution and cooling) first and then used either immediately (fresh) or stored (chilled/frozen) until being used in oestrous females. During insertion of the semen (particularly stored ones), it is necessary to place the inseminate in the vicinity of the ovum (Graafian follicle) as close as possible. Hence, fertility results of insemination are always higher when semen is deposited intrauterine. However, this is not as easy as we would expect, due mainly to the anatomical structure of females (cat, dog, sheep, and hen) or viability of semen (fish and camel).

To overcome this female- or male-originated limiting factors of fertility, various novel approaches have been reported towards acceptable rates of fertility outcome. Hence, these recent insemination techniques are outlined herein.

Keywords: Animal, Artificial insemination, Breeding, Intrauterine, Livestock, Review.

INTRODUCTION

In a broad sense, reproduction aims to sustain the life of living creatures via bearing viable and productive offspring [1]. In animals, the duration of natural mating takes a very short time, only a couple of seconds (cattle, sheep, horse, hen) or so (camel, cat, turkey, fish). The exceptions are mating-lock ('tie-to-tie') in

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dogs (5-25 minutes long), a long mating period in camels (generally 7-15 minutes or occasionally day-long), or several matings (by 7-10 males) during 'mating fly' in honeybees (a couple of minutes) [2, 3]. Alternatively, however, the AI technologies are also practiced largely in livestock animals (mostly in cow, ewe, doe, sow, mare as well as in turkey, fish, honeybees, and silkworm). The most common practice of AI is seen in cattle (Figs. **1a**, **1B**) due mainly to achieving genetic improvement in calves (Figs. **1c**, **1d**) by spreading especially males' desirable genetic characteristics (high milk yield and heavier calves born) and preventing mating-related diseases (Brucellosis, IBR/IPV, Tuberculosis, *etc.*) [4]. AI is one of the fastest ways to control diseases and increase individual (offspring) genetic characteristics on a national scale [5].

Fig. (1). AI caves in Brown Swiss (1a), Simmental (1b), or their crossed breeds (1c and 1d, resp.).

Conventional AI technologies include mostly the rectovaginal transcervical route in large animals (cattle, buffalo, camel), while the transcervical route is used both in large (horse, cattle) and small animals (dog). However, surgical methods of AI are also practiced mainly in small animals (dog, cat, sheep, goat).

Herein, a brief explanation of AI, relevant species, and people involved are given below.

DESCRIPTION OF AI

In short, AI may be described as the placement of an adequate dose (inseminate) of viable semen collected from the sire male animal to the relevant part of the genital tract of the oestrous female by using appropriate insemination techniques and methods [6, 7]. Globally, it is most commonly applied in cattle in numerous

countries (USA, UK, Denmark, Germany, Russia, Turkey, Israel, Japan, Australia, *etc.*) [2 - 4]. For guiding the routine conduct of AI practices in livestock animals [3], reproductive physiological values (oestrus, ovulation time, viabilities of sperm and ovum, optimal insemination time) of different species (cow, mare, ewe, doe, sow, bitch, queen, hen/turkey) are given in Table **1**.

Table 1. Reproductive physiological values of various livestock species*.

	Physiological values in livestock				
Species	Oestrus	ovulation time	Viabilities of gametes		optimal insemination time
			Sperm	Ovum, h	
Cattle	12-18 h	10-12 h after oestrus ends	24-36 h	20-24	The second half of oestrus or final 1/3 period
Horse	4-7 day	1-2 days after oestrus begins	72-96 h	6-8	1 day before ovulation or near ovulation
Sheep	30-36 h	24-30th h after oestrus begins	30-48 h	16-24	16-24th h after oestrus begins
Goat	30-36 h	24-30th h after oestrus begins	30-48 h	16-24	16-24th h after oestrus begins
Pig	60-72 h	36-48th h after oestrus begins	36-48 h	8-10	16-24th h after oestrus begins
Dog	9 day	36-48th h after oestrus begins	4-6 day	48-72	48th and 96th h after oestrus begins
Cat	6 day	48-72nd h after mating	24-36 h	8-16	nearly 40 h after hCG administration
Poultry (a hen)	none	Every day at certain times	30 d in the female genital tract	1-2	Once a week (afternoon)

*Slightly modified from [3].

Species Concerned

AI can be performed mainly in ruminants (cattle, buffalo, sheep, and goats), pigs, and horses as well as other species such as bees and silkworms. Further, it is also performed in certain scale fish (rainbow trout) [1 - 3, 8, 9]. However, AI is prohibited especially in thoroughbred (British) horses and AI foals are not registered officially [1, 4]. An exception to this is the disease control for dourine (venereal trypanosomiasis) and infectious anemia by equine AI in some eastern European countries [4].

