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Factors that affect the *in vitro* production of bovine embryos: A review¶

Factores que afectan la producción in vitro de embriones bovinos: una revisión.

Fatores que afetam a produção in vitro de embriões bovinos. A revisão.

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Summary

In vitro embryo production (IVP) represents a way to increase gamete use from animals with high zootechnical value. In spite of the advances obtained in IVP over the last few years, production of transferable embryos is still low. The aim of this review is to discuss ways to produce in vitro embryos, as well as oocytes formation and maturation processes that can be related to the effectiveness of obtained results. Some studies show the influence of follicular growth factors, gonadotropins, steroids and other hormones on the follicular development and the quality of the cumulus oocyte complex (COC). The follicular phase of slow growth is critical for the development of the oocyte capacity to reach the final competence and diameter. Information about endocrine influences, or likewise, the dependence of growth of small antral follicles when a loss in the oocyte or follicle functionality occurs is scarce in the literature. A variable number of different techniques and protocols for treatment of oocytes donors are described with the aim of improve the results, the COCs recovering rate and the developmental competence in vitro of collected oocytes. From the considerations presented in this review, it is possible to verify the importance of better understanding the factors involved in the IVP process, with the aim of allow new alternatives to increase the results obtained in programs of animal assisted reproduction.

Key words: *bovine cattle, in vitro embryo production, in vitro oocyte maturation*

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Resumen

La producción in vitro de embriones (PIV) representa una manera de aumentar el uso de gametos de animales con alto valor zootécnico. A pesar de los avances obtenidos en PIV en los últimos años, la producción de embriones tranferibles sigue siendo baja. El objetivo de esta revisión es discutir maneras de producir embriones in vitro, así como los procesos de formación y de maduración de los oocitos que se pueden relacionar con la eficacia de los resultados obtenidos. Algunos estudios demuestran la influencia de los factores foliculares del crecimiento, gonadotrofinas, esteroides y otras hormonas en el desarrollo folicular y la calidad del complejo del cumulus oocito (CCO). La fase folicular del crecimiento lento es crítica para el desarrollo de la capacidad del oocito de alcanzar la capacidad y el diámetro final. Información sobre influencias endocrinas, o además, la dependencia del crecimiento de pequeños folículos antrales cuando ocurre una pérdida en la funcionalidad del oocito o del folículo, es escasa en la literatura. Un número variable de diversas técnicas y los protocolos para el tratamiento de oocitos de las donantes son descritos en esta revisión, con lo objetivo de mejorar los resultados, el índice de la recuperacion de CCOs y la capacidad de desarrollo in vitro de oocitos recogidos. De las consideraciones presentadas en esta revisión, es posible verificar la importancia de entender los factores implicados en el proceso de PIV, para permitir el desarrollo de nuevas alternativas que mejoren los resultados obtenidos en programas de la reproducción animal asistida.

Palabras claves: *ganado bovino, producción in vitro de embriones, maduración in vitro de oocitos*

Resumo

A produção in vitro (PIV) de embriões representa uma maneira de incrementar o uso de gametas de animais de alto valor zootécnico. Apesar dos avanços obtidos na PIV nos últimos anos, a produção de embriões transferíveis ainda é baixa. O objetivo desta revisão é discutir maneiras de produzir embriões in vitro, assim como o processo de formação e maturação de oócitos, que pode estar relacionado a eficácia dos resultados obtidos. Aguns estudos demonstram a influência de fatores de crescimento, gonadotrofinas, esteróides e outros hormônios no desenvolvimento folicular e na qualidade do complexo cumulus oócito (CCO). A fase folicular de crescimento lento é critica para o desenvolvimento da capacidade do oócito de atingir a competência e o diâmetro finais. Informação sobre as influências endócrinas, ou seja, da dependência do crescimento de pequenos folículos antrais quando ocorre perda da funcionalidade do oócito ou folículo são escassas na literatura. Um número variável de diferentes técnicas e protocolos para o tratamento de doadoras de ovóctios são descritos com o objetivo de melhorar os resultados, a taxa de recuperação de CCOs e o desenvolvimento da competência in vitro dos oócitos coletados. Das considerações apresentadas nesta revisão é possível verificar a importância do conhecimento dos fatores envolvidos no processo de PIV, com o objetivo de possibilitar que novas alternativas incrementem os resultados obtidos em programas de reprodução animal assistida.

