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Natural antioxidants during in vitro culture improve embryo quality in cattle

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CHAPTER 1: Biotechnology of reproduction

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1.1 INTRODUCTION

The term “biotechnology” (biological technology) means all applications that manipulate biology in order to obtain goods and services. Among the various definitions, the most complete is that of UN Convention on Biological Diversity: “Biotechnology means any technological application that uses biological systems, living organisms or derivatives thereof, to make or modify products or processes for specific use”. Nowadays, the competitiveness in the livestock sector depends on the ability to respond as quickly as possible and with a little outlay to the consumer needs, who is increasingly attentive and sensitive to the quality of the product. Thus, in this area the use of veterinary biotechnologies is important to take advantage of biological systems. In recent years, many livestock holdings started to use veterinary biotechnologies and, in particular, reproductive biotechnologies, so that farmers can increase production performance and address the production towards an unthinkable quality, while at the same time obtaining the reduction of risks of disease transmission, in order to achieve a more appropriate response to the market needs and specific demands. When we talk about reproductive technologies, we are referring to techniques ranging from the simple artificial insemination (AI) to the complex cloning of animals, without forgetting the multiple ovulation and embryo transfer (MOET), the ovum pick-up (OPU) combined with *in vitro* embryo production (IVEP), cryopreservation of genetic material (gametes and embryos) and embryo/semen sexing.

All of these techniques can be used individually or simultaneously because no technique is exclusive of the others, even if they have some limitations such as the economic impact.

However, rapid genetic improvement in cattle in the developing world has been possible by the application of biotechnology that resulted in significant economic returns (Madan, 2005). Undoubtedly, further researches on both *in vivo* and *in vitro* bovine embryos production will be the impetus for additional reviews to document new knowledge in this area.

In addition to the improvement of agro-food production, reproductive biotechnologies are also used to improve the quality of human life, to maintain biodiversity and to preserve numerous endangered animal species. Furthermore, the combined application with cellular and molecular biology and genomics techniques, make reproductive technologies valid research tools for the characterization of the genes and the production of transgenic animals. In the human field, these technologies have allowed to face successfully cases of infertility, to fight against cancer, cellular aging, pre- and post-natal diseases. In effect, the increased worldwide research in the field provided valuable information about biological and pathological processes of the human organism.

Assisted reproductive technologies (ART) involving the in vitro production of preimplantation stage embryos are imperative for the treatment of infertility and/or for fertility management in both human and veterinary reproductive medicine (Rodriguez-Martinez, 2012; Clarke, 2006).In fact, these in vitro techniques gave new opportunities for cattle producers, particularly in the dairy industry, to overcome infertility and to increase dissemination of animals with high genetic merit.

The total number of in vitro produced (IVP) bovine embryos transferred to recipients worldwide in 2011 alone was 373,836 (Stroud, 2012). Despite this widespread use, the fertilization and culturing of early livestock embryos in suboptimal, foreign

microenvironments leads to a high frequency (50–70%) of early embryonic demise during about 5-7 days of in vitro development (Keskintepe and Brackett, 1996; Leidenfrost *et al*., 2011). In addition, numerous studies have shown that *in vitro*-produced bovine embryos differ from their *in vivo*-derived counterparts. These differences include timing of development, alterations of morphology (Boni *et al*., 1999), metabolism (Khurana and Niemann, 2000), intactness of zona pellucida (Duby *et al*., 1997), blastocyst numbers (Leibfried-Rutledge *et al*., 1987), cryotolerance (Leibo and Loskutoff, 1993), relevant gene expression (Niemann and Wrenzycki, 2000) and, ultimately, pregnancy rates (Hasler, 2000).

It is a self-evident truism to refer to the *in-vitro* environment as 'inappropriate', 'stressful' and 'evidently inferior' compared with the *in-vivo* counterpart. Ambitions are usually restricted to narrowing the gap between the two systems, although a more daring goal may also be outlined. Indeed, there are many examples even in reproductive biology that the natural way is not necessarily the optimal or the most efficient one. In cattle, the overall efficiency of *in-vitro* embryo production is about 100-fold higher than the *in-vivo* process if the developmental rate is measured from the antral follicle stage or from the spermatozoon to the blastocyst. The latter difference can be even higher in humans when intracytoplasmic sperm injection is applied. This thesis argues that mammalian embryo culture in vitro should not be regarded as an imperfect copy of the *in-vivo* procedures, but an artificial process with its own frames, limitations and possibilities. Acknowledging the current limitations must not restrict the search for future perspectives. (Vajta *et al*., 2010)

By using *in-vitro* systems, endless choices are offered (within and especially behind the traditional medium, gas, temperature and oil frames) to establish conditions and involve steps that fully exploit all resources of mammalian embryo development, including those

not used by the natural process, or to defend embryos from a potentially or definitely harmful maternal effect.

Nothing proves that the future cannot be brighter.

1.2 ARTIFICIAL INSEMINATION (AI)

The artificial insemination was one of the first techniques, in the context of reproductive biotechnology, to be used in breeding animals and it is the main operational tool for genetic selection in cattle. In little more than half a century this procedure has become a routine technique for almost all of the farms, particularly in dairy cattle.

Artificial insemination of semen into the uterus was developed in 1930s for cattle and within a decade became an asset to the dairy industry all over the world (Hamilton and Symington, 1939; Shelton, 1946). The method, timing in relation to the onset of estrus, and the site of semen deposition were compared and reviewed (Vandemark, 1952). For optimum results, a rectovaginal method of AI is performed during the interval between the middle to the end of estrus, and semen is deposited in the lumen of uterine body. The most important advantage of AI is to allow the production of multiple generations of offspring per bull in a short time; this permits rapid genetic evaluation of the genetic merit of the animal through the performance of the daughters (Progeny Test). In fact, AI is the best tool to increase the paternal contribution to the genetic improvement, as well as it is considered a relevant advantage to prevent the sexually transmitted diseases (STDs).

The turning point which led to the large use of this technique occurred in the ‘60-70 years, when the technique of semen cryopreservation was improved, resulting in the possibility to store semen for many years and to fertilize animals that were situated several thousand

kilometers away from the bull (Pickett and Berndtson, 1974). The availability of genetic material from tested bulls has provided a starting point necessary to continue more effectively the selection process began empirically by farmers. In the last 60 years, milk production has doubled while concurrently the dairy cattle population has been reduced to half. This paradigm shift in productivity and number of cattle has been possible in large part by artificial breeding techniques.

Actually, according to Baruselli (2007), AI in buffalo has a limited use worldwide especially due the difficulties in the estrous detection and hence, in identifying the most adequate time for insemination. These phenomena led invariably to decreased efficiency when the traditional AI is employed. This way, the use of fixed time artificial insemination (FTAI) is an advantage because it can be scheduled to pre-determined hours of the day, simplifying the management currently required in the traditional AI program.

In an earlier trial (Neglia *et al*., 2003*a*) it was demonstrated that Ovsynch was a more effective synchronization protocol than PRID followed by PG2α and PMSG for FTAI in cyclic buffalo cows, as indicated by the greater pregnancy rates (44.4 vs 30%).

Furthermore, in addition to freezing, the possibility of sexing semen (Rath and Johnson, 2008; Garner and Seidel, 2008) in the AI programs has given impetus to this phenomenal growth.

1.3 MULTIPLE OVULATION AND EMBRYO TRANSFER (MOET)

The MOET is the technology currently used to get more than 80% of the embryos produced for commercial purposes in the world. This technique consists of the administration of exogenous hormones in order to obtain multiple ovulations in animals that normally ovulate a single follicle. Generally, superovulation is performed between the 9th and the 11th day of the bovine estrous cycle, in coincidence with the emergency of the second follicular wave. Subsequently, on day 6-7 after AI embryos are recovered by flushing the uterine horns of the donors with isotonic buffered solutions. The embryos can be transferred in synchronized recipients or frozen and transferred at a later time.

1.3.1 Superovulation (SO)

Since the late 1970s, nonsurgical embryo recovery techniques have been used in combination with hormone-induced superstimulation (Peippo *et al*., 2011) of ovaries leading to multiple ovulations, for *in vivo* embryo production in cattle (Hasler, 2006).

Several regimens have been used for superstimulating cattle (Mapletoft *et al*., 2002). Most often superstimulation is induced with FSH. Purified ovine or porcine pituitary extracts used for superstimulation induction usually contain both FSH and LH, albeit in variable ratios. Whereas FSH is responsible for follicular growth during the early stages of follicular growth (<9 mm in diameter), LH is necessary for follicular growth in the final stages (Mihm *et al*., 2006). Because FSH has a short biological half-life (approximately 5 h), both the frequency of administration and dose affect embryo production rates (Monniaux *et al*.,

1983). Division of the daily dose between at least two injections instead of a single injection and use of declining doses of FSH, e.g., using eight declining doses, administrated twice a day starting 5 days before AI (between Days 8–11 of the estrous cycle) improved embryo production rates (Monniaux *et al*., 1983). However, it was subsequently reported that a single treatment of FSH, diluted in a slow-release formulation, resulted in embryo production rates comparable with those obtained using the traditional twice-daily protocol (Kimura *et al*., 2007; Bó *et al*., 2010).

Nevertheless, there are several limiting factors that affect the response of animals to superovulation: these can be intrinsic, such as age and race, and extrinsic factors such as the season, the environment where animals are located, nutrition of donors and recipients, the amount of repeated treatments, as well as the health and physiological conditions of the genital tract, such as tubal patency and cycles regularity.

The number of ovulated oocytes is dependent on the number of follicles >2 mm in diameter available at initiation of superstimulation (Singh *et al*., 2004). Protocols that control follicular wave emergence and ovulation have great impact on the feasibility of the use of superstimulation treatment, as it can be started at a self-appointed time. The number of small follicles can be increased by manual ablation of a dominant follicle (Huhtinen *et al*., 1992). Alternatively, follicular development before superstimulation can be controlled with exogenous hormones. For example, progesterone treatment of donors before FSH treatment inhibits development of a dominant follicle and increases the number of small follicles (3–4 mm in diameter) in the ovary (Callejas *et al*., 2008).

Aging is associated with a reduction in the number of 2- to 5-mm follicles in a follicular wave, as well as follicular and ovulatory response to superstimulation (Malhi *et al*., 2008).

The number of small follicles within a follicular wave is variable among animals, but relatively constant within individuals (Ireland *et al*., 2007). This observed relationship is not dependent on breed, age, management or physiology of cattle, but is inversely associated with serum FSH concentrations; in fact, nutritional supplementation strategies reported to date have not significantly improved the superstimulation outcome of well-fed donors (Velazquez, 2011). Variations in superstimulation response among individual animals result from hormonal status of the donors which can also be subjected to manipulation. Furthermore, plasma anti-Müllerian hormone concentrations before superstimulation treatment can predict potential to produce transferable embryos (Rico *et al*., 2009; Monniaux *et al*., 2010).

Superstimulation in combination with AI, embryo recovery, and embryo transfer, hastened genetic improvement relative to application of conventional AI per se. Furthermore, these techniques offer the advantage of utilizing valuable cows and heifers as embryo donors, while concurrently relegating their less valuable counterparts for use as embryo recipients.

1.3.2 Embryo transfer (ET)

The term embryo transfer refers to the process of "transferring" an embryo, collected either from a donor or grown in vitro to the reproductive tract of a recipient animal. Regardless of the species, this technique has been widely studied or applied; in general, it is a valuable tool in animal biotechnology with many applications (Dziuk, 1975). In livestock the outcome of embryo transfer depends on the quality of both recipient and embryo. In dairy breeds, heifers are better recipients than cows, especially for manipulated (e.g., biopsied and/or frozen-thawed) embryos (Hasler, 2006; Hasler *et al*., 2002). This observation could

be partly explained by blood progesterone concentrations, which are higher in heifers than in cows (Sartori *et al*., 2004).

