Workshop 5: Inter-relações da reprodução humana e veterinária

*The interface between human and animal reproduction*
Human Assisted Reproduction

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ABSTRACT

Background: Infertility is defined as the failure to conceive after one year of unprotected intercourse, and this time has been lowered to 6 month if the female partner is older than 35 years. Infertile couples are offered to low and high complexity treatments are available according to their cause of infertility. Low complexity treatments comprise timed intercourse, intrauterine insemination with or without controlled ovarian stimulation. High complexity treatments comprise standard in vitro fertilization (IVF) and IVF by intracytoplasmatic sperm injection (ICSI). Additionally to these treatments, infertile patients might be benefited by accessory techniques such as preimplantation genetic screening and diagnosis. These techniques aim to detect the most common human aneuploidies related to abortion and chromosomal syndromes; and to identify embryos carriers of genetic diseases like talassemia, Huntington's disease, fragile-X syndrome among others. Human assisted reproduction is a dynamic branch of Medicine performed by several medical specialists from gynecologists to surgeons, and professional as nurses, biologists and veterinarians.

Review: Initially, human infertility was treated by intracervical insemination due to the risk of endometrial insemination and the occurrence of pelvic inflammation, but the development of better insemination techniques and instruments led to the report of a safe intrauterine insemination method in 1974. However, insemination was restricted to oligozoospermia or cervical factors and it was not able to overcome ovarian and tubal infertility factors, neither severe male infertility factor. The range of infertility treatment was tremendously broadened in 1978 with the report of the first IVF baby birth. Since then infertile couples due to tubal factors, ovarian and moderate male infertility factors started to be treated with IVF. Several changes in embryo culture conditions and instruments for IVF were developed and the technology was world wide spread. The first IVF baby was a girl born in 1984 in Brazil. Even though, some ovarian conditions and severe male factor infertility couldn't be treated until 1992. This landmark was the report of the first babies originated by in vitro fertilization with ICSI. In parallel to those scientific and medical evolutions, drugs and new protocols of controlled ovarian stimulation increased the number of oocytes available for fertilization and, consequently, the number of exceeding embryos. These spare oocytes and embryos required the development of cryopreservation techniques for long term storage. Slow rate freezing and vitrification of embryos were reported approximately 25 years ago, but just recently vitrification of oocytes and embryos became the first option for fertility preservation and/or embryo storage in humans. Preimplantational genetic screening and diagnosis were complementary techniques developed during the same period. These tests require precise and meticulous micromanipulation techniques and skills for the obtainment of a single blastomere with intact genetic material for screening of aneuploidies by fluorescent in situ hybridization or the detection of a deleterious allele by single cell PCR. New drugs, protocols, instruments and techniques for Human Assisted Reproduction are still under development in many Universities, infertility clinics and private companies aiming to increase the efficiency of infertility treatments.

Conclusion: Human assisted reproduction is constantly evolving with the development of more precise diagnostic tests and with the improvement of clinical approaches to infertility and better conditions in embryology laboratories and this constant evolution is the result of the synergic interaction of clinical infertility specialists with researchers of different backgrounds.

Keywords: Assisted reproduction, in vitro fertilization, IVF, intracytoplasmatic sperm injection, ICSI, human reproduction.

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I. INTRODUCTION

Infertility is a disease defined as the failure to conceive after one year of unprotected intercourse, and this time has been lowered to 6 months if the female partner is older than 35 years [1]. Approximately, 10% of the couples in reproductive age might require infertile care [2]. The last report on assisted human reproduction in Europe indicated that 359,110 treatments were performed in a population of 422.5 million (850 treatments/million) [3]; in Latin America, 34,102 assisted reproduction treatments were performed in 2007 and 42.3% of those were carried out in Brazil [4].

Treatments of assisted reproduction are indicated according to the infertility factor of the couple. Male infertility factors comprise a myriad of malfunctions from retrograde ejaculation to impaired production or release of sperm such as in oligozoospermia, teratozoospermia, asthenozoospermia, and obstructive and non-obstructive azoospermia [5]. Conformably to male infertility, several diseases are enrolled in female infertility. Didactically, female infertility can be classified as ovarian/ovulatory factors, tubal factors, uterine factors and endometriosis [6-8]. Male, female and the combination of these infertility factors may be treated with Human Assisted Reproduction of low and high complexity according to the patients' needs (Table 1)[8-9].

Low complexity treatments comprise timed intercourse, intrauterine insemination with or without controlled ovarian stimulation. High complexity treatments comprise standard in vitro fertilization (IVF) and IVF by intracytoplasmatic sperm injection (ICSI). Additionally to these treatments, infertile patients might be benefited by techniques such as preimplantational genetic screening and diagnosis.

II. HISTORICAL OVERVIEW OF HUMAN ASSISTED REPRODUCTION

The treatment of infertility has been recorded since the late years of the 18th Century; at that time, by archaic techniques of artificial insemination. Despite its availability for more than 200 years, artificial insemination remained controversial in many countries until the 1970’s [10].

Modern artificial insemination consisted in the intracervical delivery of prepared semen to minimize the risk of occurrence of pelvic inflammation. Nevertheless, since 1974 the use of a of a safe intrauterine insemination method has replaced intracervical alternative [11].

Intrauterine artificial insemination was restricted to oligozoospermia or cervical factors [12]; however, it was not able to overcome ovarian and tubal infertility factors, neither severe male infertility factor. In vitro fertilization is the appropriate treatment for these conditions, and despite of the publication of attempts of in vitro fertilization of animal oocytes in the begging of the 20th Century, the fertilization of human oocytes was first reported in 1969 [13]. Several developments in embryo culture techniques were developed and in 1978 the birth after of the first IVF baby was announced and it broadened the range of treatable infertility causes; thence, infertile couples due to tubal, ovarian and moderate male infertility factors could experience parenthood through IVF [14].

In vitro fertilization treatment became worldwide spread and the first IVF baby born in Brazil was in 1984. The word technology industry took a giant leap and physicians and scientist increased performances, established treatments with both higher quality standards and have observed an even increasing pregnancy and birth rates. In 1992, Palermo and colleagues [15] reported a births of babies originated by in vitro fertilization with intracytoplasmatic sperm injection assuring that man that produced spermatozoa despite ejaculation could become parents.