Palavras chave: *embriões bovino, maturação in vitro de oocitos, produção in vitro de embriões*

Introduction

The increase in the demand for cattle embryos for commercial goals, as well as for research aims, resulted in an increased use of reproductive biotechniques, as *in vitro* embryo production (IVP). In this context, IVP is an alternative that accelerates the research in cattle reproduction and genetics (86). Some applications of the IVP technology include animal genetic improvement, production of a larger number of embryos for commercial usage, for example in high value cows with infertility problems, production of transgenic animals and embryo sexing (19, 20).

Despite the progress obtained in IVP during the last few years, the production of transferable embryos is still low (86). Many of these results have been attributed to the in vitro maturation and fertilization conditions. However, these poor results may be related to the developmental competence of oocytes collected from small follicles (2 - 6 mm diameter) (12).

To improve IVP efficiency, several works are suggesting hormonal treatments for donor cows, to synchronize follicular wave emergence, to have larger quantity of follicles and, consequently, oocytes, and to obtain better quality of oocytes and

better recovering rates (15, 48, 65). The aim of this work is to discuss alternatives to *in vitro* embryo production, as well as study the oocyte formation and maturation mechanisms that can be related to the efficacy of the results obtained in programs of assisted animal reproduction.

Physiological aspects and factors that can affect oocyte quality

The study of folliculogenesis and follicular growth factors is important in order to develop alternatives to increase the efficacy of biotechniques, such as estrous synchronization, embryo transfer and IVP. Some studies show the influence of follicular growth factors, gonadotropins and steroids on the follicular development and cumulus oocytes complex (COCs) (34, 70, 88).

The antral follicle growth in cattle, as well as in other species, presents two stages. A slow growth stage which lasts for around 30 days, from the antrum formation with 300 μm of diameter up to 3 to 5 mm of diameter (67). This stage is critical for the development of oocyte capacity, in which it reaches the final size and competence (35, 36). The fast growth stage occurs for 5 to 7 days and it extends from the follicular antrum detection with 3 mm, up to a possible ovulation of this follicle (92).

Information about endocrine influences or dependence on the growth of small antral follicles when follicular or oocyte functionality is compromised is scarce (27, 87). Experiments carried out in cows in which the release of gonadotropins regulated by hypothalamic GnRH was abolished by GnRH immunization (26, 44, 45) demonstrated that the first stage of the antral follicular growth can occur in an environment characterized by basal levels of follicle stimulating hormone (FSH) and without luteinizing hormone (LH) pulses. It is not clear how the growth of small antral follicles is possible under basal levels of FSH. The follicular wall, in this stage, is not responsive to FSH, once the follicles do not demonstrate progression from pre-antral stages up to development of small antral follicles in mice (2). However, the follicular wave during the second stage of antral follicular growth is absolutely dependent of increasing concentrations of FSH and adequate LH pulses (70).

Ovogenesis

The follicles and oocyte development in mammals starts in fetal life. Summarizing, the earliest (primordial) germinal cells multiply through mitosis until the ovogonias formed become primary oocytes. The oocytes progression to the first meiotic prophase begins, in cattle, between 75 and 80 days after conception (33). The meiotic prophase is composed by several stages: proleptotene, leptotene, zygotene, pachytene, diplotene (stage where the meiosis is interrupted). In cattle, the diplotene is reached in average 170 days after conception (9). Each stage presents a certain characteristic in the chromosomes (8).