Embryonic mortality after ET is highest during the first 2 to 3 weeks of pregnancy (Berg *et al*., 2010). During the preattachment period, embryo-maternal communication is crucial for successful establishment of pregnancy (Wolf *et al*., 2003), when synchrony between the embryo and the uterine endometrium is essential. Asynchronous conditions result in failure of the embryo to attach, leading to early embryonic mortality, among other complications (Barnes, 2000). Insufficient priming of the uterus or reduced developmental competence of the embryo may also affect signaling between the embryo and endometrium. For example, embryo-secreted interferontau induces changes in endometrial function necessary for attachment (Klein *et al*., 2006). Transfer of a Day 7 embryo to a Day 7 embryo recipient followed by its recovery on Days 14 to 16 provides an efficient way to determine its developmental competence (Berg *et al*., 2010).

Embryo recipients may have spontaneous or induced ovulation before transfer, as evidenced by the presence of a corpus luteum (CL). The embryo is transferred into the uterine horn ipsilateral to the ovary where ovulation has occurred. In cattle, synchrony between embryo and recipient is important, allowing only ±1 day of asynchrony (Gordon, 2003). This requires precise estrus detection, or predictable and synchronous induction of ovulation.

Synchrony between donor and recipients, superovulation protocols, embryo quality, and many other factors that contribute to success have been exhaustively examined over the years (Machaty *et al*., 2012) However, experienced embryo transfer practitioners also recognize the importance of suitable husbandry and management practices on success. Technical skills during the transfer procedure are also important to minimize endometrial

damage. It is evident that success in bovine embryo transfer requires a marriage of reproductive physiology, basic animal husbandry, and veterinary science to produce consistent and acceptable results" (Stroud and Hasler, 2006).

1.4 OVUM PICK-UP (OPU)

The advent of Ovum Pick-Up (OPU), i.e. the in vivo oocyte collection from live donors, has dramatically increased the worldwide interest in IVEP procedure, as a valid alternative to the *in vivo* embryo production. The OPU is a non invasive and repeatable technique for the recovery of immature oocytes from live donors by ultrasound-guided transvaginal follicular puncturing. In 1987 OPU technique, which was already used in the human field, was first adapted in cattle in an attempt to overcome the disadvantages linked to the MOET programs. Indeed, OPU can be carried out on a wider range of donors, such as acyclic, prepubertal animals, pregnant cows up to the 3^{rd}-4^{th} month of gestation, animals with non perfect conditions of the reproductive tract or at the end of their productive career and cows that are not sensitive to the hormonal stimulation treatments. In addition, unlike the MOET method, it has no negative impact on the treated animal and may have a therapeutic effect in animals with ovarian cysts or similar diseases, because it does not interfere with animal reproductive and productive cycles and does not require a pretreatment with gonadotropins.
The oocyte collection can be performed once or twice a week without negative impact on the fertility of animals (Chastant-Maillard *et al*., 2003). It has been observed that the number of good quality oocytes, i.e. oocytes included in IVEP system that are able to develop to the blastocyst stage, is higher when the collection is carried out twice a week at a distance of 3-4 days, compared to that performed once a week (Hanenberg and van

Wagtendonk-de Leeuw, 1997; Merton *et al*., 2003). In the latter case, there is a larger number of oocytes with cumulus expansion and atresia (Garcia and Salaheddine, 1998). On the contrary, with the repetition of collection for each animal twice a week, it is possible to get a more homogeneous population of oocytes with an improvement of their quality (Merton *et al*., 2003, Hanenberg and van Wagtendonk-de Leeuw, 1997). In effect, through the aspiration of all visible ovary follicles the follicular population is reduced to zero, a new follicular wave starts and the subsequent follicular aspiration 4 days later does not permit the establishment of dominance, resulting in improved oocyte quality and increased embryo yields (Boni *et al*., 1993). This is one of the factors determining the improved competence of OPU-derived compared to abattoir-derived oocytes. In addition, when OPU is applied, collected *in vivo* oocytes are transferred in a maturation medium (Caracciolo di Brienza *et al*., 2001) more rapidly than with using abattoir-derived ovaries. It has also been speculated that the better developmental competence of OPU-derived *vs* abattoir-derived oocytes is related to the shorter exposure to environmental stress (Neglia *et al.,* 2003*b*). Indeed, abattoir-derived oocytes spend a longer time between excision of ovaries from the peritoneal cavity and laboratory processing and are probably affected by cellular damages due to autolytic processes, especially when they reside in excised ovaries for prolonged periods. It follows that, in the latter case, another important factor to consider is the time interval between ovary collection and processing in the laboratory. In our setting, the time lapse between collection of ovaries at slaughter and their arrival at the lab usually varies between 3 and 6 hours.

Furthermore, OPU is more competitive than MOET in terms of embryo yields; in fact, although the number of embryos produced per OPU session is lower (on average 1) than

that collected with MOET (5 on average), the repeatability, i.e. the shorter interval between sessions (3-4 days vs 75 days, respectively) results in a significant increase of the number of embryos produced in the medium-long term (Zicarelli, 2001). Therefore, within a certain period of time, OPU/IVEP method permits a larger embryos production per donor compared to any other available technique: in cattle it has been estimated that on average it is possible to get one calf per year per donor with the IS, 20-25 and 80-100 calves per year, respectively with MOET and OPU/IVEP (Zicarelli, 2001).

Therefore, OPU opens new perspectives allowing an increase of species reproductive efficiency. In addition, the possibility of using this technique on animals with a high productive merit results in a significant reduction of generational interval and, consequently, in a major acceleration of genetic progress.

1.5 IN VITRO EMBRYO PRODUCTION (IVEP)

Developments in bovine ART are more substantial and in much broader use than those in any other nonhuman species. Further, based on these advances, cattle are often used as a model to study ovarian function and embryogenesis. In this regard, bovine embryos have many characteristics that make them a good model for the human. For example, cattle and humans are both typically mono-ovulators, and their embryos have similar size and energy metabolism (Baumann *et al*., 2007).

In the first experiments, in vitro embryo production was limited to the so-called genetic rescue of prestigious animals that were dead or slaughtered because at the end of their productive career. The first success of the assisted reproduction technologies occurred in humans with the birth of Louise Brown in 1978 (Steptoe and Edwards, 1978). Also in the

context of animal production we did not have to wait too long for success of equal importance; in fact, in 1981 Virgil, the first calf born from in vitro fertilization, was born (Brakett *et al*., 1982).

Currently the oocytes can be recovered from abattoir-derived ovaries by aspirating the follicular fluid using a hypodermic needle attached to a syringe or a vacuum system or from live animals through the OPU technique. Afterwards, collected oocytes are introduced in the IVEP system, which includes different steps such as *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) to the stage of transferable embryos, which may be intended for different uses, such as embryo transfer (ET), sexing and/or freezing.

1.5.1 Oocytes collection and evaluation

Considering the difficulties related to working with live animals, and higher costs linked to OPU technique, in the laboratory routine to reduce costs and time and to increase oocytes availability, is preferable to use abattoir-derived ovaries, as a source of oocytes for experimental purposes aimed to improve the efficiency of the different steps of the IVEP.

The Cumulus-oocyte complexes (COCs) can be collected from slaughtered ovaries by various methods, such as follicular dissection, the "slicing" (shredding), the "Transillumination-Aspiration Ovary" (TAO) and the follicular aspiration. The follicular dissection is a technique that permits individual follicles isolation, allowing the recovery of a considerable number of good quality oocytes, but it has the drawback to be a time-consuming technique and to be not suitable in certain species, such as buffalo, that have deep follicles in the ovarian stroma. The slicing of ovarian tissue provides a higher number of oocytes, but they show an extremely heterogeneous quality; furthermore, it is long and

impractical method because the use of scalpel blades is necessary and also because a longer time may compromise the oocytes viability, as well as during follicular dissection. Generally, this technique is used for animals with a very small ovarian follicles size, which does not allow the aspiration by needle, such as in rodents.

As regards the TAO, recovery times are not so long and this technique allows to recover about 50% of oocytes more than suction, thanks to the direct visualization of cortical follicles to aspire through transillumination. However, the TAO is not yet very widespread and at present the most frequently used method is the aspiration, because it is more practical and more efficient in terms of numerical ratio oocytes/time. The follicular aspiration allows to recover all oocytes of surface follicles with diameter between 2 and 8 mm; subsequently, the oocytes can be visually assessed to select those with a good morphology and discard the poor quality ones. Overall, by follicular aspiration of bovine ovary it is possible to collect from 6 to 10 good quality oocytes.

Recovered COCs are classified and divided according to the cytoplasm and cumulus morphology, because there is a definite correlation between the morphology and competence, that is considered the oocyte ability to be fertilized and develop to the blastocyst stage (Wurth *et al*., 1992). According to the cytoplasm homogeneity and to the number of cumulus cell layers, oocytes were classified into different categories (Wurth *et al*., 1994; Boni *et al*., 2002). However, it has also been shown that both the *in vitro* maturation and the developmental competence of bovine oocytes with heterogeneous cytoplasm are higher than those of oocytes with homogeneous cytoplasm (Blondin and Sirard, 1995; Fukuda and Enari, 1993). This was further confirmed by Nagano *et al*. (1999), who observed that oocytes with heterogeneous cytoplasm show a greater ability to be

fertilized, and speculated that a degree of cytoplasm heterogeneity is due to a better distribution of cortical granules, which leads to a reduced incidence of polyspermy.

Another important factor in the oocytes classification is the cumulus cells layers surrounding the oocyte; the cumulus cells play a fundamental role during the maturation process (Cox *et al*., 1993). Cumulus cells are also important during fertilization, because they attract and select the sperm (Chian *et al*., 1996), facilitate the processes of sperm capacitation, acrosome reaction, and penetration (Fukui, 1990; Cox *et al*., 1993) and prevent premature the zona pellucida (ZP) hardening (Katska *et al*., 1989).

In conclusion, the oocyte morphology can be used, with a certain reliability, to predict the gamete developmental competence; according to our classification, a progressive decrease of efficiency is recorded from Grade A to Grade D oocytes, with Grade A and B considered suitable for IVEP (Figure 1). As well as the recovery rate, oocytes quality can also be influenced by several factors, such as the suction pressure during collection, the source of gametes, the time between collection and processing, the temperature during transportation and the season (Di Francesco *et al*., 2007).

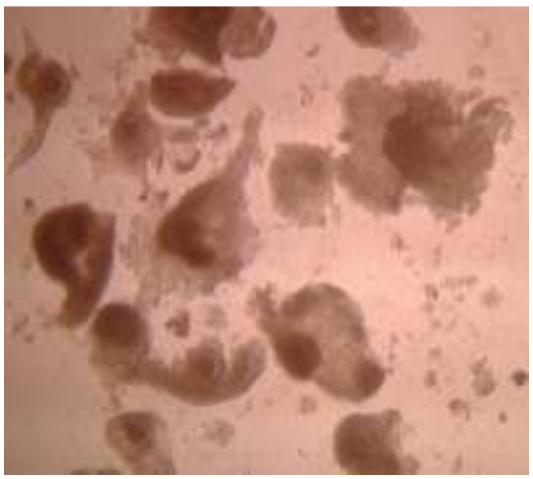


Figure 1. Grade A and B bovine oocytes

1.5.2 In Vitro Maturation (IVM)

The results obtained in the field of *in vitro* embryo production were possible thanks to studies about *in vivo* physiological conditions of animals. The hypothalamic-pituitary-ovarian axis, with the production respectively of Gn-RH (gonadotropin releasing hormone), gonadotropins (LH and FSH) and ovarian steroids such as 17-β estradiol and progesterone (Seren and Parmeggiani, 1997), controls the ovarian cycle and, thus, the reproductive function of the animal.