In parallel to those scientific and medical developments, drugs and new protocols of ovulation induction increased the number of oocytes available for fertilization and, consequently, the number of exceeding embryos.
These spare oocytes and embryos required the development of cryopreservation techniques for long term storage. Successful slow rate freezing of embryos were reported approximately 25 years ago [16]. Recently vitrification of oocytes and embryos became the first option for fertility preservation for female gametes and embryos storage in humans [17-18]. In fact, vitrification of oocytes has provided quality oocytes at warming resulting in pregnancy rates similar to those obtained with fresh oocytes. This methodology has been chosen in Spain as the main storage method for oocytes in an ovdonation program [19-20].

Preimplantation genetic screening and diagnosis were complementary techniques developed during the same period [21]. These tests require precise and meticulous micromanipulation techniques and skills for the obtainment of a single blastomere with intact genetic material for screening of aneuploidies using fluorescent in situ hybridization or the detection of a deleterious allele by single cell PCR [21]. Preimplantation genetic diagnosis is particularly interesting for the prevention of allelic disorders such as thalassemia, sickle cell anemia, cystic fibrosis, Duchenne’s Muscular Dystrophy, among several others [22-24].

III. PERSPECTIVES FOR HUMAN ASSISTED REPRODUCTION

Recently, we proposed several approaches for new ovulation induction methods based on the description of follicular waves in women [25] described originally by Baerwald et al. [26-27]. These protocols are currently under clinical evaluation and it is our expectation that it might enhance pregnancy rates after IVF.

Additionally, remarkable modifications on embryo culture systems are forthcoming as technologies as proteomics and genomics come into place. The in vivo environment for embryo development is chemically and physically dynamic; however, embryo culture systems currently used in Human Assisted Reproduction is static [28-29]. The establishment of physically dynamic conditions through tilting embryo culture media [30] or, in a more sophisticated approach, through a complex series of channels creating microfluidic stream of media [28-29] are still on progress, but they are promising technology on embryo culture techniques and they may have a great impact on pregnancy rates.

Complementary techniques such as PGD are also becoming more sophisticated through the implementation of feasible microarray based comparative genomic hybridization [31]. This technique allows high resolution investigation of the embryo genome and detection of deleterious copy number variation of genes, causes of miscarriage and it still allows the determination of chromosomal numbers [32-33].

IV. CONCLUSIONS

Human Assisted Reproduction is constantly evolving with the development of more precise diagnostic tests and with the improvement of clinical approaches to infertility and better conditions in embryology laboratories and this constant evolution is the result of the synergic interaction of clinical infertility specialists with researchers of different backgrounds.

REFERENCES

The Role of the Veterinarian on Human Assisted Reproduction

André Monteiro da Rocha

ABSTRACT

Background: Infertility is a disease affecting 10% of the human population in reproductive age and couples experiencing difficulties to achieve pregnancy may benefit from assisted reproduction treatment. According to the Red Latinoamericana de Reproducción Asistida, approximately 34,100 in vitro fertilization (IVF) treatments were performed in Latin America in 2007 and 42.3% (14,428) of these treatments were conducted in Brazil. Standard techniques and treatments for infertility were developed within the last 4 decades and some of them are still under technological improvements or they are frequently considered experimental or “state of art” technology. Usually, these treatments and techniques to overcome infertility were developed by professionals with different backgrounds including physicians, biologists, veterinarians and nurses. We aimed to review the role of veterinarians in translating animal biotechnology to human assisted procreation, developing new assisted reproduction technologies and treatments, and finally acting in clinical embryology laboratories as embryologists or supervisors.

Review: Human assisted reproduction landmarks were: (i) the birth of the first in vitro fertilization (IVF) child; (ii) the implementation of intracytoplasmatic sperm injection; and, more recently, (iii) the development of successful protocols of vitrification for cryopreservation of embryos and oocytes. Veterinarians’ contribution and participation in the field of human assisted procreation could be distinguished between technological development and/or clinical embryology. Despite of the 3 year delay on the birth of the first in vitro fertilization calf in relation to the birth of the first IVF baby, many steps and protocols of these technological advancements relied on the knowledge acquired from animal models or biotechnologies now used for commercial purposes in animal production and breeding. Furthermore, new embryo culture technologies such as microfluidics environments and controlled atmospheres are initially evaluated in animal models. Another example of the role of veterinarians on the development of human assisted reproduction is the emergence of the widespread concept of ovarian dynamics pattern of follicular waves in monovulatory animals from Theriogenology to Reproductive Medicine in the last decade. The translation of this concept to Reproductive Medicine might allow the development of different approaches of controlled ovarian stimulation. In a human assisted reproduction clinic, the clinical embryology laboratory is responsible for selecting oocytes after transvaginal ovum pick-up, fertilizing these oocytes, cultivating the supposed zygotes until the stage of 8 cell embryos or blastocysts, and embryo preparation for transference to the uterine cavity. Several tasks enrolled in these steps of human IVF are identical to those performed by veterinarians in an animal IVF facility; however, a veterinarian must be familiar to the specific guidelines and needs of a human IVF laboratory.

Conclusion: Assisted reproduction treatments are performed by groups of professionals with different academic backgrounds. Veterinarians have been playing an important roles in the development and/or adaptation of several biotechnologies utilized for human procreation; additionally, veterinarians working on animal in vitro fertilization facilities have an adequate formation and skills to act on and/or supervise clinical embryology laboratories after specific education, practice and certification.

Keywords: assisted reproduction techniques; in vitro fertilization; embryo culture; controlled ovarian stimulation.

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I. INTRODUCTION

Infertility is a disease [1] affecting 10% of the human population in reproductive age and couples experiencing difficulties to achieve pregnancy may benefit from assisted reproduction treatment.

Human assisted reproduction comprises a series of methods and techniques to treat infertility from timed intercourse to in vitro fertilization (IVF) by intracytoplasmatic sperm injection (ICSI) [2]. Approximately, 55,500 human embryos were transferred in 21,285 IVF cycles in Latin America according to the 2007’s Registro Latinoamericano de Reproducción Asistida [3]. Centers for treatment of infertility located in Brazil had performed a considerable amount of these IVF cycles (14,428; 42.3%) confirming the great experience of the Brazilian infertility specialists and embryologist in ovulation induction, ovum pick-up, and clinical embryology [3].