The chromosomes, during the interruption of the meiosis become relaxed and a nuclear structure known as germinal vesicle (GV) is formed. The interruption of meiosis persists until the puberty, when one or more oocytes re-starts the reductive division, the GV in these oocytes disappears, the chromatin is recondensed, the pairs of homologous chromosomes are separated and half of them are expelled forming the first polar body. At this point the meiosis is interrupted again (in metaphase II). These events, started by the GV breaking and completed by the formation of the first polar body, lead to the production of a mature and fertile oocyte (28, 38, 54). The hormone responsible for the resumption of meiosis in vivo is the LH. Studies have demonstrated that oocyte maturation, in cattle, is closely related to the moment of the pre-ovulatory LH peak (101) and that the oocytes from small follicles (< 8 mm) do not resume meiosis due the lack of LH receptors in granulosa cells (10, 106).

The oocytes included in primordial follicles form a finite stock which only leaves this stage when are stimulated (33). However, results reported by Johnson *et al* (59) indicate that young adult rats have mitotic activity in germinative cells, necessary to maintain the follicular pool. The factors that regulate this growing are not yet well known.

Activation of pre-antral follicles and growth of antral follicles

The primordial follicles activation is characterized by transformation from flat to cuboid cell shape, as well as by the multiplication of the granulose cells, which surround the oocyte. The factors that stimulate granulosa cells multiplication are considered promoters of primordial follicle activation (21, 35).

A variety of factors are involved in the growth of pre-antral follicles. Lee *et al* (63) suggested that bone morphogenetic protein-7 (BMP-7) stimulates the primordial factors by inducing granulosa cell division, similar to insulin-like growth factor-I (IGF-I) (75) and epidermal growth factor-like (EGF) (55). Other factors are involved in the control of activation of primordial follicles, such as the anti-mullerian inhibiting hormone (AMH) (30). Several other growth factors are being associated to the pre-antral follicular growth, including kit ligand (81), basic fibroblastic growth factor (bFGF) (78), leukemia inhibitory factor (LIF) (77) and bone morphogenetic protein-15 (BMP-15) (80).

Recently, a hypothesis postulating that the oocyte develops since the stage of primary follicle (32) has been proposed. This is based on the action of the growth and differentiation factor-9 (GDF-9), the GDF-9 is apparently related to the ovarian factors of BMP regulation, which is mediated by the quantity of BMP-2 receptors (BMPR-II) (96). The GDF-9 mRNA expression has been detected in primary follicles of mice, rat and human (1, 52, 58) and in the primary and subsequent stages of cattle and sheep (13). These reports suggest that the primary action of GDF-9 is to promote the progression of primary follicles to antral stage, this mechanism, consequently, indirectly stimulate the transition of primordial follicles to the primary stages (78, 97). Infertile mice with GDF-9 deficiency cease follicular development in the primary follicles stage. Even though the oocytes of mice with reduction in GDF-9 levels grow in an accelerated way, the somatic cells cannot develop beyond the primary follicle stage (29). This blocking coincides with the beginning of the expression of GDF-9 and BMP-15, both detected in oocytes from primary follicles up to ovulatory follicular stages. The GDF-9 can also promote follicles progression by stimulating kit ligand expression at granulosa cells. However, the interaction between GDF-9 and kit ligand seems to be highly complex. Elvin *et al* (31) registered an increase in the expression of the *Kitl* gene (kit ligand) in mice follicles without GDF. Also, Eppig (32) suggests that this factor, produced by granulosa cells, promotes oocyte growth until the specie-specific diameter is reached. At this point GDF-9 is secreted by the oocyte and kit ligand expression is suppressed in the cumulus cells, reducing the growth rate or ending the oocyte growth (34).