A fundamental requirement for a successful fertilization is undoubtedly the appropriate maturational status of the oocytes at the time they encounter the sperm (Gasparrini *et al*., 2008). The oocyte maturation process, that involves both the nuclear and cytoplasmic compartments, is fundamental for the acquisition of full developmental competence (Gasparrini *et al*., 2008). During late fetal development, bovine oocytes arrest at prophase I of meiosis, also defined as germinal vesicle (GV), for the typical appearance of the nucleus. Oocytes recovered by OPU or by abattoir-derived ovaries are just immature oocytes, still arrested at this stage of prophase. Under physiological conditions, completion of the first meiotic division occurs after puberty is attained. Before each ovulation, meiosis of the oocyte in the dominant follicle resumes after the LH surge; the resumption of meiosis takes place, starting with germinal vesicle break down (GVBD), then half of the chromosomes are extruded in the form of the first polar body and the cell cycle is arrested again, this time at the metaphase stage of the second meiotic division (MII).

Resumption of the first meiotic division, progression to metaphase II, the cumulus expansion, and the accompanying cytoplasmic changes that are required for the oocyte to become capable of normal fertilization are collectively termed oocyte maturation (Smith,

2001). Complete functional oocyte maturation, including nuclear, cytoplasmic, and membrane components, is necessary for the ooplasm to become capable of reprogramming the nucleus of the fertilizing spermatozoon, promoting male pronuclear formation, and initiating the first mitotic cell division in the newly formed embryo (Eppig, 1996). Nuclear maturation comprises the resumption and progression of meiosis until the second metaphase stage and is easy to detect. Conversely, cytoplasmic maturation refers to processes that prepare the oocyte for activation, which is more difficult to evaluate directly.

In 1972, Hunter *et al*. have reported the first in vitro maturation (IVM) of bovine oocytes, and since then subsequent studies have always tried to formulate a medium that could mimic natural conditions for the oocytes development. Among factors affecting mammalian embryo development in vitro, the duration of IVM plays a critical role, since an inappropriate timing of maturation results in abnormal chromatin (Dominko and First, 1997), oocyte aging (Hunter and Greve, 1997) and reduced development (Marston and Chang, 1964). Furthermore, although sperm can penetrate oocytes prior to completion of oocyte maturation (Chian *et al.,* 1992), subsequent development is generally reduced. Therefore, it appears that the optimum time for in vitro fertilization is at completion of meiosis, that occurs at different times in different species, varying from 18-24 h in cattle (Sirard *et al.,* 1989; Neglia *et al.,* 2001) to 36-48 h in pig (Prather and Day, 1998). Galli *et al*. (2003) confirmed that *in vitro* maturation of bovine oocytes involves removal of prophase I oocytes (together with surrounding cumulus cells) from antral follicles and their culture for 20 to 24 h; during this interval, oocytes extrude their first polar bodies and reach metaphase II (Gilchrist and Thompson, 2007). Supplementation of the maturation medium with selected serum, such as fetal calf serum (FCS), hormones and other active factors

markedly enhanced quality of the matured oocyte (Brackett and Zuelke, 1993). Among the various factors of the serum, glucose has a very important role in the maturation, given that the oocytes resume meiosis but fail to reach metaphase II in its absence (Kimura *et al*., 2008). Because maturation was better aided by the presence of proestrous or estrous cow serum, which contained high concentrations of LH (Brackett *et al*., 1989; Younis *et al*., 1989), it was replaced in the media with purified bovine LH, containing FSH (<1%) and thyroid stimulating hormone (<4%), leading to development of a defined maturation medium (Zuelke and Brackett, 1990). Further refinement of conditions for in vitro maturation to improve oocyte and embryo quality included addition of biologically active agents, e.g. FSH and various growth factors (Brackett, 2001).

Numerous growth factors involved in the signaling between the developing oocyte and the surrounding granulosa cells have been identified; these are classified, according to their structure and biological activity in: Epidermal Growth Factor (EGF); Fibroblast Growth Factor (FGF); Insuline-like Growth Factor (IGF) and Transforming Growth Factor-β (TGF-β). Most recent experimental data indicate that EGF-like growth factors (e.g., amphiregulin, epiregulin, and betacellulin) accumulate in the follicle at ovulation and are potent stimulators of oocyte maturation and cumulus expansion (Hsieh *et al*., 2009); therefore, the inclusion of such factors in the maturation medium may improve the quality of matured oocytes.

It is important to remember that the oocyte is affected by environment in which it develops and that *in vitro* maturation of oocytes is influenced by specific physical culture conditions and others not well defined (Holm and Callesen, 1998). The osmolarity (Yamauchi *et al*., 1999), ionic composition, temperature (Lenz *et al*., 1983), pH, CO_2 (Geshi *et al*., 1999), and

oxygen tension, as well as, the ratio between the number of oocytes and the volume of the medium, are important parameters that are part of the first category, while the serum and somatic cells are examples belonging to the second category (Gordon, 1994). Nowadays, in optimal conditions of *in vitro* maturation more than 90% of the oocytes reach metaphase II (Figure 2), showing an improvement of quantitative aspect of IVEP system.

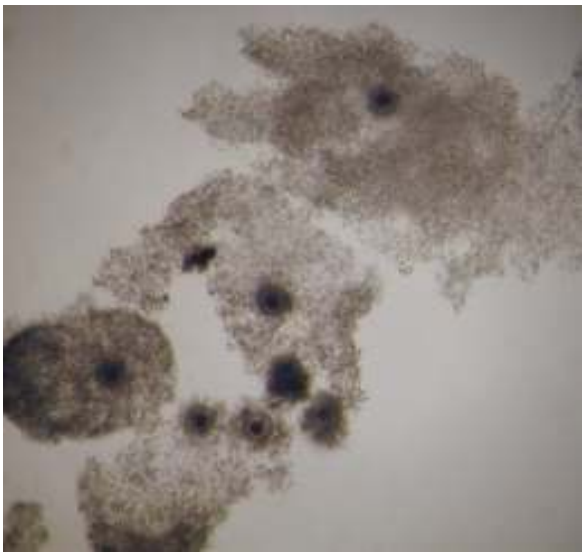


Figure 2. Matured bovine oocytes

1.5.3 In Vitro Fertilization (IVF)

The next step after maturation is *in vitro* fertilization (IVF), which consists in co-incubation of the two gametes, male and female; this is a complex process which leads to the fusion of the two gametes, returning to the number of somatic chromosomes, followed by the beginning of embryonic development. In particular, to facilitate union of the male and female gametes to form a zygote under laboratory conditions, selected and capacitated sperm are co-incubated with mature cumulus-oocyte complexes (Peippo *et al*., 2011).

Many factors may affect the IVF efficiency, such as the adequate in vitro environment for gametes survival, the sperm viability and capability, the appropriate time of insemination, the duration of gametes co-incubation, the presence of cumulus cells and also the acquisition of the oocyte developmental competence during the complex process of cytoplasmic maturation. In fact, it is likely that the fertilization failure is related to inadequacies of the IVF system, but a previous inappropriate maturation of the oocytes should not be ruled out.

Sperm must undergo a series of physiological changes in the female reproductive tract to become capable of fertilizing oocytes (Austin, 1951; Chang, 1951), a process termed capacitation (Austin, 1952); this process must be induced in vitro. Although several agents have been proven to induce sperm capacitation *in vitro*, heparin, a highly sulfated biomolecule, is still the most efficient method in the majority of the domestic species (Parrish, 2014). During capacitation, bound proteins from the cauda epididymis and the seminal plasma are removed from the sperm surface; this is essential for a series of events leading to fertilization (Rodriguez-Martinez, 2007). Proteoglycans and glycosaminoglycans were identified as factors present in the uterine tube with a role in capacitation. This led to the development of an effective method to induce capacitation of bovine sperm using heparin, a well-known glycosaminoglycan, more effective in vitro than other compounds present in uterine tubal fluid (Parrish *et al*., 1986).

Under physiological conditions sperm swim from the low bicarbonate milieu of the epididymis to the bicarbonate-rich environment of the uterine tube (Mastroianni and Komins, 1975). Therefore, cleaned sperm are most often incubated in media containing high bicarbonate concentrations that stimulate a protein kinase A-dependent protein

phosphorylation cascade closely associated with capacitation (Harrison, 2004). Bicarbonate also induces a disorder in the phospholipid packing of the sperm plasma membrane that increases membrane fluidity (Harrison and Miller, 2000) contributing to an albumin-driven efflux of cholesterol from the plasma membrane and translocation of seminolipid from the apical to the equatorial area of the sperm head (Gadella *et al*., 1994). Seminolipid is a sperm-specific glycolipid in the outer leaflet of the plasma membrane that normally prevents the acrosome reaction; its translocation destabilizes the membrane and renders it fusogenic, thereby facilitating the acrosome reaction after contact with the ZP (Gadella *et al*., 1995). Hyaluronic acid was also effective for capacitating bovine sperm. In that regard, it was suggested that by swimming through a hyaluronic acid containing medium, sperm are exposed to high shearing forces that probably remove de-capacitation factors from their surfaces (Shamsuddin *et al*., 1993). Additional treatments have also been described as effective in the stimulation of the capacitation process, including a combination of heparin and caffeine (Niwa and Ohgoda, 1988) and treatment with the calcium ionophore A23187 (Hanada, 1985) or adenosine (Breininger *et al*., 2010).

Most frequently either a Tyrode's albumin lactate pyruvate (TALP) based medium (Bavister and Yanagimachi, 1977) or a synthetic uterine tubal fluid based medium (Tervit *et al*., 1972), both without glucose and with heparin supplementation, are used for IVF (Galli *et al*., 2003). Others substances in addition to capacitation agent are added to fertilization medium to improve motility and fertilizing ability of semen, such as the complex penicillamine, hypotaurine and epinephrine (PHE). The penicillamine is able to increase the percentage of spermatozoa that undergo the acrosome reaction when used in the presence of epinephrine (Monaco, 2007). The hypotaurine increases sperm motility and, in combination

with adrenalin, is responsible for an increase in the percentage of oocytes penetration (Monaco, 2007).

Treatment of bull sperm before IVF generally involves selection of cells with highest progressive motility. Concurrently, this step also removes undesirable sperm, seminal plasma, cryoprotective agents, and other factors. Selection is sometimes achieved by allowing sperm to swim up into a medium for 30 to 60 min (Parrish *et al*., 1986). Another method commonly used for sperm separation involves a discontinuous Percoll gradient centrifugation (Lessley and Garner, 1983). Percoll consists of colloidal silica particles coated with polyvinylpyrrolidone; centrifugation of sperm through a gradient of 45% and 90% Percoll separates sperm according to their density. Because sperm with good nuclear morphology and cellular integrity are denser, they are deposited in the Percoll layer with greatest density (Le Lannou and Blanchard, 1988). Because motile sperm align their movements with centrifugal force, they also deposit faster during centrifugation (Rhemrev *et al*., 1989).

Ideally, the optimal sperm concentration is determined for each bull in preliminary experiments to achieve maximum in vitro fertilization with the lowest level of polyspermy; however, in our experience sperm concentration used is usually 10^6 per mL in cattle, but it depends on the quality of the semen. In addition, it has been suggested that prolonged gamete co-incubation under the conditions of IVF, in which high concentrations of spermatozoa are incubated in small volumes of medium, results in the production of high levels of hydrolytic enzymes (Rehman *et al*., 1994) and free radicals (Aitken, 1994) that damage the oocytes. The time required for fertilization varies depending on species and on fertilizing capacity of semen; however, it does not ever exceed 20 hours of co-incubation.