Coincidentally, Brazil is the world leader in in vitro production (IVP) of bovine embryos. According to Sociedade Brasileira de Tecnologia de Embriões there was a 27% increase in the number of IVP embryos from 2003 to 2007. Additionally, in 2007, 245,257 bovine embryos were produced in the world and the Brazilian yield accounted for 86.6% of this amount [4]. Ninety four percent of these embryos were originated from Zebu cows with technology specifically designed for these breeds [4]; thus Brazilian theriogenologist are also developing new protocols and technologies.

Apparently, animal and human assisted reproduction are parallel fields; nevertheless, there are several intersections in the progress of these disciplines, and the interchange of knowledge between veterinarians practicing animal reproduction and those professionals enrolled in human reproduction such as biologists, physicians and nurses might contribute to the development of more efficient protocols and techniques in both areas.

II. ASSISTED REPRODUCTION EDUCATION IN VETERINARY MEDICINE AND MEDICINE

The Ministry of Education of Brazil established guidelines for the instruction of undergraduate students in several traditional careers, among them Veterinary Medicine. These documents determine the minimal knowledge and skills of the professionals of each area; for Veterinary Medicine, the freshly graduated professional is expected to manage and perform assisted reproduction techniques [5].

Veterinary Medicine Schools line up Theriogenology knowledge in several disciplines from physiology to biotechnology of reproduction to accommodate the Veterinary Medicine teaching to Federal guidelines. The specific duration of Theriogenology training varies from 4 to 8% of the educational duration [6-8]; however, principles of reproductive physiology and management are applied in various other subjects as in medicine and breeding of several species of companion and production animals [6-9]. Furthermore, there are several graduate student programs in Animal Reproduction and Reproductive Sciences held by outstanding Universities [10].

Conversely, the guideline for the instruction of future medical doctors determines that the students should acquire several abilities; nevertheless, it does not mention the need to treat infertility or to perform or manage assisted reproduction treatments [11]. Thus, assisted reproduction and infertility treatment comprise a minimal portion of the medical education and these issues would be addressed in the 3rd year of the medical residency in Obstetrics and Gynecology, but they are limited up to 15% of the time spent in the Obstetrics ambulatory [12]. Consequently, few physicians have received education to deal with reproductive issues and assisted reproduction.
III. THE INTERSECTION BETWEEN ANIMAL AND HUMAN ASSISTED REPRODUCTION

Despite of the 3 year delay on the birth of the first in vitro fertilization calf in relation to the birth of the first IVF baby [13-14], many steps and protocols of these technological advancements relied on the knowledge acquired from animal models [15].

Veterinarians’ contribution and participation in the field of human assisted procreation could be distinguished between technological development and/or clinical embryology.

New embryo culture technologies such as microfluidics environments and controlled atmospheres are initially evaluated in animal models [16-18], as well as, some quality control programs for IVF laboratories are performed with the evaluation of mouse’s embryo development [19].

Another example of the role of veterinarians on the development of human assisted reproduction is the emergence of the widespread concept of ovarian dynamics pattern of follicular waves in monovulatory animals from Theriogenology to Reproductive Medicine in the last decade [20-24]. The translation of this concept to Reproductive Medicine might allow the development of different approaches of controlled ovarian stimulation [25].

In human assisted reproduction clinics, the clinical embryology laboratory is responsible for preparing media and dishes, selecting oocytes after transvaginal ovum pick-up, fertilizing these oocytes, cultivating the supposed zygotes until the stage of 8 cell embryos or blastocysts, and embryo preparation for transfer to the uterine cavity [26-27]. Several tasks enrolled in these steps of human IVF are identical to those used by veterinarians for commercial purposes in an animal IVF facility for production and breeding; however, a veterinarian must be familiar to the specific guidelines and needs of a human IVF laboratory [26-27].

The mutual interaction between human reproduction professionals and theriogenologists might also bring new protocols and technologies for animal reproduction, such as in oocyte cryopreservation.

The successful cryopreservation by slow rate freezing of human oocyte was initially described in 1986 [28]. However, this technique had low efficiency and several improvements were developed in Italy due to legal constrains [29-34]. However, several reports indicate that vitrification of oocytes is superior to slow rate freezing [35] yielding laboratorial and clinical outcomes similar obtained with fresh oocytes [36-37]. Over than 900 normal babies were born after oocyte cryopreservation [38] and the transposition of the experience in oocyte vitrification from human reproduction to animal IVF might be beneficial and meet the commercial requirements for animal production purposes.

IV. CONCLUSIONS

Veterinarians have been playing important roles in the development and/or adaptation of several biotechnologies utilized for human procreation; additionally, veterinarians working on animal in vitro fertilization facilities have an adequate formation and skills to act on and/or supervise clinical embryology laboratories after specific education, practice and certification.

REFERENCES

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Application of the Concept of Follicular Waves in Human Assisted Reproduction

Paulo Homem de Mello Bianchi

ABSTRACT

Background: Ovulation induction (OI) is one of the cornerstones in human assisted reproduction treatments. OI is based on the understanding of ovarian follicular physiology. Current knowledge of human follicular dynamics relies on hystologic observations of ovaries in distinct moments during one menstrual cycle (interval between two menses). According to this data follicles in different stages of maturation are seen at any time during a menstrual cycle. Initial recruitment and grow of the primordial follicles is still a poorly understood process, but continued development of antral follicles, selection of the dominant follicle and ovulation depends on follicle stimulating hormone (FSH) and luteinizing hormone (LH) serum levels, which are influenced by estradiol and progesterone concentrations. With the degeneration of the corpus luteum at the end of the luteal phase, serum estradiol and progesterone concentrations fall, leading to menstruation and an increase in FSH levels. All this observations have led to the formulation of the current theory of human follicular dynamics by which follicles continuously grow from the primordial pool randomaly, but only those which happen to be at the antral stage (FSH sensitive) during menses (FSH elevation) may continue to develop until the pre ovulatory stage and ovulate. Accordingly, in current OI protocols for human in vitro fertilization (IVF) stimulation usually begins 2 to 5 days after the first day of menstruation.