CHAPTER 6

Artificial Oocyte Activation

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Abstract: Against all expectations, the presence of a carefully selected normal spermatozoon does not guarantee oocyte activation/fertilization. In contrast, some ICSI cycles will have to face no or low fertilization in several consecutive cycles. Both sperm- and oocyte-derived problems may account for such a dilemma. In case of physiological activation problems, any artificial increase in ooplasmic calcium could rescue the fertilization process. Such approaches are summarized under the term artificial oocyte activation (AOA). AOA can be achieved by modified ICSI techniques, piezoelectrical manipulation, or chemical stimuli. Amongst these approaches, the latter is the currently most accepted one in IVF laboratories around the world and particularly the Ca^{2+}-ionophores ionomycin and calcimycin are the most extensively studied agents. Recently, a ready-to-use ionophore (A23187) has been introduced which is CE-marked and as such will assist in the standardization of AOA techniques. There is growing evidence that for proper indications usage of AOA can be considered quite safe. This conclusion is based on studies on morphokinetics, chromosome segregation, and gene expression. More importantly, available neonatal and neurodevelopmental data are reassuring. However, since artificial oocyte activation rarely results in physiological Ca^{2+} oscillations and is not beneficial for all patients with a suspected activation deficiency these techniques should not be used without profound indication.

Keywords: Activation failure, Calcimycin, Fertilization failure, ICSI, Ionomycin, Ionophore.

INTRODUCTION

In a recent report, the ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine [1] stated that in a clinical laboratory setting it is highly recommended that Performance Indicators (PI) are evaluated. On the one hand, this would allow for the systematic analysis of the lab´s contribution to patient care and, on the other, these PIs could represent an important instrument within an existing Quality Management System. More importantly, particular Key

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Performance Indicators (KPI) such as ICSI degeneration rate, fertilization rate, or blastocyst development rate would prove useful to perform internal and external quality control. By ascertaining such KPIs embryologists could not only set minimum competency limits but also aspirational goals which would assist benchmarking with others.

According to these figures [1] normal (two polar bodies and two pronuclei) fertilization rate after conventional IVF should be between 60% (competency value) and 75% (benchmark value). Obviously, higher fertilization rates are expected for ICSI (65%-80%). These rational expectations are based on the mere fact that with the ICSI technique a single spermatozoon is directly injected into the ooplasm [2] which avoids some of the physiological barriers of the cumulus-oocyte-complex. ICSI, however, bears the risk that gametes are accidentally chosen for injection which shows deficits in its genetic constellation, phospholipase C zeta (PLCζ) content, protamine ratio, the integrity of centrosome, and/or DNA methylation. Since such a scenario would have an impact on further outcomes, embryologists tend to select sperms considered for usage in the most physiological way [3]. Such selection processes aim for sperms with optimal morphology at higher magnification (IMSI), high sperm head birefringence, completed maturation (so-called pICSI), and/or DNA intactness.

However, even in the presence of a presumably functional spermatozoon that has been processed and identified by a "physiological" selection method oocyte activation and fertilization cannot be taken for granted. In fact, approximately 10% of all ICSI cycles show fertilization problems and 3% result in complete fertilization failure. As a consequence, some patients suffer from repeated fertilization failure which leads to cycle cancellation in the presence of normal sperm parameters and good ovarian response.

For affected couples, artificial oocyte activation (AOA) is the method of choice to restore fertilization and in fact, sometimes is the only way to achieve embryo transfer and eventually pregnancy. It should be kept in mind that AOA techniques should not be applied routinely since they are no physiological approaches at all.

PHYSIOLOGICAL AND ARTIFICIAL OOCYTE ACTIVATION

Before one could further focus on AOA and understand its mechanisms it is of importance to bring physiological oocyte activation to mind. In contrast to the natural conception or conventional IVF ICSI bypasses several physiological steps such as cumulus cell penetration, binding to the zona protein 3 receptor, active passage through the perivitelline space, and Izumo 1 to Juno binding once the membranes of the gametes fuse. A sperm entering the oocyte causes an elaborated

reaction in the same. In detail, time-lapse sequences of early preimplantation development revealed that the first sign of oocyte activation is the extrusion of the second polar body (on average 3.3h post-injection) followed by the formation and juxtapositioning of the pronuclei (6.3h and 8.5h, respectively) [4].

These events observed at light-microscopical resolution parallel physiological processes at the molecular level. Of note, the sperm-derived oocyte activation factor, an enzyme known as PLCζ, enters the ooplasm upon fusion or injection. It then binds to membrane-bound phosphatidylinositol-biphosphate (PIP2) which in turn cleaves into two molecules, diacylglycerol (DAG) and inositol-3-phosphate (IP3). While DAG is involved in zona reaction to prevent polyspermy the latter compound is driving oocyte activation.