It has been demonstrated that the optimal sperm-oocyte co-incubation time for maximizing the blastocyst yield in buffalo is 16 h (Gasparrini *et al*., 2008). Shortening the gamete co-incubation length to 8 h has resulted in a significant reduction of oocyte cleavage, similar to that reported in cattle (Ward *et al.*, 2002; Kochhar *et al.,* 2003). During this interval, sperm pass through the cumulus cell layers surrounding the oocytes, attach and bind to the ZP, and after undergoing the acrosome reaction, penetrate the ZP. The fertilizing spermatozoon will then bind to and fuse with the oocyte plasma membrane, followed by activation of the oocyte, and formation of the male and female pronuclei (Schultz and Kopf, 1995). However, in most laboratories co-incubation time of bovine gametes is usually 18 to 20 h, for practical reasons. Finally, it is worth noting that there are marked differences in the kinetics of sperm penetration of different bulls; this parameter is related to the blastocyst rate (Rubessa *et al*., 2009) and can be a useful indicator to predict the ability of in vitro fertilization of the bulls. The great variability in the rate of penetration suggests to include this assessment in the preliminary screening of the bulls before their use for *in vitro* fertilization programs, also in order to determine the ideal time of co-incubation to maximize reproductive performance.

Based on several reports, the use of sperm from bulls with proven fertilizing ability affects the success of bovine IVF (Aurich and Hahn, 1994; Yang *et al*., 1995). In some cases, it may be advantageous to use semen derived from a limited number of bulls with proven fertility, although this cannot be applied in cattle-breeding programs where embryos must be derived from specific animals (Gordon, 2003). Furthermore, a bull with best morphological and genetic traits has not often a high fertility, and it may also happen that that the fertility in the field does not correspond to that *in vitro*. This problem is very

limiting the relationship between embryo production laboratories and private farms; normally the farmers choose the semen referring to bulls catalogs possessing all the characteristics of the offspring but hardly shows the percentages of *in vitro* fertility.

1.5.4 In Vitro Culture (IVC)

Although IVEP is currently successfully carried out in most domestic species, the knowledge of epigenetic factors, which regulate embryonic development, is still poor and an optimal medium for in vitro embryo culture has not yet been formulated. In fact, only 30-45% of matured oocytes reach the blastocyst stage following IVF and embryo culture, with pregnancy rates of 40–60% following embryo transfer (Hasler *et al*., 1995). Furthermore, embryos have a high capability to adapt to suboptimal culture conditions *in vitro*, but the sensitivity to the environment can result in long-term alterations in fetal and postnatal growth (Hoelker *et al*., 2014) .The optimization of the IVC systems is therefore necessary, as it is known that the optimal conditions of culture result in an increase of quality, vitality and resistance to freezing of embryos, and in a reduction of birth defects and of embryonic and neonatal mortality incidence (Lonergan, 2007; Kane, 2003).

There are two fundamental morphological events that characterize development of preimplantation embryos: (a) compaction, represented by progressive reduction of the space between the dividing cells and the increase of the perivitelline space; (b) the initial formation of the blastocyst. Within pre-implantation embryo development it is also possible to identify an early stage, which corresponds to the presence of embryo into the oviduct, and a late stage that coincides with its passage into the uterus (Figure 3); in the first stage, the embryo is a undifferentiated entity, non-vascularized, not sensitive to hormones and growth

factors and which divide repeatedly itself by mitosis without, however, showing any signs of growth (Turner *et al*., 1992). In contrast, in the late stage of development, the embryo is no longer an undifferentiated element, as confirmed by formation of the first embryonic epithelium, the trophectoderm, and the inner cell mass, and shows a significant increase of growth and responsiveness to hormones and growth factors.

The culture system initially started with the in vivo culture in an intermediate host, such as ligated oviduct of sheep (Galli *et al.,* 1998); this system provides the in vitro culture of zygotes up to 48 hours after fertilization and their subsequent transfer into the oviduct of the intermediate host. However, this process has high costs, requires appropriate facilities, is questionable from an ethical point of view and it is not practical for the production of embryos on a large scale. Later, a co-culture system was introduced using a complex tissue culture medium with various cell types, such as oviductal epithelial cells and cumulus cells; in other co-culture systems, the developing embryos were incubated together with a wide variety of somatic cells, including BRL (buffalo rat liver) cells (Hasler *et al*., 1995; Reed *et al*., 1996) and Vero cells (Carnegie *et al*., 1997). In those years, much effort to improve the IVC system has been concentrated on the type of co-culture cells, but then their supplementation was identified as part of the reason for the large offspring syndrome (LOS), which is characterized primarily by higher birth weight coupled with congenital malformations (Walker *et al*., 1996; Young *et al*., 1998). The syndrome is caused by the exposure of pre-elongation embryos to unusual environmental conditions. It is not clear exactly what environmental changes are important but a major cause may be embryo culture conditions, and in some cases at least, the use of serum and co-culture (Sinclair *et al*., 1999). A better understanding of the necessary culture conditions led to the development of semi-

defined media, with embryos incubated in the absence of feeder cells with little or no serum added. Numerous studies have been done to improve the efficiency of embryo production and eventually the synthetic oviductal fluid (SOF) medium with BSA (bovine serum albumin), originally based on the biochemical composition of sheep uterine tubal fluid (Tervit *et al*., 1972), as well as Charles Rosenkrans medium (Rosenkrans *et al*., 1993), which became the most popular for embryo culture, were developed. Finally, novel embryo culture systems based on microfluidic technology offer unique advantages. Such systems would facilitate the gradual change of the culture medium to meet the precise requirements of the developing embryo, while overcoming substantial limitations of conventional culture systems (Krisher and Wheeler, 2010). However, the costs and practical issues have not allowed these new system to be widely used.

Several factors are known to affect the in vitro embryo development, such as ammonia, oxygen radicals, growth factors, etc. Among these, the increased oxidative stress is a major factor impairing mammalian in vitro embryo development (Gasparrini *et al*., 2000). Preventing oxidative damage by maintaining a physiological O_2 tension (5% to 8%) coupled with low glucose concentrations was later found to enhance early embryo development (Iwata *et al*., 1998), and it was also discovered that less dilute conditions are beneficial because of autocrine and/or paracrine factors (Gardner, 1994). Completely defined media, developed by modifications of the SOF medium with glutamine or citrate and nonessential amino acids (Keskintepe *et al*., 1995), and by replacing BSA with polyvinyl-alcohol (PVA), also support development to blastocyst (Keskintepe and Brackett, 1996). Thus, defined conditions not only facilitate the improvement of culture media to provide a better, more consistent environment for the developing embryo, but are also critical in disease control

(Brackett, 2001). There is evidence that embryos cultured in a defined medium such as SOF with PVA without serum or co-culture are much less susceptible to develop the LOS (Jacobsen *et al*., 2000). Thus, the approach to solving this syndrome may be to concentrate on modifying simple media such as SOF, with the object of increasing the yield of viable blastocysts while at the same time checking that the modifications do not give rise to later developmental problems.

In order to standardize methods of embryo production, defined culture media still require further refinement; currently, despite many efforts to improve in vitro embryo culture systems in different species, the oviduct remains irreplaceable for embryo development. Nevertheless, other approaches have included the use of antioxidants supplementation such as thioredoxin and superoxide dismutase (Nonogaki *et al*., 1991), EDTA (Nasr-Esfahani *et al*., 1992), catalase (Nasr-Esfahani and Johnson, 1992) and vitamins C and E (Vermeiden and Bast, 1995). Recently, Seidel's laboratory examined the effects of EDTA and vitamins E and C on culture of IVP cattle embryos; vitamin E markedly improved blastocyst development but EDTA and vitamin C had no obvious beneficial effect (Olson and Seidel, 2000). Among factors that are toxic to embryo culture, there are also some other than medium constituents. For example, the number of times and the conditions under which embryos are taken out of the incubator for examination may be important during IVEP steps. Taking embryos out of the incubator exposes them to conditions of temperature, light and gas phase CO_2 to which they would not be exposed in vivo. There is evidence that exposure to light and room temperature negatively influences embryo development (Hegele-Hartung *et al*., 1991; Noda *et al*., 1994). In addition, as well as the oocyte quality is crucial in determining the proportion of immature oocytes that form blastocysts, post-fertilization

culture environment has a major influence on the quality of the blastocyst; in fact, it was reported that the embryo quality and viability is mainly affected by the culture system following IVF (Rizos *et al*., 2002*a*). A substantial amount of evidence exists to demonstrate that the culture conditions to which the embryo is exposed, particularly in the post-fertilization period, can have perturbing effects on the pattern of gene expression and on the embryo quality, with potentially important long-term consequences (Lonergan, 2007). In accordance, it was reported that the expression of seven genes involved in apoptosis, oxidative stress, gap junction formation and differentiation in blastocysts was different between *in vitro* produced and *in vivo* cultured embryos, i.e. using the ewe oviduct (Rizos *et al.* 2002*b*). Also, the different culture systems have profound effects on embryo freezability (Rizos *et al*., 2003). For example, if the in vitro produced zygotes were implanted into the sheep oviduct they had the greatest potential to survive freezing and thawing; on the contrary, if zygotes were cultured with serum-supplemented SOF, they exhibited low cryotolerance (Thompson, 1996).

Collectively, in vivo and in vitro studies support the notion that the environment of the embryo is critical for its future. The identification and characterization of the short-term effects of in vitro culture raises the question about long-term consequences and safety of assisted reproductive technologies (Lonergan, 2007). Currently almost all embryo transfer in cattle is carried out non-surgically using frozen blastocysts and this necessitates the use of a reliable culture system. In combination with optimal methods of in vitro maturation (IVM) and IVF, optimal methods of embryo culture would greatly facilitate the use of IVP embryos. In dairy cattle this would lead to a better use of the genetic merit of high yielding dairy cows.

Figure 3. IVP bovine embryo at different developmental stages

1.6 EVALUATION OF EMBRYO QUALITY

Over the past 30 years, basic and applied studies on classical and advanced embryo technologies have generated a vast literature on factors regulating oocyte and embryo development and quality. In addition, over this period, commercial bovine embryo transfer has become a large international business.

It is generally accepted that bovine embryos produced *in vitro* have lower developmental capacity and quality than *in vivo*-produced embryos (Hasler *et al.,* 1995; Galli and Lazzari 1996; van Wagtendonk-de Leeuw *et al.,* 2000). Relatively little has changed in the techniques of producing embryos *in vivo* although there is increasing evidence of the importance of, for example, peripheral and follicular endocrine profiles for the subsequent developmental competence of the embryo. Most of immature bovine oocytes fail to develop to the blastocyst stage during the process of *in vitro* production, with developmental rates being generally limited to approximately 35-40%. Furthermore, pregnancy rates after ET on cryopreserved in vitro produced embryos are lower than those recorded with in vivo embryos, due to their poorer quality (Gomez *et al*., 2009). These unsatisfactory low

developmental capacities represent a major hurdle for further implementation of embryo technologies in the bovine (e.g. ovum pick-up followed by *in vitro* embryo production). Therefore, accurate assessment of embryo quality is crucial to increase the efficiency of *in vitro* embryo production.

However, it is important to define the term 'quality' at the outset. The ultimate test of the quality of an oocyte or zygote or cleavage-stage embryo remains its ability to develop to the blastocyst stage, to establish a pregnancy and to produce live and healthy offspring. Thus, it is necessary to identify reliable parameters that reflect developmental competence.