Review: Recently a new model of human folliculogenesis, based on the occurrence of follicular waves, has been described. Major development of medical ultrasound technology has allowed for the precise detection of small antral follicles in the transvaginal route. Using this technology and shifting the landmark for observations from menses to ovulation, synchronous grow of a group of antral follicles (follicular wave) has been observed two or three times in an inter ovulatory interval. Emergence of a wave is preceeded by an elevation in FSH levels. The first wave emerges around ovulation and the last is the ovulatory follicular wave. This is in accordance with previous observations in other mono ovulatory animals like the cows and mares, but in contrast with the current model of human folliculogenesis. Application of follicular wave knowledge has proved to be very usefull to the development of more effective OI protocols used in Veterinary Medicine. It has been shown that synchronization of OI with wave emergence results in more oocytes and embryos with better quality in cows. Protocols to control wave emergence are currently standart of practice for in vitro production of some animals' embryos. The description of the follicular waves in humans gives the opportunity to develop new OI protocols for IVF. Synchronization of OI with wave emergence could be achieved through mechanical (aspiration of the dominant follicle folowed by ovarian stimulation) or chemical interventions (initiate ovulation or using high doses of estradiol and progesterone in any phase of the cycle, followed by ovulation induction). However some differences between in vitro production of animal embryos and Human IVF should be enfasized and could challenge the application of this knowledge. For instance, Human embryos are usually obtained and transfered to the same subject, therefore a synchronization of embryo and endometrial development is a major concern.

Conclusions: Description of follicular waves in Human ovaries could have profound implications in OI for IVF, but some challenges should be expected to transpose the animal model to the Human setting.

Keywords: Human follicular waves; ovulation induction; in vitro fertilization.
I. INTRODUCTION

II. CURRENT THEORY OF HUMAN FOLLICULOGENESIS AND OVULATION INDUCTION PROTOCOLS

III. DESCRIPTION OF THE FOLLICULAR WAVES IN THE HUMAN OVARIES

IV. POSSIBLE APPLICATIONS OF THE FOLLICULAR WAVE CONCEPT TO HUMAN ASSISTED REPRODUCTION

V. DISCUSSION AND CHALLENGES

I. INTRODUCTION

Despite much development in laboratory techniques in the past years, ovulation induction is still one of the cornerstones in assisted reproduction treatments in humans [10]. The proper use of exogenous gonadotropins to increase the number of mature oocytes available at one time relies on the understanding of key events of reproductive physiology. In this article we are going to review the theory of human ovarian follicular dynamics on which current ovulation induction protocols are based, indicate evidence of the occurrence of follicular waves in human ovaries and speculate on the impact of this alternative physiologic paradigm to ovulation induction protocols and human assisted reproduction.

II. CURRENT THEORY OF HUMAN FOLLICULOGENESIS AND OVULATION INDUCTION PROTOCOLS

Since menses are a very distinct event during the women's reproductive years, menstruation has been used as a landmark in the studies of the female reproductive system. Cyclic changes in reproductive organs, tissues and sexual hormones between two menses are called the menstrual cycle. Didactically there are two distinct phases during an intermenstrual interval. In the follicular phase, just after menses, follicles grow, produce estradiol and ultimately ovulate. The ovulated follicle becomes the corpus luteum, which produces progesterone and dominates the next phase, the luteal phase. If pregnancy did not occur, corpus luteum degenerate in fourteen days, hormonal levels fall and a new cycle begins [25].

The current theory of human follicular dynamics is derived from histologic observations of excised ovaries in different moments during the menstrual cycle [16] together with serum and follicular fluid steroid measurements of women and animal models in several physiologic and pathologic conditions [11].

Germ cells can be identified in ovaries of human embryos since the 16th week of gestation. Through mitotic divisions the number of germ cells in the ovaries reach its maximum at around 20 weeks of gestational age (maximal ovarian reserve) [25]. Germ cells in the fetal ovaries are surrounded by flattened cells from the germinative epithelium (early granulosa cells) constituting the primordial follicle. From the 20th week of gestational age on mitotic activity of germ cells in the fetal ovaries ends and their number decrease progressively [11]. Gradually primordial follicles activate and begin the process of maturation. Granulosa cells become cuboidal and proliferate to form a multilayer surrounding the oocyte [11,17]. Stromal cells adjacent to the granulosa transform to constitute the theca, early sensitive to the actions of the luteinizing hormone (LH) [9]. Later a cavity is formed in the center of the follicle, the antrum, which separates two groups of granulosa cells, the cumulus oophorus, surrounding the oocyte, and the parietal cells, close to the theca [11]. At this stage granulosa cells acquire follicle stimulating hormone (FSH) receptors [18,9]. A few follicles are able to progress beyond this point, reach full maturation (with a big antrum) and finally ovulate. The great majority of follicles actually suffer atresia through apoptosis [11]. Primordial follicles that begin the process of maturation during the fetal stage and childhood are fated to suffer atresia in the early stages of the process [19]. Only during the menarche (reproductive years) a small proportion of follicles are able to reach full maturation and ovulate.
Morphometric studies showed that ovarian follicles in different stages of development are present throughout the menstrual cycle [16]. The transition from primordial to mature follicles takes several months [19]. The mechanisms responsible for the activation of the primordial follicles and beginning of follicular development are still unknown, but it appears that the initial steps of folliculogenesis are not under the control of gonadotropins [24]. From these observations it has been postulated that activation of the primordial follicle and development until the early antral stage occurs continuously and randomly [11].

Final follicular development (from the early antral stage on) and ovulation, on the other hand, is clearly dependent on FSH and LH actions [11]. Follicles in the antral stage demand different concentrations of FSH to continue to grow [11,8,26]. FSH concentration increase above the stimulatory threshold in the late luteal phase [20]; at this time follicles that happen to be in the antral stage grow and produce estradiol. Estradiol has a negative feedback effect on the production and secretion of FSH and ultimately FSH concentration fall below the threshold again. The time period in which FSH concentrations are above the stimulatory threshold is called FSH window [11]; during this period all follicles that happen to be FSH sensitive (antral stage), are able to grow.

When FSH concentrations fall below the threshold and the FSH window is closed, usually just one follicle continues to grow, becoming the dominant follicle, and eventually ovulates. Actually this follicle is sensitive to lower concentrations of FSH [11] and, during the common follicular growth phase, its granulosa cells also acquire LH receptors [27]. Since during the follicular phase small concentrations of LH are present, the dominant follicle is able to further develop also under the stimulation of LH. This is the mechanism of dominance responsible for the selection of just one follicle to complete the development and ovulate each cycle [11].