The growth of antral follicles, differently from pre-antral follicles, is totally gonadotropins-dependant. Bovine estrous cycle presents two or three follicular waves, each of them preceded by an increase in FSH concentrations (3). This increase in FSH levels starts the growth of a cohort of FSH-dependent follicles $\geq$ 3 mm of diameter. The first wave starts in day 1 (D1) of the estrous cycle and the day 0 (D0) is considered the ovulation day. During the following three days, follicles $\geq$ 3 mm continue to grow until the follicular population in the ovary reach 4–8 mm of diameter in D3 (41, 51). At this stage, one of the follicles with a larger diameter starts to grow quicker and is selected as the future dominant follicle (DF). The remaining follicles will undergo atresia (41). This is due to the direct inhibitory effect that DF exerts on the development of subordinate follicles of cattle and sheep (62, 79, 105), causing their atresia (104). The factors involved in DF selection are more complex and also related to follicular growth factors, as well as to the presence of LH receptors in granulose cells, among other factors. However, as it is not the main purpose of this review, these aspects will not be addressed.

On D6, DF reaches its maximum diameter and remains functional from 2 to 4 days. When DF loses its functionality (regression phase), a new wave emerges. With luteal regression, DF from the second or third follicular wave remains functional followed by the increase in LH pulses, until LH peak occurs stimulating the final follicular and oocyte maturation and causing the DF ovulation (56).

LH has a characteristic of inducing changes in follicle and oocyte structure, being the main hormone responsible for the final follicular growth in the pre-ovulatory stage. The mechanism which is apparently associated with follicular deviation (selection), is the LH-mediated induction of LH receptors in granulosa cells, the increase in circulating estradiol levels and the FSH plasmatic decrease (88). In other words, the moment when follicular deviation is maximal is when the LH pulses start to increase.

Influence of follicular dominance in oocyte competence

During the growth of oocytes inside the follicles, some factors influence the quality and development of their competence. These factors include follicular diameter, day of the estrous cycle, atresia levels and influence of other follicles as DF (51). A higher number of blastocysts are observed when the oocytes are collected during the follicular growth phase than those collected during the follicular dominance phase (51).

Dominant follicle has an inhibitory effect on the development of subordinate follicles causing their atresia, mainly through inhibin and estradiol 17-β secretion (68, 104). In studies carried out by Hagemann *et al* (50) the suppressive effect of DF on the diameter and competence of oocytes from subordinate follicles was registered. In this study the development and the in vitro competence of oocytes collected during the growth phase of follicles from 3 to 5, 6 to 8, 9 to 12, and 13 mm, were 44, 47, 55, and 70%, respectively, higher than those recovered during the dominance phase (31, 27, 30, and 44%, respectively), showing the negative influence of the DF on the competence of oocytes from subordinated follicles.

Few reports demonstrate the DF influence on folliculogenesis. Bungartz and Niemann (22) and Lussier *et al* (66) evaluated, in embryo transfer programs, the DF removal at the moment of superstimulation, comparing with treatments in the presence of a DF. These studies indicated a higher number of ovulations and viable embryos when the DF is absent.

Follicles dissected during the dominance phase had more atresia than those dissected during the follicular growth stage. Reports from studies performed in sheep and cattle indicate that a moderate atresia does not affect negatively the oocyte competence, which can be better in follicles with low level of atresia (23, 68). This can be explained by structural changes observed in association with the degeneration of oocytes that happens in the subordinated follicles (11) similar to what occurs in pre ovulatory follicles close to the LH peak (4). This condition can give some advantages when atresia is recent (34).

In order to better evaluate these statements, more studies are necessary, comparing the quality of COCs submitted to different levels of follicular atresia and elucidating which via of atresia influences the oocyte competence: the increase in the estradiol levels or the reduction of LH pulses through use of exogenous progesterone.