The produced IP3 interacts with its receptors located at the membrane of the smooth endoplasmic reticulum (sER) whereupon the release of Ca^{2+} ions from inside the sER is initiated. This Ca^{2+} flux is modulated in a time-dependent manner so that it is literally presented in an oscillating mode. sER Ca^{2+}-depletion is unlasting and in order to avoid complete loss of calcium the oocyte uses Ca^{2+} sensors that monitor available Ca^{2+} within the sER and in case a limited or even drastic depletion of the same is detected calcium entry from outside the egg is triggered to refill sER storages. In order to maintain the oscillatory activity of calcium over a longer period of time mechanisms have to exist which ensure restoration of the Ca^{2+}- level to baseline after every single peak. This is mostly done *via* ion pumps, exchanger proteins, or calcium uptake into mitochondria (for review see [5]). Finally, oscillations cease once the two pronuclei are formed and they are noticed again immediately before every mitosis [6].

ARTIFICIAL OOCYTE ACTIVATION

Any deviation in the above-mentioned crucial biochemical processes and molecules involved, such as PLCζ, $PIP_{2,}$ or IP_3, would automatically cause a reduction or loss of intracellular Ca^{2+}, in particular the absence of calcium oscillations. Such a scenario could also be the result of oocyte-related problems, *e.g.* in case the ooplasma is immature (which in turn would result in maldistribution of sER) or in case IP3-receptors are not functional. However, irrespective of the fact whether cytosolic calcium deficiency is caused by sperm- or oocyte-derived problems it will have a profound impact on cell physiology, and as a consequence, the egg remains unfertilized. As long as calcium is the underlying cause of the dilemma any AOA technique would assist oocyte activation and fertilization by literally requisitioning extracellular Ca^{2+} from the culture medium to compensate for the observed loss. It has to be kept in mind that the vast majority of such artificial methodologies do not generate physiological

CHAPTER 7

Advances in the Human Preimplantation Embryonic Culture System

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Abstract: The embryonic culture system is the main part of *in-vitro* embryonic development. The culture system consists of chemical factors such as the chemical composition of the culture media and physical factors such as temperature, pH, oil overlay and, incubation systems. The optimal culture conditions are needed to support embryonic development *in-vitro*, increase pregnancy, implantation rates, and decrease the level of pregnancy loss. Any suboptimality in culture conditions may impair embryonic development and affect subsequent viability. In this chapter, we will focus on the current culture system in the field of assisted reproductive technology (ART), passing by current culture media formulations, deficiencies, culture devices used, and the areas that need further investigations.

Keywords: Culture dishes, Culture media, Culture system, Embryo culture, Oil overlay, pH.

THE EMBRYONIC CULTURE SYSTEM: A CHEMICAL PERSPECTIVE

Culture system is the surrounding environment of the gametes and embryos growing *in vitro* based basically on the normal composition of the female reproductive tract, in which embryos would normally be exposed [1, 2]. Mimicking nature could support preimplantation embryonic development *in vitro*, which might result in higher post-implantation outcomes (pregnancy, implantation, and live birth rates).

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Culture Media Formulation

Many commercial media are developed for assisted reproductive technologies (ART), to support embryonic development [1, 3, 4]. Basically, the chemical composition of *in-vitro* fertilization media is composed of:

1. Water, which is the major component, about 99% of the media [5].
2. Ions such as potassium, which has an important role in sperm capacitation, chloride ions, which affect mRNA or protein synthesis, and calcium as an essential ion for the compaction stage of the embryos [6].
3. Carbohydrates and other components such as amino acids, vitamins, nucleic acid precursors, chelators, antioxidants, antibiotics, buffer system, protein/macromolecules, hormones, and growth factors [1, 4, 7].

Each component of the media has a specific role to complete the orchestra. Different commercial culture media have different compositions or nutrient concentrations [3, 4]. The exact media formulations and their concentrations are usually not disclosed by the manufacturers due to commercial competition [3]. Up to date, the optimum medium formulation is still unknown and needs more investigations [3]. Hence, each IVF laboratory should choose and validate the media that match their needs.