1.6.1 Non-invasive techniques

An accurate assessment of oocyte and/or embryo developmental competence is crucial for high *in vitro* developmental rates, for successful establishment of pregnancy after transfer to recipients to enable improvements in terms of the overall whole efficiency and economy of assisted reproductive techniques. Traditional methods predict the developmental competence of an oocyte or embryo indirectly on the basis of scoring morphological properties and developmental kinetics or on the basis of the origin of the embryo or oocyte, which indicates that the developmental environment in which the embryo has developed is correlated to subsequent developmental capacity.

Numerous morphological criteria have been reported to be correlated with oocyte and/or embryo quality (Camargo *et al.,* 2006; Wang and Sun, 2007). Morphological factors related to the quality of cumulus-oocyte complexes (COC) and/or oocytes include COC morphology (Blondin and Sirard, 1995; de Wit *et al.,* 2000; de Wit and Kruip, 2001; Boni *et al.,* 2002), the number of cumulus cell layers (Blondin and Sirard, 1995; Zeuner *et al.,* 2003;

Warriach and Chohan, 2004; Yuan *et al.,* 2005), oocyte diameter (Fair *et al.,* 1995; Fair, 2003) and oocyte coloration (Nagano *et al.,* 2006). Furthermore, the size of the perivitelline space (De Sutter *et al.,* 1996) and the thickness and organization of the ZP (Talevi *et al.,* 1997; Gabrielsen *et al.,* 2001) have been correlated with developmental capacity. Similar studies in cattle established ZP birefringence as a marker of the developmental capacity of unfertilized (Held *et al.,* 2012) and fertilized (Koester *et al.,* 2011) oocytes and zygotes. Recently, a correlation between ZP birefringence and *in vitro* development was reported for equine oocytes (Mohammadi-Sangcheshmeh *et al.,* 2013). Additional characteristics indicative of oocyte quality in various species, including cattle, consist of mitochondrial distribution (Bavister and Squirrell, 2000; Stojkovic *et al.,* 2001; Au *et al.,* 2005) and glucose-6-phosphate dehydrogenase (G6PDH) activity measured by Brilliant cresyl blue (BCB) staining of immature oocytes (Pujol *et al.,* 2004; Alm *et al.,* 2005; Bhojwani *et al.,* 2007). Blastocyst development is already one step along the road to the production of live offspring. However, it is essential that the embryos reaching the blastocyst stage are of the highest quality possible to ensure optimal pregnancy rates after transfer. Nowadays, reliable markers of the developmental competence of blastocysts are rare, assessment of embryo quality is still a challenge and morphological assessment is at present the most popular method for embryo selection prior to transfer. Embryo quality assessment is most commonly performed by non-invasive morphological evaluation of embryos based on embryo color, blastomere symmetry, cytoplasmic granulation and fragmentation, although this technique is prone to subjectivity (Lindner and Wright, 1983; Merton, 2002).

The quality of bovine blastocysts has been described simply on the basis of morphological criteria, such as the darkness of the cytoplasm (Pollard and Leibo, 1994) caused by a higher

lipid content (Abe *et al.,* 2002) that is correlated with metabolic changes (Khurana and Niemann, 2000). In human embryos, assessment of the diameter of Day 5 blastocysts revealed that blastocyst diameter correlated positively with subsequent developmental capacity (Racowsky *et al.,* 2003). Accordingly, bovine blastocyst diameter was identified as being correlated with cell count and hatching probability (Hoelker *et al.,* 2006).

Although there is a consensus that morphological assessment is subjective, the grading of bovine oocytes according to morphological criteria still provides some basic information for the preselection of oocytes to maximize *in vitro* embryo development. In contrast, reliable morphological predictors at the blastocyst stage for viability after embryo transfer are completely lacking, which could be explained by the suggestion that attainment of the blastocyst stage is considered more a reflection of past achievements than a guarantee of future fitness (McEvoy *et al.,* 2000). Despite this, the most important criterion to determine the quality of embryos to be transferred is the timing of development to specific morphological stages (Leibfried-Rutledge *et al.,* 1987; McKiernan and Bavister, 1994; Bavister, 1995; Van Soom *et al.,* 1997). Extensive research to find a better non-invasive, rapid and simple embryo quality parameter involved studies on developmental kinetics (Van Soom *et al*., 1997; Lonergan, 2000) and oxygen consumption measured by respirometer (Lopes *et al*., 2007). In fact, oxygen consumption of preimplantation embryos is another important parameter that may provide valuable information on metabolic processes and subsequent embryo viability. The kinetics of development of early bovine embryos have been explored extensively (Grisart *et al.,* 1994; Van Langendonckt *et al.,* 1997; Holm and Callesen, 1998; Holm *et al.,* 1998; 2002; Lockwood *et al.,* 1998; Lonergan *et al.,* 1999). A clear relationship exists between the time of first cleavage after insemination *in vitro* and

developmental competence, with the bovine oocytes cleaving earlier (~30 h after insemination) being more likely to reach the blastocyst stage compared with their later cleaving counterparts (~36 h after insemination) (Dinnyés *et al.,* 1999; Lonergan *et al.,* 1999). This finding has been confirmed for bovine embryos and for many other species, including rhesus monkeys (Bavister *et al.,* 1983), humans (McKiernan and Bavister, 1994; Sakkas *et al.,* 1998; Shoukir *et al.,* 1998; Fenwick *et al.,* 2002), buffaloes (Totey *et al.,* 1996) and mice (Warner *et al.,* 1998), highlighting that the timing of the first cleavage division is linked to developmental ability of embryos. In addition, the duration of the first cell cycles before embryonic genome activation are shorter in *in vivo*-derived that *in vitro*-derived zygotes (Holm *et al.,* 2002). Accordingly, it is generally accepted that early blastocyst formation and expansion is positively correlated with pregnancy rates after transfer, representing the method of choice for the selection of the best *in vitro* derived blastocysts (Figures 4, 5 and 6) before transfer to recipients.

Figure 4. Expanded blastocyst

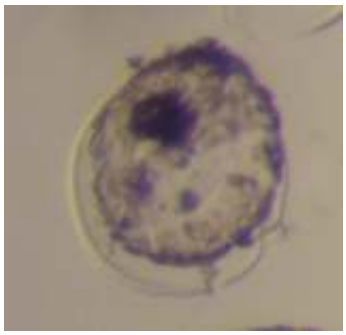


Figure 5. Hatching blastocyst

Figure 6. Hatched blastocyst

1.6.2 Invasive techniques

Invasive techniques need extensive manipulation and/or fixation, which impede further embryo culture or transfer. Nevertheless, they are very valuable in fundamental research and are very frequently used in cattle IVP in combination with other techniques in order to fine-tune embryo quality assessment (Overström, 1996). Several invasive techniques are available for embryo quality evaluation, such as total cell number determination by nucleus staining using propidium iodide (PI) or Hoechst (Pursel *et al*., 1985); evaluation of differentiation in inner cell mass (ICM) and trophectoderm (TE) by differential staining (Van Soom *et al*., 2001; 2002); cryotolerance (Shehab-El-Deen *et al*., 2009) and detection of apoptotic cells using different staining techniques (Wydooghe *et al*., 2011).

In addition, quantitative examination of gene expression is an additional valuable tool to assess the viability of cultured embryos. Groups of embryos of different morphologies, reproductive types (*in vivo* - *in vitro*) and developmental kinetics, which may have developed in contrasting environments, not only exhibit different developmental competence, but also display differences with respect to mRNA expression patterns (Hoelker *et al*., 2014). Thus, it is reasonable to consider these mRNA patterns as molecular signatures of embryo developmental competence. Furthermore, some research has shown that fast cleaving bovine embryos have an altered gene expression (Lonergan, 2000; Ward *et al*., 2001; Fair *et al*., 2004; Gutiérrez-Adán *et al*., 2004; Dode *et al*., 2006) or altered polyadenylation status of several developmentally important gene transcripts (Pocar *et al*., 2001; Brevini-Gandolfi *et al*., 2002) and a higher chance of reaching advanced developmental stages in comparison with late cleaving embryos (Van Soom *et al*., 1992; Dinnyés *et al*., 1999; Lonergan *et al*., 1999; Lequarré *et al*., 2003; Favetta *et al*., 2004;

Gutiérrez-Adán *et al*., 2004). A possible hypothesis is that fast cleaving embryos contain all the necessary gene products and proteins to support further development without any problem, whereas an aberration of some gene products and proteins in slow cleavers could be related to a higher chance to go into developmental arrest or apoptosis (Vandaele and Van Soom, 2011).

Studies focusing on the relationship between bovine blastocyst quality and transcript abundance (Lonergan *et al.,* 2006; Wrenzycki *et al.,* 2005; 2007; Duranthon *et al.,* 2008) provide valuable information about bovine embryonic gene expression, but the conclusions that can be drawn from a small number of differentially expressed candidate genes are limited because it is hard to interpret what the consequences are for subsequent *in vivo* development.

Development of embryos produced by IVF is characterized by a proportion showing abnormal development, delayed cell division or blastomere fragmentation, with many embryos either arresting growth prior to the blastocyst stage or failing to implant following transfer (Trounson and Bongso, 1996). However, counting the total number of blastomeres, the ratio of ICM to TE and the percentage of apoptotic nuclei provide more detailed information on embryo quality and the developmental potential of embryos after transfer to recipients.

It is now well recognized that the ICM differentiates into all tissues of the developing fetus and the TE forms the placenta and embryonic membranes and is involved in maternal recognition of pregnancy (Roberts *et al*., 1990). The quality of these two cell components of the developing embryo determines the outcome after transfer to recipient females. Different methods have been applied for differential staining (DS) of blastocysts in different species,

including isolation of ICM by micromanipulation (Gardner *et al*., 1973), or antibody-based immunosurgery using complement mediated membrane lysis in TE cells (Solter and Knowles *et al*., 1978). These methods are not only time consuming, they are also expensive and depend on the efficiency of the antibody, which is often inconsistent. Further scientific studies have showed that attempts to develop a chemically defined system, using calcium ionophore to permeabilize TE cells (de la Fuente and King *et al*., 1997), as well as permeabilization of TE cells by brief exposure of blastocysts to a non-ionic detergent solution containing propidium iodide (PI), followed by fixation and DAPI staining, permitted the differential visualization of ICM and TE compartments (Thouas *et al*., 2001). The proportion of ICM cells in blastocysts is crucial for implantation (Iwasaki *et al*., 1990), and increased cell death in these cells will compromise subsequent development, since critical threshold numbers of ICM cells are required for normal post-implantation development (Tam, 1988).

Apoptosis is thought to be the default pathway of defective cells in embryos (Raff *et al*., 1993), and cell death by apoptosis occurs predominantly in the ICM in both mouse (Hardy, 1997) and bovine embryos (Byrne *et al*., 1999). Bovine embryos undergo apoptosis predominantly at the morulae stage and during blastocyst development (Byrne *et al*., 1999). Hence, apoptosis may be associated with cell population control and maintenance of cellular quality in the ICM lineage, by eliminating abnormal or defective cells that possess damaged DNA or chromosomal abnormalities (Brison, 2000). The percentage of apoptotic cells is significantly higher in blastocysts produced *in vitro* as compared with in-vivo derived embryos (Bergeron *et al*., 1998), and apoptosis is regulated by the activity of pro- and anti-apoptotic genes during pre-implantation development (Bergeron *et al*., 1998). The

identification of apoptotic cells relies mainly on morphological changes such as chromatin condensation and nuclear and cytoplasmic fragmentation into apoptotic bodies. However, apoptotic cells are not easily distinguishable by light microscopy from other elements with condensed chromatin, such as mitotic cells in telophase (Migheli *et al*., 1995). The in-situ terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) technique is commonly used for detection of apoptosis in cells. However, the reliability of TUNEL has been questioned, as major DNA fragmentation is a late event in apoptosis (Collins *et al*., 1997). Therefore, a combination of morphological assessment of blastocysts showing the presence of fragmented and condensed nuclei and TUNEL labelling is used to assess apoptosis rate in blastocysts. Nevertheless, this method does not permit a clear determination of the cell types undergoing apoptosis in blastocysts, i.e. whether or not the ICM or TE are equally affected. However, using DS method in combination with TUNEL technique, it has been possible to carry out both a qualitative and quantitative analysis of *in vitro* embryos produced. The two different methods provides a means of assessing the effect of culture conditions on cell number of both embryo compartments (ICM and TE), as well as providing information on the localization of apoptotic nuclei within the blastocyst (Fouladi-Nashta *et al*., 2005).In conclusion, development of additional accurate laboratory methods and future efforts to assess embryo quality will improve the efficiency of embryo production from in-vitro culture systems.