LH pulsatility on the development of small antral follicles

LH is responsible for a series of physiological reproductive events, as mentioned before. Stimulation of theca cells by LH is essential for androgenesis as a precursor for estradiol, and higher levels are associated with the viability of small antral follicles and the progress into the follicular wave growth (5). When antral follicles emerge and are maintained by an exogenous source of FSH, atresia of subordinated follicles is prevented and DF selection does not occur. The small antral follicles do not present atresia, showing a reduction of LH receptors in theca cells and of mRNA levels from steroidogenic enzymes when compared to DF (71, 72). When LH doses are administered concomitantly to exogenous FSH and the FSH steroidogenic potential is kept, the subordinated follicles produce steroids levels similar to the DF (25). Thus, LH is essential for DF selection and for the development of small antral follicles. In fact, LH receptors are expressed in granulosa cells during the differentiation of DF and in other antral follicles. However, the FSH-dependant follicular growth phase, before the DF selection, does not depend on LH. The small antral follicles function

is subtly affected by intrafollicular concentrations of estradiol, inhibin, free IGF-I and molecules of low molecular weight, IGF binding proteins, when the LH pulses are reduced or completely abolished using steroids treatment (6, 42). Therefore, LH is essential to stimulate antral follicles above 9 mm of diameter (45), when the switch from FSH to LH dependency occurs.

Development of oocyte competence in relation to follicular pattern

One mean to evaluate oocyte competence is by the follicular diameter (64, 94, 103). However, follicles with similar diameters can be found in different physiological phases (94), showing that this evaluation method does not confer a good accuracy to evaluate the IVP rates.

Lequarre *et al* (64), working with oocytes from slaughter animals, evaluated the oocyte developmental competence from oocytes collected from antral follicles with different diameters. The follicles were separated according to their diameter in three groups: small follicles, smaller than 4 mm; medium follicles, between 4 and 5 mm of diameter; large follicles, more than 6 mm of diameter. In this experiment the number of blastocysts obtained from follicles larger than 6 mm was higher than from those obtained from follicles smaller than 4 mm.

In other study, Wit *et al* (103) investigated the effect of the follicular environment in the quality and developmental competence of COCs from slaughter ovaries. The COCs were collected from follicles without atresia, with low level of atresia, with atresia and with advanced atresia, and were classified according to their quality in A, B and C. The COCs of A quality were obtained mainly, but not exclusively, from follicles without atresia. The quality B ones were obtained from all classes of follicles with atresia and the ones with quality C came exclusively from follicles that presented advanced atresia. The COCs B produced more blastocysts than the COCs A and C, indicating that a low level of atresia can present satisfactory results in IVP (103).

A study carried out by Vassena *et al* (94) investigated the influence on morphology and oocyte development regarding the follicular development stage and the presence of CL or DF, in COCs collected from ovaries of slaughtered cows. The animals were submitted to synchronized ovulation and were slaughtered at days 2, 3, 5 or 7 of the estrous cycle (day 0 = day of follicular emergence). The COCs proportion that developed to blastocyst stage was higher in COCs collected at day 5 (23%) after emergence, than that collected at days 2 (12%), 3 (13%) or 7 (16%). These data, according to the authors, do not support the hypothesis that the CL affects the embryonic development, however there is a positive correlation between recent follicular atresia and oocyte competence. These studies demonstrate that oocyte morphologic characteristics, subjectively evaluated, can not be used to predict oocyte competence.

Based on the knowledge of how the oocytes formation and activation occurs, several reports have been published with the aim to increase the IVP rates, either using methods to improve in vivo oocyte quality or by modifying the IVP protocols. Other alternatives are the increase of oocyte recovery, using hormones to manipulate the follicular wave. Despite all alternatives to improve IVP rates, an adequate marker to evaluate competence and development of oocytes has not been described yet (64), even though the follicular diameter (12, 82), the level of atresia (51, 102) and the progesterone concentration in the follicular fluid (53) could be good indicators. The oocyte competence level is also correlated with morphology of the COCs (12, 16) and the morphology of the corona radiata (61).