Based on the observations above it has been postulated that primordial follicles continuously and randomly activate and start to grow since the intra-uterine life until menopause [16]. Most follicles suffer atresia but those, which happen to be sensitive to FSH (antral stage) during the rise in FSH concentrations, that occurs in the late luteal and early follicular phases, continue to grow and eventually ovulate. This model of folliculogenesis has been called the Propitious Moment Theory [2]. Final follicular development until ovulation is expected to occur only during the follicular phase of the cycle [25]; the presence of the corpus luteum after ovulation is thought to inhibit follicle development to a mature follicle.

This model of folliculogenesis has been the base for current ovulation induction protocols in human assisted reproduction treatments. Administration of exogenous gonadotropins takes place in the early follicular phase, beginning more precisely between the second and fifth day of the menstrual cycle. The objective is to extend the FSH window allowing for more follicles to complete all stages of development and produce a greater number of mature oocytes for fertilization [11].

III. DESCRIPTION OF FOLLICULAR WAVES IN THE HUMAN OVARIAN

Ultrasound is a valuable tool to investigate folliculogenesis since it allows for noninvasive, in vivo and dynamic imaging of the ovaries, contrasting with the still images provided by histological evaluations. With the development of high definition ultrasound technology, currently it is possible to see follicles as small as 2 mm in diameter, through the transvaginal route.

Intrigued by previous ultrasound observations of follicular activity during the luteal phase, which challenged the prevailing theory of human folliculogenesis, Dr Angela Baerwald and co-authors decided to investigate ovarian physiology using serial high definition transvaginal ultrasound and serum hormonal determinations in an interovulatory interval, instead of an intermenstrual interval, of fifty healthy, normo-ovulatory women [3,4].

Changing the landmark for observations from the menstrual to the ovulation and using serial ultrasound imaging provides a dynamic, ovarian centered view of folliculogenesis. Their data shown that follicles rather develop in coordinated and synchronous groups appearing two (68% of women) or three (32% of women) times during an interovulatory interval [3]. The first group of follicles starts to grow on the day of ovulation; the interovulatory interval is shorter for women with two groups of follicular development comparing to those with three; in the last group a follicle always ovulate [3,4]. The coordinated growth of a cohort of follicles has been previously described in other mono ovulatory animals, like the equines and bovines, and has been called follicular waves [12,13,14,15,21].
Wave emergence is preceded by a rise in FSH concentrations. Selection of the dominant follicle occurred when a follicle reached 10 mm or more in diameter; it only takes a few days from wave emergence to selection of the dominant follicle. Only a small proportion (15%) of women with two wave pattern had a dominant follicle in the first wave. Nineteen percent of women with three waves pattern had a dominant follicle in all three waves. Dominant follicles of anovulatory waves didn’t reach an ovulatory diameter (20 – 22 mm). The interval between wave 1 and 2 in women with two waves pattern was approximately 14-15 days [3,4], meaning that the last follicular wave emerges just before or in the beginning of menstruation. In women with three waves pattern the second wave emerges 11-13 days after the first [3,4] (before menstruation) and the last one emerges 6 – 7 days after the second [3,4] (after menstruation).

All this observations are in accordance with studies in other mono ovulatory animal species like the equines and bovines [12-15]. On the other hand it is in sharp contrast with the current theory of random follicular growth; moreover, it shows that follicular development occurs also in the luteal phase, not only in the follicular phase as thought until now.

IV. POSSIBLE APPLICATIONS OF THE FOLLICULAR WAVE CONCEPT TO HUMAN ASSISTED REPRODUCTION

The follicular wave concept has long been used in Veterinary Medicine for in vitro production of animal embryos, particularly in cows. It has been shown that when ovarian stimulation with exogenous gonadotropins starts at the begging of a follicular wave, there are more mature oocytes at the egg retrieval and embryos are of better quality [23,1].

Detection of ovulation and consequently the emergence of the first wave would be the most obvious approach, but since ovulation detection is a time consuming process considering a herd, protocols to synchronize wave emergence with initiation of gonadotropin administration have been developed. There are mechanical and pharmacological strategies to determine wave emergence. The mechanical strategy relies on the ablation of the dominant follicle before ovulation through aspiration. Shortly after aspiration serum concentrations of estradiol and inhibin fall; without the inhibitory effect of estradiol and inhibin on the hypothalamus and pituitary, FSH concentrations rise followed by a wave emergence [5]. However, this strategy is not common in theriogenology because it is an invasive and time consuming procedure [22].

The pharmacological strategy consists in administer a boost of parenteral estradiol and progesterone anytime during the estrous cycle. The consequent rise in serum concentrations of estradiol and progesteone lowers both FSH and LH concentrations, causing atresia of the dominant follicle and, in the luteal phase, luteolysis. In either scenario, after metabolization of the exogenous estradiol, endogenous FSH rises initiating a new follicular wave [7]. Therefore ovulation induction could be initiated four days after the hormonal boost [22].

Current ovulation induction protocols for human assisted reproduction, based on the prevailing theory of human folliculogenesis, are not synchronized with follicular wave emergence but rather with menstruation. Starting gonadotropin administration in the second to fifth day of the menstrual cycle probably does not coincide with the beginning of a follicular wave, particularly for women with a three wave pattern. Actually, by that time several women have probably already selected a dominant follicle and the subordinate ones could have initiated the atresia process.

Since the follicular wave phenomenon in humans appear to be very similar to the cows, similar protocols to control wave emergence and synchronize it to the beginning of ovarian stimulation could theoretically increase the number of mature oocytes, reduce the requirements of exogenous gonadotropins and improve embryo quality, and consequently pregnancy rates.

We have recently published a reiew article where we speculate on possible protocols for synchronize ovulation induction with wave emergence in humans [6]. Briefly, aspiration of the dominant follicle (or induction of ovulation with hCG) followed some days after by gonadotropins administration are probably the easier strategies to do this synchronization, but rely on the identification of a clearly dominant follicle on the follicular phase of the cycle. Conversely, exogenous estradiol and progesterone boost is expected to be a more flexible strategy since it could be used anytime during the cycle, but concerns might rise regarding possible side effects of a hormonal boost and optimal timing and hormonal requirements to induce wave emergence [6].
V. DISCUSSION AND CHALLENGES

Application of the follicular wave concept in human assisted reproduction is very promising based on the results observed in farm animals. A particular useful application of this concept is for fertility preservation in women with cancer, when the time span between diagnosis and initiation of chemotherapy or radiotherapy often is not sufficient to wait for menstruation and perform a conventional ovulation induction protocol.