Another important parameter for pregnancy establishment and maintenance after ET is the embryo cryotolerance. This is of the utmost importance, as most of the embryos transferred around the world are cryopreserved. For this reason, we believe that it is worth to discuss the topic of cryopreservation in a separate paragraph.

1.7 CRYOPRESERVATION

The concept of preserving life in a suspended animation state with the use of low temperature is not new. Cryobiology history can be traced back to antiquity. As early as in 2500 BC low temperatures were used in Egypt in medicine and the use of cold was recommended by Hippocrates to stop bleeding and swelling. With the emergence of modern science, Robert Boyle studied the effects of low temperatures on animals and in 1949 bull semen was cryopreserved for the first time by a team of scientists led by Christopher Polge. The study on cryopreservation moved into the human world in the 1950s with pregnancies obtained after insemination with frozen sperm. The maintaining of the potential viability of living reproductive cells, embryos and tissues of several mammalian species after long-term storage represents a tool of great opportunity for both human and animal reproductive applications (Woods *et al*., 2004; Vajta and Kuwayama, 2006). This is possible with the use of cryopreservation, a process where cells or whole tissues are preserved by cooling to low sub-zero temperatures such as −196 °C (the boiling point of liquid nitrogen). At these low temperatures, any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped.

Cryopreservation occurs naturally. Antifreezing is actually required for ensuring survival of various organisms. However, the simple immersion in liquid nitrogen for certain samples, such as embryos and oocytes, does not maintain the necessary viability to make them usable after thawing. Increased understanding of the mechanism of freezing injury to cells empathized the importance of cooling methods to obtain maximum survival on thawing of the living cells. Because embryos are viable at 37°C and no biological activity takes place at -196 °C, the time of greatest danger during cryopreservation appears to be during the

transitions of temperature: during cooling to -196 °C and during subsequent rewarming to 37°C. When water is cooled below its freezing point, it solidifies in a crystalline structure known as ice. Because ice is less dense than liquid water, it necessarily follows that ice crystals occupy a greater volume than does the liquid water from which they were formed. As adjacent volumes of liquid water within a cell solidify, their expansion into ice causes pressure and shearing forces on intracellular organelles, which can suffer considerable damage. Avoidance of ice crystal formation therefore is one of the principal goals of successful cryopreservation. As water transitions from liquid to ice, any solutes in the liquid phase are excluded from the solid. This lowers the freezing point of the remaining unfrozen solution. As the temperature drops and the solid form proliferates, the concentration of electrolytes and other solutes can reach very high levels (Lovelock, 1954). These concentrations can be quite toxic to intracellular proteins, and thus avoidance of these solution effects is a second major goal of successful cryopreservation. During rewarming, the solid ice melts and releases free water, resulting in decreasing osmolarity of the surrounding solution. When rewarming is slow, there is danger of free water thawing and recrystallizing, thus causing further damage. When rewarming is rapid, sudden drops in extracellular osmotic pressure may lead to rapid shifts of free water across and into the cell, leading to swelling and cell damage (Mazur, 1980). This is called osmotic shock, and its avoidance is a third major goal of successful cryopreservation. Therefore, simply immersing embryos into liquid nitrogen is not an effective strategy for successful cryopreservation. All successful methods must avoid these three issues: ice crystal formation, solution effects, and osmotic shock.

Up to this point in time, all cryopreservation strategies, including all methods of embryo cryopreservation, have used additional chemicals to avoid cell damage. These chemicals are called cryoprotectants and generally can be divided into two categories: permeating and non-permeating.

Permeating cryoprotectants are small molecules that readily permeate the membranes of cells. They form hydrogen bonds with water molecules and prevent ice crystallization. At low concentrations in water, they lower the freezing temperature of the resulting mixture. However, at high enough concentrations, they inhibit the formation of the characteristic ice crystal and lead to the development of a solid, glasslike, so-called vitrified state in which water is solidified, but not expanded. In this manner, the permeating cryoprotectants satisfy the first goal of successful cryopreservation, the avoidance of ice crystals. Permeating cryoprotectants play a second important role in cryopreservation, and this is to protect the cell from solution effects. They achieve this goal by remaining in solution and by thus effectively diluting the remaining electrolytes. This effect is described by the phase rule, which states that in a two-phase system, such as liquid water and ice at a given pressure, the total solute concentration in the liquid phase is constant for a given temperature (Friedler *et al*., 1988). The most commonly used permeating cryoprotectants are: Propylene Glycol (PROH), Ethylene Glycol (ET), Glycerol and Dimethyl sulfoxide (DMSO).

In contrast to the permeating cryoprotectants, nonpermeating cryoprotectants remain extracellular. They act by drawing free water from within the cell, thus dehydrating the intracellular space. As a result, when they are used in combination with a permeating cryoprotectant, the net concentration of the permeating cryoprotectant is increased in the intracellular space. This further assists the permeating cryoprotectant in preventing ice-

crystal formation. The nonpermeating cryoprotectants play an important role during thawing. During thawing, the water generated by the melting ice rapidly decreases the extracellular osmotic pressure. Osmotic shock may occur if the intracellular cryoprotectant cannot diffuse out quickly enough to prevent excessive influx of free water and the swelling, or even rupture, of the cell. Therefore, freezing and thawing protocols commonly use a high concentration of nonpermeating cryoprotectants during the thawing phase. The most commonly used nonpermeating cryoprotectant is sucrose, but other disaccharides and other nonpermeating agents also may be used.

In conclusion, different are the advantages to use cryopreservation: the progress in cryopreservation would improve domestic animal breeding by genetic selection programs (Vajta, 2000), to contribute at the maintenance of biodiversity through wildlife species conservation (Pukazhenthi and Wildt, 2004) and preserve the human fertility.

1.7.1 Cryopreservation methods

All oocytes and embryos suffer considerable morphological and functional damage during cryopreservation. The extent of the injury depends on factors including the size and shape of the cells, the permeability of the membranes, and the quality and sensitivity of the oocytes and embryos. All these factors may be highly variable depending on the species, developmental stage and origin (e.g., in vitro produced or in vivo derived embryos). However, oocytes and embryos also have a remarkable, sometimes surprising ability to repair this damage fully or partially, and, for optimal cases, to continue normal development. The purpose of cryopreservation procedures is to minimize the damage and help cells to regenerate. Almost all cryopreservation strategies are based on two main

factors: cryoprotectants and cooling–warming rates. Since the first successes of mammalian embryo cryopreservation in the sixties of the last century, two major groups of methods can be delineated: traditional slow-rate freezing and vitrification. Storage, warming and rehydration, i.e. removal of cryoprotectants differ only slightly between the two procedures (with some exceptions) and the main difference exists in the addition of cryoprotectants and cooling. Traditional slow-rate freezing was introduced first, and for the majority of domestic animal and human embryologists this remains the only acceptable approach. Over time, methods have become highly standardized with a considerable industrial and commercial background. Traditional slow-rate freezing can be interpreted as an attempt to create a delicate balance between various factors causing damage, including ice crystal formation, fracture, toxic and osmotic damage. The controlled cooling rate allows solution exchange between the extracellular and intracellular fluids without serious osmotic effects and deformation of the cells (this fact is reflected in the other name of the procedure: equilibrium freezing). Controlled-rate and slow freezing are well established techniques pioneered in the early 1970s which enabled the first birth from a human frozen embryo in 1984. Since then many studies have developed to preserve human and animal samples from freeze damages until the development of a new technique, the vitrification, that provides the benefits of cryopreservation without damage due to ice crystal formation.

The phenomenon of the solidification of water without ice crystal formation is also called vitrification. However, the term “vitrification” in cryobiology refers to the other group of cryopreservation methods, where the main purpose is to ensure ice free solidification of the whole solution containing the sample. The process itself can be described as an extreme increase in viscosity of solutions. The benefits are unquestionable: ice crystal formation, one

of the most dangerous causes of cryoinjury, is entirely eliminated except for a transitional and very short freezing of the solutions during warming that is generally regarded harmless to the oocytes and embryos. Unfortunately we have to pay the price of this benefit. Vitrification can only be induced in exceptional situations: with dangerously-high concentrations of cryoprotectants and/or with extreme increase of the cooling and warming rates. One characteristic of vitrification is that as the cooling rate chosen increases, the cryoprotectant concentration can be lowered and vice versa. Extensive research in the past 20 years has resulted in new, sometimes radically-new approaches and created an acceptable compromise between the positive and negative by decreasing cryoprotectant toxicity and increasing cooling rates. As a result, vitrification has become a competitive alternative to slow-rate freezing. Moreover, the high cooling and warming rates applied at vitrification provide a unique benefit compared to the traditional freezing: the possible partial and sometimes total elimination of chilling injury, as the sample passes through the dangerous temperature zone quickly enough to disallow sufficient time for damage to develop. During vitrification, permeating cryoprotectants are added at a high concentration while the embryo is at room temperature. Because the toxicity of this high concentration of permeating cryoprotectant is substantial, the embryo cannot be kept at this temperature for long. Instead, a very short time is allowed for equilibration, after which the embryos are plunged directly into liquid nitrogen. To further protect against ice-crystal formation, an extremely rapid rate of cooling is used. For this reason, novel cryovessels have been used that allow direct contact between liquid nitrogen and the embryo-containing solution and that have in common a very high surface-to-volume ratio (Park *et al*., 2000; Mukaida *et al*., 2003). This

form of ultrarapid freezing must be followed by ultrarapid thawing to prevent ice recrystallization. To achieve vitrification, three important factors should be considered:

1) Cooling rate, which is achieved with liquid nitrogen or liquid nitrogen slush. When using liquid nitrogen, the sample is plunged into liquid nitrogen resulting in cooling rates of hundreds to tens of thousands degrees Celsius per minute, depending on the container, the volume, the thermal conductivity, the solution composition, etc. (Yavin and Arav, 2007). It was shown for oocytes and embryos that increasing the cooling rate would improve survival rates by up to 37%.

2) Viscosity of the medium in which the embryos are suspended. This is defined by the concentration and behavior of various cryoprotectants and other additives during vitrification. The higher is the concentration of cryoprotectants, the higher is the glass transition temperature (Tg), thus lowering the chance of ice nucleation and crystallization. The combination of different cryoprotectants is often used to increase viscosity and Tg, and reduce the level of toxicity.

3) Volume; the smaller is the volume, the higher is the probability of vitrification (Arav, 1992; Arav *et al*., 2002). Smaller volumes allow better heat transfer, thus facilitating higher cooling rates. Many techniques have been developed to reduce sample volume with an explosion of methods appearing in the literature during the last decade (Yavin and Arav, 2007).

1.7.3 Vitrification techniques

Vitrification was first introduced in embryology for cryopreservation of mouse embryos (Rall and Fahy, 1985). In the subsequent decade, a moderate number of publications dealing

with successes in cryopreservation of oocytes and embryos from several domestic animal species were published (Pereira and Marques, 2008). The latest development in the practical application of ultrarapid vitrification methods was based on the concept of minimum volume cooling (Hamawaki *et al*., 1999). Different carrier tools were applied to minimize the volume and to submerge the sample quickly into the liquid nitrogen.