Back to Nature and Let the Embryo Choose Theories

The development of the embryonic culture media passed with many evolutionary steps [6]. Starting with: The introduction of the culturing idea, *in vitro* first successful fertilization of human gametes in simple media by Robert Edwards, and further modifications to the current complex human culture media forms [6, 8]. Now, the design of culture media emerged from two distinct main theories. The first one is a “back to nature” or “sequential culture media”, which developed to mimic the oviduct and uterine component as the natural environment surrounding the embryos. This approach involves refreshing the embryos from one media, which supports pre-compaction embryo development to another that supports post-compaction development regarding the physiology and embryonic need [6, 9]. Although the beneficial use of the sequential media; raises the concern about the stress that evolved from moving the embryos from one media to another [9]. To eliminate this concern, the other approach has been emerged as “let the embryo choose” or “single-step media.” In this approach embryo, itself will choose its requirements from the surrounding media. As the media contains the concentration of nutrients and adequate substrates required for embryonic development from fertilization to the blastocyst stage with no need for media change [6, 9]. Both single-step and sequential media are commercially available

and have their unique concentrations of energy substrates to support preimplantation embryonic development as well as clinical outcomes (Fig. **1**) [10]. Many studies compare those two types of media, their effect on the embryological outcomes, clinical outcomes, aneuploidy, or even the live birth rate [11 - 15]. However, it was difficult to identify which media is superior due to many cofounders that may affect the results [10, 16]. Emerging from those main approaches, many protocols can be used for embryo culture such as (I) interrupted culture system with the sequential culture media by refreshing the embryos in new media on day 3, (II) daily refreshment system with sequential culture media, (III) uninterrupted culture system with no refreshments using single-step media, (IIII) interrupted culture system either by refreshing the embryos on day 3 or with daily refreshment using single-step media.

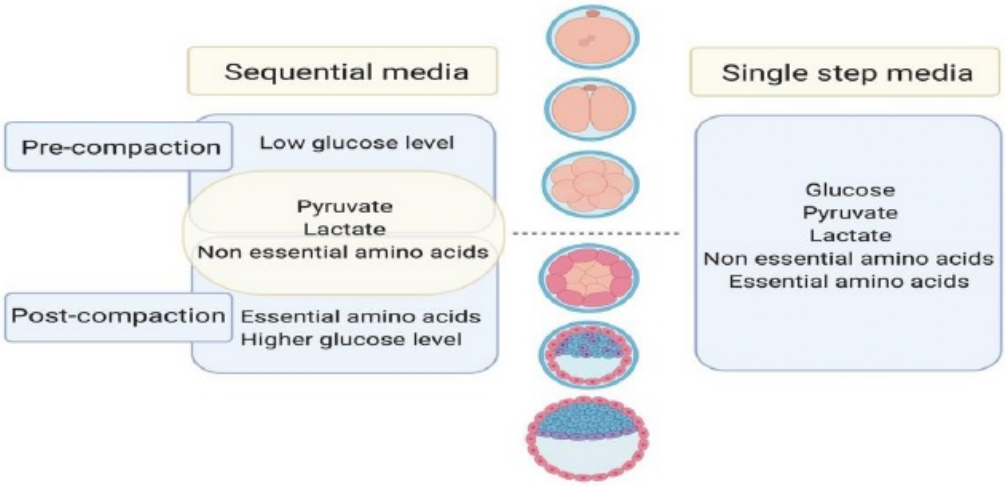

Fig. (1). Main energy substrates of a single step and sequential media for pre-compacting and post-compacting embryos.

Oxygen

The gas phase, especially oxygen is considered one of the most important factors for embryonic development [1, 17]. Oxygen has an important role in the energy production process, although the high concentration may lead to the formation of the reactive oxygen species, which had a toxic effect on gametes and embryos [18]. Its concentration within the uterus and oviduct is approximately 2%, 5-8%, respectively which differs from the oxygen concentration of the air 20% (Fig. **2**) [17, 19]. In 1971, Wes Whitten found no blastocyst formation for mouse embryos cultured under 20% O_2, whereas 100% blastocyst formed under 5% O_2 [17]. He reported that excessive oxygen concentration may harm the surface membrane of the embryonic cells [17]. Also, in the case of human embryonic cells, different O_2