However, some challenges and differences between human treatments and animals’ should be discussed.

Assisted reproduction in animals, particularly in cows, is intended to produce an elevate number of animals with high genetic potential. Donor animals are usually young and healthy individuals; embryos produced are transferred to several recipients in order to increase the number of descendants at each time. In humans, on the other hand, assisted reproduction is intended to help patients with some degree of subfertility/infertility; embryos are often transferred to the same woman that was submitted to ovarian stimulation. This carateristics have two main consequences: first, results of treatment could be inferior in women comparing to animals; second, concerns should be raised with the endometrial quality of the patient. Ovulation induction synchronized with wave emergence could be asssynchronous to the endometrial development with possible impairment of implantation. Vitrification of embryos for posterior endometrial preparation and transference is an option if endometrium assynchrony occurs.

Protocols to determine follicular wave emergence based on animal models have to be tested in humans. For instance if aspiration of the dominant follicle occurs before complete dominance has been established, ovulation induction can cause the subordinate follicles in the same wave to grow and not a new wave to emerge. The exact size of the dominant follicle when complete dominance is stablished in humans should be investigated to avoid this situation. Moreover, the interval between follicle aspiration and wave emergence in humans should be determined to set the ovulation induction.

The dose and duration of administration of exogenous steroids to cause atresia of the dominant follicle and lutheolysis should also be determined, as long as the time interval between steroids administration and wave emergence.

Also, side effects of such pharmacological manipulation should also be weighted in cost benefits analysis before it become standard of practice.

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Diagnóstico Genético Pré-Implantacional

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ABSTRACT

Background: The ranks of patients seeking preimplantation genetic diagnosis (PGD) to identify embryos with monogenic disorders like cystic fibrosis or thalassemia are growing rapidly. Even so, the most common indication for preimplantation embryo testing remains the risk of chromosomal imbalance. In most cases, the PGD strategy employed for chromosomal testing involves biopsying a single cell (blastomere) from each embryo at the 6 to 10-cell stage, 3 days after fertilization. The cell is placed on a microscope slide, fixed, and then subjected to cytogenetic analysis. While the biopsied cell is being analyzed, the rest of the embryo is maintained in culture. Most infertility clinics prefer to transfer the embryos no later than day-5 post fertilization. Consequently, PGD methods need to be extremely rapid, providing a result in less than 48 hours.

Review: Most chromosomal PGD protocols employ fluorescence in situ hybridization (FISH). This approach involves the hybridization of chromosome specific DNA probes, labeled with different colors, to nuclei spread on a microscope slide. The method is rapid, performs equally well whether applied to interphase nuclei, and permits enumeration of up to 10 chromosomes per cell. Initially, the PGD for aneuploidy was envisioned that most of the patients seeking PGD for aneuploidy would be those who carry a chromosomal rearrangement. Couples in which one of the partners carries a chromosomal rearrangement frequently experience miscarriage or bear children due to chromosome imbalance. However, in recent years the vast majority of patients requesting PGD for aneuploidy have in fact been chromosomally normal individuals undergoing IVF. Early methods employed five FISH probes for PGD, focusing on the chromosomes most often found to be abnormal in prenatal samples (13, 18, 21, X, and Y). Aneuploidies for these chromosomes are sometimes compatible with survival to term, leading to aneuploid syndromes. This strategy was successful in reducing the number of such syndromes, but a statistically significant improvement in embryo implantation could not be shown. Later PGD studies expanded the number of chromosomes assessed to eight. This was achieved by performing two sequential rounds of FISH analysis, assessing five chromosomes in the first round and three more in the second. The three new chromosomes added to the PGD screen (15, 16, and 22) are frequently found to be aneuploid in miscarriages. The eight-chromosome PGD protocol led to a doubling of embryo implantation rates in two separate studies and reduced the number of spontaneous abortions. Although this problem can be partially overcome by performing sequential FISH experiments on the same cell, the accuracy of the method declines with each additional hybridization. The good news is that there is a method related to FISH—comparative genomic hybridization (CGH)—that can detect aneuploidy that affects any chromosome within a sample. In addition to offering comprehensive detection, CGH can be used on interphase cells.

Conclusion: In summary, the use of FISH for the purpose of PGD has already improved IVF outcome for several groups of infertile patients, including women aged 37 and above, those with recurrent miscarriages, women with a previous comprehensive screening for aneuploidy will likely further increase the benefit of PGD to these patients and maybe even a broader range of patients. The widespread availability of CGH for PGD is nearly at hand, but while we await final refinements and validation, PGD strategies can still be improved by making the best use of current methods and reassessing the chromosomes selected for screening.

Keywords: PGD, aneuploidy, FISH, CGH.

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I. INTRODUÇÃO

a. Diagnóstico Genético Pré-Implantacional (PGD)

Estudos citogenéticos por meio da técnica de FISH mostraram que mais de 50% dos embriões em estágio pré-implantacional possuem células aneuplóides [1]. Considerando que a maioria dessas alterações impede o desenvolvimento embrionário e consequente implantação, a identificação de embriões geneticamente normais é crucial para a obtenção de gestações viáveis [2]. A identificação confiável de embriões euplóides (cromossomicamente normais) só pode ser determinada pelo Diagnóstico Genético Pré-implantacional (PGD). O PGD envolve a interação entre as técnicas de reprodução assistida, a biópsia de blastômero ou do corpúsculo polar e posterior análise da célula única, possibilitando o diagnóstico de doenças geneticamente herdadas em embriões humanos [3,4].

A aplicação clínica desse método proporciona aos casais com alto risco reprodutivo, maiores chances de terem filhos não afetados, identificando os embriões portadores de alterações cromossômicas e evitando que os mesmos sejam transferidos. Segundo Ludwig e colaboradores (2001), a técnica permite diminuir o risco de transmissão de alterações genéticas em mais de 95% [5]. Além de evitar descendentes afetados pelas principais Síndromes Genéticas, o diagnóstico pré-implantacional é uma alternativa para o diagnóstico pré-natal, principalmente nos países em que o aborto terapêutico não é permitido.