Some alternatives are described below, which use different methods to improve the *in vitro* production rates.

Alternatives to improve IVP efficiency

A variety of different techniques and protocols for treatment of oocyte donors have been described to improve in vivo COCs recovering rates and the

developmental competence of oocytes collected in vivo. Aiming at improving cattle IVP rates, Ward *et al* (99) showed the influence of the aspiration vacuum pressure to obtain oocytes and in the quality of recovered oocytes, using ovum pick up (OPU) guided by ultrasound. In this study, it was verified that pressure above 50 mm Hg (millimeters of mercury) decreases the oocyte recovering rate and the recovery of quality I oocytes, increasing the number of denude oocytes. Among the protocols used in oocyte donors, treatment with gonadotropins (98), GnRH (18), anti-inhibin immunization (60) and gonadotropins with bovine somatrotophin (bST) (57, 86) can be mentioned. Treatments using different doses of FSH before OPU improve the number of collected oocytes and embryo production compared with cows that have not been stimulated (49, 91, 98).

Other studies suggest different days of the estrous cycle to perform the OPU (days 3-4, 9-10, or 15-16 days after the estrous) (85) and different intervals between the OPUs with punctions after 48 and 96 h (90), once a week (47) or twice a week (40). The period between puncture sessions influences embryo production rate (69). This study suggests that the best COCs recovery rate occurs when the interval between punctions is seven days, compared to protocols that used intervals of 3 or 4 days. However, the embryo/oocytes rate production is lower. Galli *et al* (39) observed that OPU, twice a week, produces a maximum number of recovered oocytes with an appropriate quality for IVP. The method of continuous punctions, twice a week, shows influence in the estrous cycle by modifications in endocrine function and mechanisms of follicular growth, leading to irregular estrous intervals (91) or absence of estrous (17, 40). Frequent sessions of serial punctions can result in absence or dysfunction of the corpus luteum (CL) and inadequate progesterone production (24, 83). Takenouchi *et al* (93) suggested that if the OPUs were restricted to days 0 and 12 of the estrous cycle, the influence in the follicular dynamics could be decreased or even abolished. Båge *et al* (7) also showed that in punction schemes, twice a week, restricted to the first half of the estrous cycle, the heifers presented normal intervals between estrous. In contrast, with the normally used punctures from 3 to 4 days of interval, OPU in intervals of 2 and 5 days did not affect the number of oocytes collected by session; however, the COCs quality and the blastocyst production rate were higher in the two days interval, which can be attributed to the DF effect in the atresia of the subordinated follicles (69).

Goodhand *et al* (46) used treatments with progesterone and oestradiol in cows submitted to follicular punctions, without differences in the number of punctured follicles, recovered oocytes and embryo production. However, when FSH was associated to these treatments, there was an increase in the number of follicles punctured and in the recovery of quality I oocytes, whereas, when the FSH was administered in multiple doses, an increase in the number of transferable embryos was found.

The rbST (recombinant bovine somatotrophin) has been used to improve the IVP rates and associated to gonadotropins to increase the population of follicles (43, 86, 100). The rbST effect seems to be mediated by the increase in IGF-I and insulin concentrations. However, some direct effects of the rbST can not be disregarded (86). Insulin on its own or in combination with gonadotropins shows effect on proliferation and steroidogenesis of bovine granulosa cells in culture (100). Pivato (86) compared cows that were punctured under gonadotropin stimulation with and without rbST and observed that animals treated with rbST presented higher number of oocytes than those not treated. However there was no difference in the cleavage and blastocysts rate between groups. Other study carried out by Bols *et al* (14), compared cows that received 640 mg of rbST weekly, for six consecutive weeks and cows with placebo. In this work it was not detected differences in the number of oocytes collected or in the number of blastocysts per group. These results show variability in the response to rbST treatment in oocyte donor cows.