CHAPTER 8

Assisted Hatching

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Abstract: The selected Spermatozoa when it reaches the ovulated Cumulus Oocyte Complex after ovulation, dispersion of the granulosa cells and corona radiata cells occur. The Spermatozoa then must cross the Zona Pellucida (ZP), fuse with the oolemma, and then subsequently fertilize the oocyte. Embryologist Karl Ernst von Baer coined the term 'Zona Pellucida' from Greek work Zone which means belt or girdle and Latin work Pellucida which means transparent or shining. This extracellular matrix is about 13-15 um thick and surrounds all the mammalian eggs and pre-implantation embryos. Zona Pellucida structure is made up of carbohydrates, specific proteins, glycoproteins, hyaluronic acid, heparin, collagen, and fibrous proteins. Human Zona Pellucida contains 4 glycosylated proteins namely ZP1, ZP2, ZP3, and ZP4. ZP plays an important role in helping oocytes to transport essential nutrients and helps in avoiding polyspermy by hardening after fertilization. The embryos must break open the protective ZP layer to the implant, the process is called hatching. It is said that in Assisted reproductive treatment (ART) factors such as the non-availability of enzymes from the endometrium which helps in hatching, extended culture, vitrification may lead to failure in the hatching of embryos from ZP. It was postulated that micromanipulation of ZP to create an opening will help the embryos to hatch and thus implant and will lead to an increase in Implantation rates (IR). This process was later called Assisted Hatching (AH). Various methods were discovered for Assisted hatching such as mechanical ZP AH, zona digestion using enzymes, and laser-Assisted hatching. This chapter will focus on the advantages and disadvantages of each method of AH and their applications in ART along with the impact of AH on clinical outcomes. The use of any method of AH should be chosen carefully to avoid damage to the embryo which will defy the whole purpose of application of AH. In any case, laser-assisted hatching is widely used for Pre- Implantation Genetic Testing (PGT) of the embryos as it is very safe if applied properly, convenient, easy to use, and faster compared to other methods of AH. Each laboratory should identify the correct time and stage at which application of AH is considered based on whether it is helping to improve clinical rates or not.

Keywords: Assisted hatching, Chemical hatching, Laser-assisted hatching, Mechanical hatching, Zona Drilling, Zona Pellucida.

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INTRODUCTION

The mammalian female gamete-Oocyte is surrounded by cumulus and corona cells and is called Cumulus Oocyte Complex (COC). This COC is released during ovulation. When the selected spermatozoa, after passing through various barriers of the female reproductive tract, reaches and penetrates the ovulated COC the dispersion of tightly bound granulosa cells and corona radiata cells occurs. The spermatozoa then have to cross the Zona Pellucida (ZP) to fuse with the Oolemma and then subsequently fertilize the oocyte [1]. In 1827, Embryologist Karl Ernst von Baer adopted the word 'Zona Pellucida' from Greek work Zone- meaning belt or girdle and Latin word pellucida meaning transparent or shining while describing the Human egg. This thick extracellular matrix (ECM) which is about 13-15 um in diameter, surrounds all the mammalian eggs and preimplantation embryos [1, 2].

The structure of ZP is made up of carbohydrates, ZP-specific proteins, and Glycoproteins. Zp also contains hyaluronic acid, heparin, chondroitin (proteoglycans) and collagen, laminins, elastins, and fibronectins (fibrous proteins). Most mammalian eggs have 3 glycosylated proteins, human ZP contains 4 glycosylated proteins, namely ZP1, ZP2, ZP3, and ZP4. All these ZP1-4 have a polypeptide chain which includes the ZP domain (ZPD). The ZPD has 2 subdomains ZP-N and ZP-C connected by the linker region, 8 conserved cysteine residues, and ~270 amino acids (aa) [1, 2].

The main events during fertilization are sperm-COC recognition, penetration of ZP, and sperm oocyte fusion. The interaction between ZP and sperm is species-specific by receptor-ligand interaction leading to activation of sperm. Molecules like murine tyrosine kinase and galactosyltransferase are believed to be the sperm's receptors for ZP. Glycoprotein ZP3 acts as a ligand for receptors present on the sperm surface. This binding of sperm results in exocytotic event releasing proteolytic enzyme which will facilitate the penetration of ZP by the sperm, the process is called Acrosome reaction, where ZP2 acts as secondary sperm binding site resulting in gamete interaction *via* contact of ZP with the inner membrane of sperm which has undergone Acrosome reaction [3]. Such a complex interaction and process may hint towards the indirect role of ZP in sperm selection. Post fertilization, exocytosis of cortical granule content in perivitelline space (PVS) happens and comes in contact with the oolema and ZP. This exocytosis and cortical reaction lead to the blocking of multiple sperms entering the oocytes and thus avoiding polyspermy [4].

Zona hardening happens post-fertilization and is helpful to avoid polyspermy, protect the preimplantation embryo during early embryo development, and

blastomere integrity and passage of embryo in the female reproductive tract before implantation. It is also argued that ZP protects the embryos from various uterine factors and immune responses. Zona hardening is also observed in mouse and human embryos cultured *in vitro* [1, 5, 6].

For the embryo to implant, it must hatch out of its protective layer-ZP. Based on time-lapse observations it was believed that repeated expansions and contractions of the blastocyst help the embryo exert pressure on the ZP to assist its hatching and implant. The volume of the blastocyst increases thereby stretching the epithelium of trophoblast cells and exerting increasing hydrostatic pressure leading to thinning of ZP. The presence of specialized plump cells called 'Zona Breakers' are also found to be present near the opening of the ZP [7]. Now there are sufficient studies in the database which suggest that various enzymes, proteolysins such as trypsin, proteases, and uterolysins, which are secreted by either the blastocyst and/or endometrium are responsible for the hatching of the embryo [7, 8, 16].