These techniques can generally be divided into two categories, surface techniques and tubing techniques.

The surface techniques include:

1) Solid Surface Vitrification (SSV) method, that combines the advantages of containerless vitrification in microdrops and the increased heat exchange of a cold metal surface (Dinnyés *et al*., 2000). A metal cube, in some cases covered with aluminum foil, is partially submerged into liquid nitrogen. Microdrops of vitrification solution, containing the embryos, are dropped onto the cold upper surface of the metal cube (Figure 7) and are instantaneously vitrified. Warming was performed by placing the drop of vitrified solution in a warming solution directly. This approach makes a shortcut to exclude entirely the vapor formation by cooling metal surfaces in liquid nitrogen, and placing the solution containing the biological sample on the surface of metal.

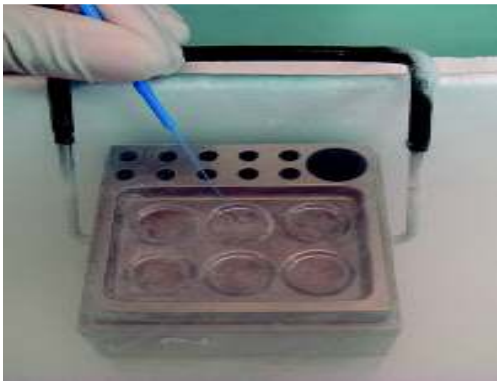


Figure 7. Solid Surface Vitrification

2) The Cryoloop method consists in the use of a solution film formed between files of a nylon loop (Figure 8) for holding the sample (Lane *et al*., 1999a; 1999b). Due to the absence of solid support and the extremely small volume, the cooling rate upon immersion in liquid nitrogen may be as high as 700,000 °C/min (Isachenko *et al*., 2003) thus also permitting cooling in liquid nitrogen vapor. However, in spite of the unquestionable benefits of this system from the point of potential disease transmission, one may have concerns regarding the safety of storage, as this very sensitive and fragile system may increase the risk of accidental warming over the safe temperature zone, especially if storage of the container is also performed in the vapor of liquid nitrogen (Fuller and Paynter, 2004).

Figure 8. Cryoloop

3) In the Cryotop procedure a thin plastic film strip attached to a plastic handle (Figure 9) is the carrier tool (Hamawaki *et al*., 1999; Kuwayama and Kato, 2000). Loading is performed with the use of a glass capillary under the control of a stereomicroscope. Before cooling, almost all medium is removed, so the embryos and oocytes are only covered with a very thin solution layer. Submersion of the Cryotop into the liquid nitrogen or into the rehydrating solution after vitrification results in extremely high cooling and warming rates,

respectively, permitting further decrease in concentration of cryoprotectants. During storage, a protective cap is applied to prevent mechanical damage of the film and the vitrified sample. The minimal volume approach of the Cryotop method increases the cooling and especially the warming rates (up to 40,000 °C/min) which may contribute to the improved and consistent survival, and both in vitro and in vivo developmental rates. According to data published (Kuwayama, 2007), the Cryotop technique is at present the most efficient method for cryopreservation of sensitive samples including human oocytes. The Cryotop vitrification method is easy to learn. Anybody with basic experience in embryology can perform it appropriately after a few hours of training period. The method is simple and reliable, provides consistent results and variations between operators are minimal.

Figure 9. Cryotops

4) The Vitri-Inga vitrification strip is an apparatus that consists of a fine, very thin polypropylene strip (0.7 mm thick) with a specially designed round tip (Figure 10), in which there is a minute hole to receive the embryo; the strip is connected to a hard and thicker plastic handle (Almodin *et al*., 2010).

Figure 10. Vitri-Inga

The tubing techniques include:

1) The Open Pulled Straws (OPS) are French mini-straws (250 µl, IMV, L'Aigle, France) heat-softened over a hot plate, and pulled manually until the inner diameter and the wall thickness of the central part decrease from 1.7 mm to approximately 0.8 mm, and from 0.15 mm to approximately 0.07 mm, respectively. Loading of sample into these straws is performed using the capillary effect by simply touching a 1- to 2-µl droplet containing the ova or embryos with the narrow end of the straw (Vajta *et al*., 1997; 1998). This end is then immediately submerged into liquid nitrogen and the liquid column solidified instantaneously. Warming is performed by placing the end of the straw directly into the holding medium. The vitrified medium becomes liquid within 1 to 2 seconds, whereupon the holding medium enters the straw. Immediately afterwards, by means of the sedimentation, the ova or embryos float out of the straw into the holding medium.

2) With CryoTip method the samples are loaded into a plastic capillary with ultrathin walls, both ends are sealed, and the capillary is submerged into the liquid nitrogen. The CryoTip also includes a protective tube preventing mechanical damage during handling and storage. For warming the capillary is submerged into warm water, then decontaminated with ethanol, cut and the sample is expelled into the rehydrating solution (Kuwayama *et al*., 2005).

3) Cryopette is a cryopreservation device that is intended to be used to contain, freeze using vitrification procedures. Consists of a polycarbonate Cryo-Straw with a silicone rubber displacement bulb. This device has been optimized as a closed system for cryopreservation procedures (Portmann *et al*., 2010).

Each of these two groups has its specific advantages. In the surface methods, if the size of the drop (~ 0.1 mL) can be controlled, high cooling rate can be achieved because these systems are open, and high warming rates are achieved by direct exposure to the warming solution. The tubing systems have the advantage of achieving high cooling rates in closed systems, thus making them safer and easier to handle. Decreasing the vitrified volume and increasing the cooling rate allow a moderate decrease in cryoprotectant concentration so as to minimize its toxic and osmotic hazardous effects (Yavin *et al*., 2009).

1.7.3 Embryo cryopreservation

The first mammalian species whose embryos were successfully cryopreserved was the mouse in the early 1970s when Whittingham *et al.* (1972) obtained live mice after the transfer of frozen-thawed morulae. The earliest successes with mouse embryo cryopreservation were achieved with slow cooling, at a constant rate, to deep subzero temperatures (−80°C). Observations of embryos during this cooling procedure demonstrated the extreme dehydration encountered during the process when ice formed from pure water as extracellular ice formed around the cells, leaving the embryos in the small, residual hypertonic unfrozen fraction (Leibo *et al*., 1974). Conditions that permitted intracellular ice to form (by cooling too fast and leaving water molecules capable of nucleating ice crystal growth) were universally found to be damaging (Mazur, 1970). It was also argued that slow

warming was necessary to avoid cell volume stress. In the intervening time, further investigations demonstrated that this low-temperature (and successful embryo cryopreservation) could be achieved if the embryos were slowly cooled only to a relatively high subzero temperature and then transferred directly to –196°C. In this case, warming needed to be rapid to achieve high viability. These findings, developed by Willadsen and Cambridge to freeze sheep embryos (Willadsen, 1977), have also laid the foundations of cryopreservation techniques widely used in commerce. The first experiment with the technique of vitrification date, however, until 1985, when Rall and Fahy applied this technique for the first time in mouse embryos, while the first pregnancy achieved by transfer of vitrified bovine embryos has been reported by Massip *et al*. in 1986.

Since then, many experiments and significant improvements were achieved in the field of embryo cryopreservation. In recent years embryos have been successfully cryopreserved in most domestic species, especially cattle (Lazar *et al*., 2000; Zhang *et al*., 1993), sheep (Ptak *et al*., 1999; Dattena *et al*., 2000), goats (Baril *et al*., 1989; El Gayar and Holtz, 2001; Traldi, 2000), horse (Hochi *et al*., 1994) and pig (Hayashi *et al*., 1989; Berthelot *et al*., 2000). Across all these different species, the protocols applied to embryo cryopreservation tend to fall into one of two broad categories, slow controlled-rate cooling and ultrarapid cooling or vitrification. Throughout these experiences, it became obvious that embryo cryopreservation would too become centrally important in reproductive technologies by allowing optimal utilization of resources in the development of breeding programs in animals (Massip *et al*., 2004) by permitting genetic banking (for example of rare animals), and by enhancing the clinical approach to infertility treatment.

EXPERIMENTAL PART

CHAPTER 2: Introduction to experimental part

2.1 ANTIOXIDANT SUPPLEMENTATION DURING IVC

The high incidence of developmental failure of in vitro produced embryos has been attributed to a wide range of non mutually exclusive dysfunctions, including poor gamete quality, chromosomal abnormalities, telomere uncapping, and suboptimal culture conditions that induce oxidative stress. Indeed, increased oxidative stress is the major factor affecting in vitro mammalian embryo development. Oxidative stress has been implicated in many different types of cell injuries, including membrane lipids peroxidation, oxidation of amino acids and nucleic acids, apoptosis and necrosis which may subsequently decrease the viability of IVP embryos (Johnson and Nasr-Esfahani, 1994; Kitagawa *et al*., 2004). To overcome this, exogenous antioxidant compounds have been frequently used to increase antioxidant capacity of embryos via increasing intracellular levels of Reactive Oxygen Species (ROS) scavengers, such as glutathione (GSH).

Reactive oxygen species, represented by superoxide anion (O_2^-), the hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), are originated from embryo metabolism (enzymatic mechanism) and/or surrounded environment. In addition, the relative contribution of each ROS source differs according to developmental stages and species. It is also well known that several exogenous factors and culture conditions could increase ROS production by embryos. ROS can induce oxidative modifications of the cell components, thus indirectly causing DNA fragmentation, protein oxidation, lipid peroxidation and mitochondrial

damage; then, ROS activity could change most types of cellular molecules and pathways, inducing block and regression of cellular development.

As a result, embryos have multiple protective mechanisms against ROS as complementary systems. The extrinsic protection of embryo presented in follicular and oviductal fluids mainly involves antioxidants such as hypotaurine, taurine and ascorbic acid. Embryo intrinsic protection involves enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and gamma glutamylcysteine synthetase (GCS). The transcripts for these enzymes were described in the oviduct. These important transcripts are saved in order to get to capability of embryonic development (Guérin *et al*., 2001). In addition to antioxidant enzymes, non-enzymatic antioxidants have important roles for keeping intra- and extra-cellular redox balance. One of such non-enzymatic antioxidants is GSH. This is a tripeptide thiol synthesized by glutamic acid, cysteine and glycine in the pathway of γ-glutamyl cycle (Cornell and Meister, 1976). GSH acts as a strong reducing agent as well as serving an electron donor for GPx; in addition, it is known to have many important functions in cells or embryos not only for protection from oxidative injuries, but also for DNA synthesis, transcription, cell cycles, cytokine activity and apoptosis (Liang *et al*., 1989; Chiba *et al*., 1996). Thus, for an effective culture of bovine embryos, it is important to regulate intracellular GSH by controlling cysteine and cysteine as a major source of GSH against the oxidative stress.

Also, sub-optimal in vitro culture conditions, specifically high glucose concentrations, exposure to light and higher oxygen concentrations, when compared with in vivo conditions, have also been associated with increased ROS generation and perturbed developmental ability (Luvoni *et al*., 1996; Hashimoto *et al*., 2000; Guérin *et al*., 2001;

Kitagawa *et al*., 2004). Nevertheless, the assumption that improved embryo development under low oxygen concentrations could be due to reduced ROS production is yet to be conclusively demonstrated (Harvey *et al*., 2002; Harvey, 2007). To reduce the toxicity of ROS for improving embryo development, antioxidants supplementation is effective to regulate intra- and extra-embryonic environments; in fact, maintaining of intra- and extra-cellular redox balance is very important for providing suitable environments for metabolic and gene expressions followed by embryo development (Takahashi, 2012). Furthermore, Lopes *et al*. (2010) showed that oxygen consumption and ROS production are increased at the time of fertilization and cell cleavage in bovine zygotes.