Os primeiros casos de Diagnóstico Genético Pré-Implantacional em embriões humanos foram descritos independentemente por Handyside e colaboradores (1990) e Verlinsky e colaboradores (1990) [3,4]. Desde as primeiras tentativas na década de noventa, centenas de PGD têm sido realizados, demonstrando uma crescente aceitação da metodologia por diferentes populações. De acordo com a classificação determinada pela ESHRE PGD Consortium, o diagnóstico genético pré-implantacional deve ser dividido em duas categorias de acordo com as características do casal: (1) PGD de alto risco, que inclui casais com anomalias cromossômicas e/ou doenças monogênicas; (2) PGD de baixo risco, que tem por objetivo aumentar a taxa de implantação em casais com idade materna elevada (em torno de 37 anos), perdas fetais de repetição ou com frequentes falhas de fertilização in vitro. Atualmente, sua indicação mais comum é o screening para aneuploidias em embriões gerados por mães com idade acima de 37 anos [6].

b. Aplicações do Diagnóstico Genético Pré-Implantacional

i. Análise do Primeiro Corpúsculo Polar

Na gametogênese feminina, durante a primeira divisão meiótica, um dos cromossomos homólogos de cada bivalente segrega para o primeiro corpúsculo polar (1CP) e o outro para o núcleo do oócito (MII) que se encontra em metáfase II da meiose. Desta forma, conclui-se que o 1CP tem o conteúdo genético complementar ao núcleo
MII, o que significa dizer que um desequilíbrio em MII implicará na alteração recíproca no corpúsculo polar.

Considerando que o 1CP por sofrer extrusão não tem função reprodutiva, a sua biópsia e posterior análise permitem a caracterização indireta da constituição cromossômica do MII e a identificação dos óocitos aneuplóides [7] sem comprometer a viabilidade do gameta.

ii. Análise de Blastômeros

Trata-se da metodologia mais empregada no PGD [3,8]. Esse procedimento envolve a dissolução de uma região da zona pelúcida e a aspiração de uma ou, no máximo, duas células embrionárias. A remoção de um blastômero em embrião com o equivalente de 6-8 células parece não afetar o seu desenvolvimento, in vitro, para o estágio de blastocisto sendo considerada uma técnica eficiente.

A vantagem mais evidente é que, nesta metodologia, se analisa tanto a contribuição paterna quanto a materna, pois neste estágio a constituição genética do embrião está completamente formada; tornando o PGD em blastômeros um método comparável ao diagnóstico pré-natal. Por outro lado, a desvantagem desta técnica é a coexistência de mais de uma linhagem celular no embrião (linhagens cromossomicamente normais e linhagens aneuplóides), fenômeno chamado de mosaicismo embrionário, que tem origem pós-zigótica e que tem sido detectado em uma porcentagem considerável de embriões [9]. A presença deste mosaicismo dificulta o diagnóstico citogenético pré-implantacional em blastômeros [10], sobretudo nos casos em que apenas uma célula é analisada. Em 2004, Sermon e colaboradores descobriram a necessidade de um estudo prospectivo e randomizado que comparese a viabilidade embrionária quando realizado a biópsia de uma ou duas células e a incidência de erros no diagnóstico citogenético dos blastômeros [11]. Atualmente esta questão parece, ainda que de forma preliminar, estar solucionada.

iii. Análise de Blastocistos

Este tipo de biópsia é a mais tardia que se pode proceder. Consiste em cultivar o embrião in vitro até alcançar o estágio de blastocisto (aproximadamente 5 dias após a fecundação) e remover de 10 a 30 células. As vantagens são: proporcionar a análise de diversas células e a seleção dos embriões mais viáveis pela manutenção do seu cultivo até a fase de blastocisto [13]. A principal desvantagem é que não restam mais do que poucas horas após a biópsia para a realização do diagnóstico, já que segundo o informe do ESHRE PGD Consortium Steering Commitee (2004), a transferência embrionária deve ser realizada no máximo no 6º dia pós-fecundação. Além disso, muitos centros de reprodução assistida têm dificuldades para cultivar os embriões in vitro até blastocisto e, por esta razão, são poucos os centros no mundo que praticam esta forma de biópsia [14].

c. Screening para Aneuploidias (PGS)

É a aplicação mais frequente do PGD. As anomalias cromossômicas numéricas estão relacionadas tanto com falhas de implantação quanto com mortalidade e perda embrionária resultando em abortos espontâneos. A incidência de anomalias cromossômicas é de 1,4% em embriões de mães com até 34 anos de idade e aumenta para 52,4% em mulheres entre 40-47 anos [15]. Da mesma forma, também foi detectada uma incidência elevada de alterações cromossômicas em embriões de casais jovens com mais de três falhas de implantação (>55% de embriões são aneuplóides) e em casais com pelo menos dois abortos espontâneos (>68%) [16].

Shahine & Cedars (2006) questionaram se a aplicação do PGS em gametas e embriões poderia ajudar a aumentar as taxas de implantação e gestação em casais com idade materna elevada, em casais com falhas recorrentes de implantação, em mulheres que apresentam abortos espontâneos de repetição e também em casais portadores de translocação equilibrada para detectar alterações dos cromossomos envolvidos no rearranjo [17]. Baseados em dados de 20.000 ciclos de PGD, Kuliev & Verlinsky (2008) relataram que o efeito positivo do PGS é evidente quando comparadas as taxas de sucesso reprodutivo dos mesmos pacientes, antes e depois da realização do diagnóstico [18].

II. TÉCNICAS DE CITOGNÉTICA-MOLECULAR

O uso das técnicas de citogenética molecular tem favorecido os estudos em embriões humanos. Baseadas na utilização de diversos tipos de sondas de DNA (centroméricas, loci, específicas ou pinturas cromossômicas) e associadas a diferentes fluorocromos, essas metodologias vêm se aprimorando com a evolução dos microscópios.
e dos sistemas de captura. Atualmente, as técnicas de citogenética molecular constituem tanto protocolos de pesquisa quanto de diagnóstico, adicionando sensibilidade e especificidade aos resultados obtidos por citogenética convencional.