ASSISTED HATCHING AND ART

There are many reasons which may lead to the failure of implantation of a viable embryo. Sometimes the embryo may be slow-growing or its inherent quality may not be good to implant. Endometrium may not provide adequate uterine factors to assist implantation or might not be receptive enough. Another reason might be due to the inability of the embryo to hatch owing to circumstances related to laboratory and clinical conditions [11]. Implantation of the embryo after hatching is based on a series of events that need to be completed in a coordinated manner. Failure in completing any one of these numerous steps might stop the embryo from implanting. Patients' ability to produce enough uterolysins may be diminished or the ability of such uterolysins to carry out their function may also be influenced due to assisted reproductive treatment cycles they are undertaking [13].

Improvement of culture conditions led to extended cultures in many IVF labs around the world to select good quality blastocyst and transfer fewer embryos. This might lead to compromise the hatching process as enzymes that are thought to be released by endometrium are present to assist the hatching in natural conception. Extended culture might also lead to disrupting the timing and mechanism of hatching as Zona hardening is observed [1, 7, 9, 13]. *In vitro* culture embryos are found to be slower in development or poor in quality or many of them may not reach the blastocyst stage or hatch when compared to the development of embryos in natural conditions [10]. FSH levels, pre-ovulatory oestradiol, cause of infertility, and advanced maternal age also have an impact on

CHAPTER 9

Oocytes and Embryos Cryopreservation

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Abstract: The availability of cryopreservation technology has extended the scope of human-assisted conception treatment and made it more convenient for patients. Freezing of oocytes and ovarian tissue is now taking place in research institutions and should hopefully become widely available for clinical use in the future if the women have to be away at a critical time during the treatment; the frozen oocyte was thawed and used when required. Good quality embryos frequently remain after the transfer of the required number into the woman; these can be frozen and used at a later date, if required, instead of the couple going through another cycle of in vitro fertilization (IVF) treatment. The conduct of cryopreservation in humans is based on information originally derived from animal work, where sperm cryopreservation, for example, has been carried out for several decades for animal breeding. This chapter will consider the principles of cryopreservation followed by a general description of the practical steps. The clinical applications of cryopreservation will also be described. A final section will deal with frozen embryo replacement.

Keywords: Oocyte and embryos cryopreservation, Slow freezing, Thawing embryos, Vitrification.

HISTORY OF OOCYTE AND EMBRYOS CRYOPRESERVATION

Early basic scientific advancements in measurements of temperature and therefore the chemistry of solutions and gases are certainly sentinel events for cryobiology. It's been suggested that original versions of the tools to live temperature were

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made by Galileo within the early seventeenth century. The primary accurate means of measuring temperature were developed in the early 1700s by the German physicist Gabriel Fahrenheit through the application of mercury in glass.

Since then, modifications of instruments to assess temperature became significantly more accurate and easier to use. Equally important were early advancements made within the nineteenth century involving understandings of liquefaction of gases and the potential use of such refrigerants to chill and store specimens at extremely low temperatures. When one traces the history of mammalian gamete cryopreservation, numerous accounts reference the start of low-temperature biology to 1866, when an Italian military physician Mantegazza documented the observation that human spermatozoa became immotile when cooled in the ice [1, 2].

Investigations in the late 1940s and early 1950s by Chang [3, 4] on low-temperature storage of rabbit oocytes, zygotes, and embryos paved the way for studies on the cryopreservation of female gametes and embryos. Subsequent experiments by Sherman and Lin [5 - 8] demonstrated that mouse oocytes could even be cooled in glycerol, stored, and subsequently fertilized in recipients; furthermore, resulting in embryos supported pregnancies.

In the 1960s and early 1970s, a merging of basic/theoretical cryobiology and practical studies ultimately gave rise to the increased success of embryo cryopreservation. Classical basic science investigations by Mazur [9 - 11] formed the inspiration for understanding cell-specific optimal cooling and warming rates which today remain a pivotal key to successful mammalian gamete and embryo cryopreservation. It had been the combined strengths of Mazur, Leibo, and Whittingham that resulted in successful cryopreservation of mouse embryos.

The cryoprotective agent used by these researchers was 1.5 M dimethylsulfoxide (DMSO), which was combined with a slow cooling rate (0.3C/min to 80C) and deposited in liquid nitrogen [12]. Slow cooling rates supported dehydration during cooling and prevented intracellular ice formation, so this technique was used. In addition, it was discovered that the cryoprotective agent (here DMSO) should be added and removed in a stepwise manner to avoid osmotic shock or injury. In the early 1980s, the same cryopreservation methods were used to successfully create the first human pregnancies after freezing and thawing [13, 14].