Some puncture schemes suggest the use of FSH to improve IVP viability (47, 48, 69). Blondin *et al* (11) reported an excellent oocyte competence when the interval between pre-stimulation with

FSH and oocyte recovery was 48 hours, which indicates that in this period there were changes in the COCs, similar to the ones occurred in the pre-maturation process. The mechanical removal of COCs from the follicles, caused by OPU, induces oocyte pre-maturation. Some studies suggest that the administration of GnRH pre-OPU, after follicular stimulation with FSH, could improve the oocyte quality through standardization of oocyte maturation stage, increasing the blastocyst production rate. However, Pivato (86) did not observe difference in the quality of oocytes collected when compared two puncture schemes using FSH and GnRH (10 or 20 hours before OPU).

Other alternative to increase the follicular population is by active or passive immunization against the biological action of inhibin. This proceeding was initially tested in sheep and resulted in an increase of the plasmatic FSH concentration (37) and in the number of ovulations per cycle (74). The active immunization against inhibin has been used to increase the double ovulation rate in cattle (89, 76).

Viana *et al* (95) worked with 3 groups of Gyr cows, using Norgestomet® as exogenous progestagen source, for 14 days. One group received FSH (G1) during the OPU sessions; other group was treated with swine anti-inhibin (G2) and the control group received only progestagen (CG). In this study it was registered an increase in the number of quality I oocytes from G1 in relation to CG. The authors related these results to the fact that G1 presents a high number of larger follicles facilitating this way the laminar flux of the collection by the increase in the follicular liquid and by presenting lower levels of atresia than CG.

In a work performed by our group (84) it was possible to observe that animals submitted to treatments with exogenous progesterone have a higher number of follicles suitable to puncture, higher number of oocytes collected and an increase in the oocyte quality in relation to cows that did not receive progesterone. This fact might be connected to different levels of atresia to which follicles and oocytes from this group were submitted. In this respect, it is still necessary more studies to understand the mechanisms involved in oocyte quality, in order to allow the implementation of new alternatives to increase the levels of IVP.

As described before there is a lot of alternatives to improve the results of the IVF, therefore, up to date there isn't a complete efficient method that is compatible with the in vitro embryo production system. We could cite as one of the reasons of this slow advance the high efficacy of the subjective evaluation of oocyte developmental competence trough nucleus and cytoplasm visualization of oocytes submitted to IVF.

As mentioned, to date there is not a totally efficient criterion to evaluate the oocyte quality and its correlation with future in vitro embryo production (64). Methods to improve such criteria, in order to have a more positive correlation between oocytes and embryos produced are still under study. Some studies have focused on new ways for oocyte quality evaluation (64, 94). Lequarre *et al* (64) evaluated the metabolization energy at the beginning and at the end of the maturation period, measuring oxygen and pyruvic acid increase as well as lactate liberation and evaluation of the transcriptome in immature oocytes. These studies focused on protein synthesis before and after *in vitro* maturation and on the evaluation of nuclear maturation kinetics, as the moment of extrusion of the first polar body, that are associated with subsequent embryonic development. Other works were been published analyzing the functional genomics of the expressed genes trough confirmation of the DNA microarray experiments by real-time PCR for a subset of genes. Studies of global transcription in bovine oocytes and early embryos by using the Affymetrix bovine-specific DNA microarray, that is the biggest available array at present, has been done too. These studies could provide molecular biomarkers for development because embryonic mortality is the biggest limiting factor in animal reproduction and production (73).

The techniques for gamete evaluation as the alternatives techniques for *in vitro* embryo production that are been developed could elucidated some aspects of the influence of oocyte quality over the *in vitro* embryo production and consequently show the real importance of the pre-maturation gamete selection.

Conclusion

From the considerations presented in this review it is possible to note the importance of a better understanding of the factors involved in IVP process, in order to allow the study of new alternatives to improve the results obtained in assisted animal reproduction programs.

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