A hibridação in situ fluorescente (FISH) se tornou a técnica padrão para a avaliação de aneuploidias, sendo possível analisar de duas a nove sondas cromossomo-específicas divididas em uma, duas ou três etapas de hibridação [19]. Cabe destacar que o número de cromossomos que pode ser analisado em cada etapa está ligado ao número limitado de fluorocromos disponíveis e à possibilidade de sobreposição dos sinais [2, 20]. Em resumo, em uma situação ideal, é possível analisar somente cinco cromossomos em cada hibridação. Além disso, a quantidade de vezes que o material genético pode ser hibridado também é limitada, sendo observada perda considerável na qualidade da amostra a partir da terceira denaturação [21]. Entretanto, o parâmetro técnico mais desfavorável é que a metodologia empregada requer a fixação dos núcleos em lâminas de vidro, o que aumenta o risco de perda cromossômica por artefato de técnica. Em geral, as técnicas que envolvem fixação celular em lâminas são eficientes em diagnosticar casos de hiperploidia, falhando na detecção de hipoploidias. Com o intuito de superar esta limitação técnica, Wells e colaboradores (1999) e Wells & Delhanty (2000) descreveram e aprimoraram a aplicação da Hibridação Genômica Comparativa (CGH) [24] em célula única [22, 23].

III. HIBRIDAÇÃO GENÔMICA COMPARATIVA

Esta metodologia permite detectar desequilíbrios no número de cópias de qualquer um dos 23 pares cromossômicos em apenas uma hibridação. A primeira aplicação da CGH no diagnóstico pré-implantacional foi descrita em blastômeros [25].

De acordo com a literatura, estudos por meio da CGH revelaram que de 25 a 30% dos embriões diagnosticados como normais após análise de FISH para nove cromossomos apresentavam aneuploidias para outros cromossomos [26]. Estes dados enfatizaram a importância da análise dos 23 pares cromossômicos na eficiência do diagnóstico pré-implantacional. A busca por umPGD altamente eficaz não apenas evita o nascimento de crianças afetadas por cromossomopatias como também favorece a transferência de um único embrião para o útero materno, contribuindo com a diminuição dos riscos envolvidos em gestações gemelares.

Atualmente a aplicação da CGH no diagnóstico pré-implantacional tem sido muito discutida e, o que parece ser consenso entre os pesquisadores europeus e norte-americanos é que o screening para aneuploidias, por meio desta técnica, favorece a implantação embrionária aumentando assim as taxas gestacionais, independentemente se o diagnóstico foi realizado no óocito ou no embrião de terceiro dia ou até mesmo blastocisto [27].

V. CONCLUSÃO

A aplicação da FISH no diagnosis pré-implantacional tem favorecido o sucesso dos tratamentos de reprodução assistida para vários grupos de paciente inférteis, incluindo idade materna avançada e abortos de recorrência. No entanto, a aplicação de um screening completo para aneuploidias permitiria que o PGD fosse realizado de forma mais efetiva e abrangente, beneficiando um número maior de casais.

A uso definitivo da CGH no PGD está muito próximo de acontecer, mas enquanto esta metodologia espera pelas últimas validações, as estratégias adotadas hoje para o PGD ainda podem ser aprimoradas, principalmente no que diz respeito à escolha dos cromossomos selecionados para o screening.

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Microenvironment, Microfluidics, and Gametes and Embryos

Gary D. Smith

ABSTRACT

Background: In vitro fertilization (IVF) remains one of the most exciting modern scientific developments and continues to have a tremendous impact on people’s lives. Since its beginning, scientists have studied and critically analyzed techniques in order to find ways to improve outcomes; however, little has changed with the actual laboratory technologies and equipment of IVF. Semen is still processed in test tubes and fertilization and culture still occurs in culture dishes. This somatic cell culture approach has also infiltrated the embryonic stem cell (ESC) field.

Review: New technologic possibilities exist with the burgeoning advancement of nano- and microfluidic technologies. Microfluidics encompasses the study of physical principles of fluid behavior in a micro-environment and its application to chemistry, molecular biology, and cell biology. Two primary advantages exits in micro-scale cell isolation, culture and analysis. Firstly, microfluidics provides size and mechanical advantages not realized at the macro-scale. Secondly, microfluidics provides a micro-environment that is inherently more physiological and permissive to modifications that more closely mimic the in vivo developmental environment. Although a young field, many developments have occurred which demonstrate the potential of this technology for IVF. In this presentation, we briefly discuss physical principles of microfluidics and highlight some previous utilizations of this technology, ranging from chemical analysis to cell sorting. We then present designs and outcomes for microfluidic devices utilized thus far for each step in IVF: gamete isolation and processing, fertilization, embryo culture, and embryo analysis. Finally, we discuss and speculate on the ultimate goal of this technology – development of a single, integrated unit for in vitro assisted reproduction techniques.

Conclusion: Benefits of microfluidics for gamete isolation, gamete maturation, embryo culture and analysis, and growth and directed differentiation of embryonic stem cells are evident and progressing.

Keywords: microfluidics, gametes, embryos.
Embryo Culture: Concerns and Perspectives

Gábor Vajta

ABSTRACT

Background: Culture of preimplantation-stage embryos plays a crucial role in both domestic animal and human embryology. During the past 30 years, a considerable advancement has been achieved in this field. The development was most intensive in the 90's of the last century in domestic animals, and in the first decade of our millennium in humans. Due to the latter advancement, extended embryo culture and single blastocyst transfer is now the preferred method to decrease the chances of multiple pregnancies while preserving the overall efficiency of the treatment. However, there are still a lot of questions to answer and a lot of possibilities to improve the overall efficiency of mammalian in vitro embryo culture.

Review: In spite of the scientific challenge and considerable commercial interest continuously stimulating research worldwide, the understanding of the requirements is insufficient, opinions and theories are controversial and the consensus is missing even in the basic principles of physical, chemical and biological environment. Composition of media, use of two-phase versus single media, need of medium exchange are constant subject of debates, and some basic principles including the right temperature of incubation have also been questioned. The purpose of this review is to summarize and confront different opinions about these issues, explain pro’s and contra’s, demonstrate the fragility of some commonly accepted arguments, and stimulate a fertile debate towards improvements and optimization. An open-minded approach expanding, sometimes breaking the traditional frames of embryo culture is suggested. Domestic animal embryology with its unlimited quantitative possibilities, legal freedom and widespread experience may play a considerable part of this process. Issues may include improvements in the culture process itself, i.e. new media and supplements, different dishes, and changing mentality during embryo handling, but may also be extended to related techniques as non-invasive embryo selection methods and active treatments to improve embryo quality.

Conclusion: Although some researchers suppose that the currently applied in vitro culture systems have already approached the biological limits, a novel, critical and pragmatic approach may result in substantial improvement and may expand considerably the possibilities of future assisted reproduction in both domestic animals and humans.

Keywords: embryo, blastocyst, mammalian, culture, in vitro, evaluation.