Testart *et al.* pioneered the use of 1, 2-propanediol (PROH) as a permeating cryoprotectant for pronuclear-stage zygotes [15]. Furthermore, as an osmotic buffer, these researchers used sucrose in the cryo-media as a non-permeable cryoprotectant. Slow cooling rates were used until the temperature reached 30°C,

after which the samples were submerged in liquid nitrogen and the warming rate was rapid.

Cryopreserving pronuclear- and cleavage-stage mammalian embryos has become a popular practice in assisted reproductive technology (ART) labs throughout the United States and around the world. As experience with cryopreservation of different cell types grew, it became clear that as cell size grows, so does the complexity of cryopreservation [11].

This principle is particularly important in the cryopreservation of mammalian oocytes and embryos slow-rate freezing and vitrification are the two approaches currently used to cryopreserve mammalian oocytes and embryos [16].

Effects on oocyte and embryonic cellular functions may impair abilities to develop normally following the cryopreservation process, regardless of the cryopreservation method used. Oocyte and/or embryo "cryo-damage" is the collective term for these damaged cellular events although the effects of cryopreservation on documented and/or theoretically specific cellular structures and functions, as well as subsequent effects on oocyte and embryonic developmental competence, have been previously reviewed [17].

Cells are subjected to a variety of stresses during cryopreservation, including mechanical, thermal, and chemical stresses [11, 18], which can disrupt cell function and cause cell death. Oocytes, in general, are more vulnerable to cryo-damage than later embryonic stages moreover slow-rate freezing attempts to control biophysical properties of freezing, such as cooling and warming rates, in conjunction with cryoprotectants to minimize adverse cellular events. This approach allows cells to be cooled to extremely low temperatures while minimizing the production of intracellular ice crystals and attempting to avoid the negative effects of increased solute concentrations and osmotic stress [19]. As a result, extracellular ice formation drives cellular dehydration into an equilibrium mechanism when slow-rate freezing is used.

In recent years the new technique of vitrification came to avoid the mistakes of slow freezing, the word "vitrification" comes from the Latin word vitreous, which means glassy or similar to glass. Vitrification is a non-equilibrium cryopreservation method that was originally designed for the cryopreservation of mammalian sperm [20] and embryos [21]. This technique used a special device known as straw-like Fig. (**1**) and is divided into three regions 1st leaf which put embryos or oocytes on its 2nd plastic mark to write the name and id of the couple and 3rd the coverslip.

CHAPTER 10

Reproductive Cloning

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Abstract: Hearing the name "Dolly" was and still stirs the minds of professionals and non-specialists towards the term "cloning", but the way of producing dolly is not the only aspect of cloning. Cloning is defined as the techniques through which identical or virtually identical individuals can be produced. Based on this definition, in this chapter, we are trying to clarify the different applications, aspects, and techniques of cloning such as gene cloning, therapeutic cloning, but to focus on reproductive cloning. Reproductive cloning is the method of making a genetically similar clone of a whole organism. Then it is needed to be discussed with all the scientific thoughts around it, advantages, disadvantages, legal or illegal, and comparing it to other aspects and this is our aim in this chapter.

Keywords: Cloning, Embryo splitting, Reproductive cloning, Somatic Cell Nuclear Transfer, Tetraploid complementation.

INTRODUCTION

The term "cloning" was and is still being widely used by biologists to express many concepts. Even though all these concepts depend on the usage of copies of the whole biological material, they vary in their precision. There are three common major applications of cloning; the first is gene cloning which means the cloning of DNA segments [1, 2], the second is cells cloning which is done to give rise to particular types of cells, and the third is individual cloning that seeks the

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mimicking of the natural process in the identical twin development specifically in multicellular organisms Table **1**. The first case to appear and to spread the concept of cloning to the public is the famously known case of Dolly, which was successfully produced by cloning after about 270 trials by the British scientist Ian Wilmut. After its success, trials have been done on many types of mammals such as mice, rats, pigs, sheep, cows, goats with an increasing success rate without reaching 100%. The spontaneous abortion was the fate of most of the cloning pregnancies [3]. In the case of fetus survival from death during pregnancies, if they did not face early death, they were seen to have serious health concerns such as distorted limbs, gross obesity, dysfunctional immune systems, and organs such as liver and kidneys, and also lungs and the progressive diseases and failures were one of the reasons of the early death of Dolly along with arthritis after living only 6 years after its development by cloning, causing its health to decay rapidly [4, 5]. With all these consequences, it was nearly impossible to apply to humans especially since there are more serious consequences that are abridged in devastating economic, health, and emotional ramifications due to the fetus's death [5].

Table 1. Different applications of cloning.

-	**Gene Cloning (Molecular Cloning)**	**Therapeutic Cloning**	**Gene Cloning (Molecular Cloning)**
Definition	The process of isolating a DNA sequence of interest to make multiple copies of it. The identical copies are clones	The technology used to produce tissues and organs from a single cell to replace damaged cells, tissues, and organs	The process of generating an organism that has the same DNA as another organism
Product of cloning	produces copies of genes or segments of DNA	produces embryonic stem cells of engineered tissues for experiments	produces copies of whole animals
Applications	1. **Main aim**: Studying the structure and function of genes in detail. 2. **Medical applications**: the synthesis of vitamins, hormones, and antibiotics 3. **Agricultural applications**: cloning in bacteria facilitate nitrogen fixation in plants	1. **Main aim**: To make use of the unique ability to generate virtually all types of cells in an organism. 2. **Medical applications:** Grow healthy tissues in the laboratory that can be used to replace damaged or diseased tissues. 3. Learn more about the molecular causes of diseases.	1. production of organs for transplantation to humans 2. Studies of human genetic diseases. 3. Basic research in the control of gene expression and function
Techniques	Recombinant DNA technology	Somatic cell nuclear transfer	1. Somatic cell nuclear transfer 2. Embryo splitting

(Table 1) cont.....

-	Gene Cloning (Molecular Cloning)	Therapeutic Cloning	Gene Cloning (Molecular Cloning)
Potential drawbacks	Nature is an extremely complex, interrelated chain. It is believed by some scientists that the introduction of genetically-modified genes may have an irreversible effect with consequences not yet known.	There are striking similarities between stem cells and cancer cells. Both cell types can proliferate indefinitely and some studies show that after 60 cycles of cell division, stem cells can accumulate mutations that could lead to cancer.	Reproductive cloning is a very inefficient technique and most cloned animal embryos cannot develop into healthy individuals. For instance, Dolly was the only clone to be born live out of a total of 277 cloned embryos. This very low efficiency, combined with safety concerns, presents a serious obstacle to the application of reproductive cloning.

The characteristics of clones are not only genetically but also environmentally controlled, the nutritional inputs during pregnancy or from parents after birth, society, or life experience while growing up and other elements can be determinants of the clone's characteristics. So, if there are two genetically identical clones as the nucleus and the mitochondria donor are the same but are grown in different environments or at different times, they will not be identical in their physical or behavioral characteristics. Even monozygotic twins are not fully genetically identical or epigenetically identical as many elements contribute to their variation such as mutations, stochastic developmental variations, and varied imprinting effects (parent-specific chemical marks on the DNA) that differ in impact on each twin [6, 7]. If twin clones do not have the same mitochondria, as there may be multiple donors or the cell is obtained from an individual and the nucleus is obtained from another, there are additional variations that will exist especially in the high energy demanding body parts such as the heart, muscles, brain, and eye. Also, some differences exist in the body systems that are controlled by mitochondria to determine cell numbers by using mitochondrial control over cell death [8 - 10].

HUMAN CLONING *VS.* ANIMAL CLONING

In the context of human cloning, there is no scientific evidence that human embryos have been cloned (Table **2**). In 1998, there was a claim from South Korean scientists to be successful in cloning a human embryo till the phase of a four cells embryo, and then the experiment was interrupted. In 2002, a kind of religious group called Clonaid claimed the birth of a girl named eve to be the first cloned human and the first of another 12 cloned humans, but also without introducing any evidence to prove their claim. In 2004, a paper was published in

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Dr. Islam M. Saadeldin obtained his Ph.D. degree from the Seoul National University, South Korea. He worked as a postdoctoral researcher at Seoul National University, a visiting scholar at Niigata University, Japan, and an associate professor at King Saud University, Saudi Arabia. Currently, he is a research professor at Chungnam National University, South Korea. He has a patent of invention regarding bovine embryo transgenesis through PiggyBac transposons and he authored more than 160 research papers, reviews, and book chapters covering the fields of advanced reproductive biotechnology such as somatic cell nuclear transfer (SCNT), transgenesis, adult and embryonic stem cells, as well as elucidating the roles of extracellular vesicles in embryo communication and the embryonic-maternal crosstalk. His current research focuses on developing CRISPR/Cas9 engineered extracellular vesicles for improving embryo implantation and pregnancy of cloned and transgenic animals. He has been awarded several national and international prizes, such as the Egyptian State Prize, Shoman Prize, Almarai Prize, Misr Elkheir prize, The Interstellar Initiative for young investigators, the International Embryo Technology Society (IETS) Early Career Achievement Award, and AUA Scholar Award.