Therefore, in order to protect the embryos from increased oxidative stress, and the consequent damages, in the last years there has been an increased interest in strategies based on the supplementation of antioxidant compounds during in vitro embryo culture. Indeed, various antioxidants such as β-mercaptoethanol (β-ME), cysteine and cysteamine have been added to the IVM (de Matos and Furnus, 2000; Gasparrini *et al*., 2000; Gasparrini *et al*., 2003; Gasparrini *et al*., 2006) and IVC media (Hosseini *et al*., 2009; George *et al*., 2008) to improve the development of pre-implantation embryos. Several protein and non-protein antioxidants have been reported to improve embryonic development (Table 1). Addition of free radical scavenger enzymes such as SOD, CAT or thioredoxin (TRX) has been proven beneficial for embryo development, by scavenging ROS and serving embryos a low oxidative stress conditions in mouse (Natsuyama *et al*., 1993), pig (Ozawa *et al*., 2006) and cow (Ali *et al*., 2003). In these species, melatonin, known as an antioxidant protein, also improved the embryo development (Papis *et al*., 2007; Rodriguez-Osorio *et al*., 2007; Tian *et al*., 2010).

In addition to GSH, other non-protein antioxidants, such as vitamins, mineral or chelating agents, supports embryo development *in vitro*. Antioxidant vitamins such as α-tocopherol, β-carotene, ascorbate and vitamin C are among the major dietary antioxidants that directly scavenge ROS and provide a condition of protection against the damaging effects of ROS. Supplementation of vitamins to culture medium improves embryo development in mouse (Wang *et al*., 2002), pig (Huang *et al*., 2011) and cow (Olson and Seidel, 2000).

Finally, supplementation of polyphenols also promoted development of pig (You *et al*., 2010), mouse (Perez-Pasten *et al*., 2010) and bovine embryos (Wang *et al*., 2007). Polyphenols are a wide variety of organic molecule contained in vegetables, fruit and plant-derived beverages such as tea, red wine and extra virgin olive oil. They are characterized by the presence of several groups involved in phenolic structures and include the flavonoids and phenolic acids; in particular, flavonoids promoted development of bovine embryos culture both in high (20%) and low (5%) O_2 tension, by reducing of intracellular ROS (Lee *et al*., 2011).

Table 1. Antioxidants that improve in vitro development of preimplantation embryos

ANTIOXIDANT	ANIMAL	REFERENCE
β-ME and Cysteamine	cow	De Matos *et al*., 2002
	pig	Kitagawa *et al*., 2004
	buffalo	Gasparrini *et al*., 2000; 2003
GSH	cow	Luvoni *et al*., 1996
	goat	Lee *et al*., 2000
	pig	Choe *et al*., 2010
GSH-ethyl ester	cow	Curnow *et al*., 2010*a*
	macaque	Curnow *et al*., 2010*b*
Cysteine	cow	Ali *et al*., 2003
	pig	Katayama *et al*., 2007
	mouse	Goto *et al*., 1993
Cystine	buffalo	Gasparrini *et al*., 2006
Vitamins	cow	Olson and Seidel, 2000
	pig	Hossein *et al*., 2007; Huang *et al*., 2011
	mouse	Wang *et al*., 2002
Polyphenols	cow	Wang *et al*., 2007; Lee *et al*., 2011
	pig	You *et al*., 2010
	mouse	Perez-Pasten *et al*., 2010
Antioxidant enzymes (SOD, CAT, TRX)	cow	Ali *et al*., 2003
	pig	Ozawa *et al*., 2006
	mouse	Legge and Sellens, 1991; Natsuyama *et al*., 1993
Melatonin	cow	Papis *et al*., 2007
	pig	Rodriguez-Osorio *et al*., 2007
	mouse	Tian *et al*., 2010
L-carnitine	Buffalo (*bubalus bubalis*)	Boccia *et al*., 2013; Gasparrini *et al*., 2013
	mouse	Abdelrazik *et al*., 2009
Sodium-selenite	pig	Uhm *et al*., 2007
	rabbit	Carney and Foote, 1991
	mouse	Abedelahi *et al*., 2010

2.2 AIM OF THE WORK

As previously described, to improve the development and quality of pre-implantation bovine embryos, different antioxidant compounds have been tested during in vitro embryo culture, in the attempt to reduce ROS generation and subsequent oxidative stress conditions. Nevertheless, despite the improvements achieved, efforts are still needed to identify the most effective supplements to improve embryo quality, that plays a critical role in determining both cryotolerance and pregnancy maintenance after ET.

Therefore, the aim of this thesis was to investigate whether supplementation of bovine culture medium with different antioxidant compounds found in several vegetal sources, such as Resveratrol (Experiment 1), L-Ergothioneine (Experiment 2) and Crocetin (Experiment 3), improves in vitro blastocyst development, viability and quality. In each experiment the latter parameter was assessed by different approaches, such as the timing of development to specific morphological stages, resistance to cryopreservation, total cells number, ratio of ICM:TE cells and apoptosis index. The rationale for the choice of the natural antioxidants that we tested is reported separately in a brief introduction of each experiment.

CHAPTER 3: Experiment 1

Effect of resveratrol supplementation during culture on the quality and cryotolerance of bovine in vitro produced embryos

Resveratrol (3,4',5-trihydroxystilbene) is a phytoalexin (Figure 11) identified in more than 70 plant species including grapes, plums, and peanuts (Baur and Sinclair, 2006). It is also an important antioxidant polyphenolic compound, which contributes to red wine's beneficial effects on the prevention of human cardiovascular disease. Recently, the interest in resveratrol has increased exponentially, following the major findings that this molecule has positive effects on cancer chemoprevention, cardioprotection, inflammatory processes and several aspects of metabolism, leading to increased lifespan of various organisms, from yeasts to vertebrates (Pirola and Fröjdö, 2008). Several studies have been conducted to identify the biologic functions and activities of resveratrol for mammalian reproduction (Kwak *et al*., 2012; Huang *et al*., 2013). A positive effect of resveratrol on in vitro embryonic development was demonstrated in swine, as indicated by enhanced blastocyst formation and improved embryo quality (Lee *et al*., 2010). Recently, Wang *et al*. (2014) demonstrated that the addition of resveratrol to the in vitro maturation medium increases progesterone secretion and decreases estradiol secretion by cumulus cells, promoting cumulus expansion and polar body formation in bovine oocytes and improves blastocyst yield. Therefore, the aim of this work was to evaluate whether supplementation of culture medium with resveratrol improves in vitro blastocyst development and embryo quality in cattle, the latter assessed both in terms of cryotolerance and allocation of blastocyst cells into the inner cell mass (ICM) and trophectoderm (TE) lineages.

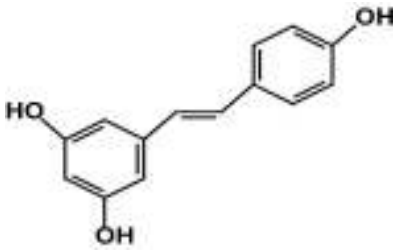


Figure 11. Chemical structure of resveratrol.

3.1 MATERIALS AND METHODS

Experimental Design

A preliminary dose response trial (0 to 10 μM) demonstrated deleterious effects of resveratrol on both cleavage and blastocyst development at concentration ≥ than 5 μM. Therefore, the range of concentrations to test was reduced in the following experiment, in which presumptive zygotes were cultured in vitro with 0 (control, n= 439), 0.25 μM (n= 422), 0.5 μM (n= 447) and 1 μM resveratrol (n= 416), over six replicates. To assess the embryo quality, the blastocysts produced (on average 153 per group) at day 7 (end of culture) were vitrified, warmed and cultured for further 2 days, and assessments were carried out both at 24 and 48 h. Finally, zygotes were cultured in the absence (control) and presence of 0.5 μM resveratrol to produce blastocysts for subsequent DS.

Considering that differences in the cell number and allocation of blastocysts may appear when the developmental stages are taken into account (Korhonen *et al*., 2010), only good quality XBl and HBL stage embryos (with a clearly visible ICM and blastocoel) were chosen for the evaluation of cell allocation by DS.

Reagents and media

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich Chemical (Milano, Italy).

The oocyte aspiration medium was TCM 199 supplemented with 25 mM Hepes, 2 mM sodium bicarbonate, 2 mM sodium pyruvate, 1 mM L-glutamine, 10 µL/mL amphotericin B (H199) supplemented with 2% bovine serum (BS) and 95.6 SI/mL heparin. The IVM medium was TCM 199 supplemented with 15% bovine serum (BS), 0.5 µg/mL FSH, 5 µg/mL LH, 0.8 mM L-glutamine and 50 µg/mL gentamycin. The IVF medium was Tyrode's modified medium (Parrish *et al*., 1986) without glucose and bovine serum albumin (BSA), supplemented with 5.3 SI/mL heparin, 30 µM penicillamine, 15 µM hypotaurine, 1 µM epinephrine and 1% of BS. The IVC medium consisted of Synthetic Oviduct Fluid (SOF) medium (Tervit *et al*., 1972), with 30 µL/mL essential amino acids, 10 µL/mL non-essential amino acids and 5 % BS. Resveratrol was dissolved in dimethyl sulfoxide (DMSO) to make a 1000X stock (1 mM) that was kept frozen until day of use, when the lower stock concentrations were obtained by dilutions. Then the relative stock solutions were diluted 1: 1000 in SOF, to reduce to the minimum the amount of DMSO during culture. Likewise, in the control group DMSO (1:1000) was added.

The base medium for the vitrification and warming solutions was H199 with 20% fetal calf serum (FCS). The equilibration medium (VS1) consisted of 7.5% DMSO and 7.5% ethylene glycol (EG) in base medium and the vitrification solution (VS2) consisted of 16.5% DMSO and 16.5% EG with 0.5 M sucrose in the base medium. The warming solutions consisted in 0.25 M and 0.15 M sucrose in the base medium.

In vitro embryo production

Abattoir-derived oocytes were matured and fertilized in vitro according to our standard procedure (Rubessa *et al*., 2011). Briefly, bovine ovaries were recovered from a local abattoir and transported to the laboratory, in physiological saline at 30 to 35 °C. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 2 to 8 mm in diameter and only those with uniform cytoplasm and multilayered cumulus cells were selected, washed twice in the aspiration medium and once in the IVM medium. Groups of 25 COCs were matured in 400 µL of IVM medium, covered with mineral oil, in four well plates (NuncTM, Roskilde, Denmark), for 22 h at 39 °C, and 5% CO_2 in air. In vitro matured COCs were washed and transferred, 25 per well, into 300 µL of IVF medium covered with mineral oil. Frozen sperm from a bull previously tested for IVF were thawed at 37 °C for 40 s and selected by centrifugation (25 min at 300 X g) on a Percoll discontinuous gradient (45 and 80%). The pellet was reconstituted into 2 mL of IVF medium and centrifuged twice, at 160 and 108 X g for 10 min. The pellet was diluted with IVF medium and added in the fertilization wells at the concentration of 1 x 10^6 sperm/mL. Gametes were co-incubated for 20 h at 39 °C, in 5% CO_2 in air, after which presumptive zygotes were vortexed for 2 min to remove cumulus cells in Hepes-TCM with 5% BS, washed twice in the same medium and randomly distributed (30 to 50 per well, into 400 µL of IVC medium) into different culture groups differing on the basis of resveratrol concentrations. Zygotes were incubated in a humidified mixture of 5% CO_2, 7% O_2 and 88% N_2 in air at a temperature of 39 °C. On Day 7 cleavage and blastocyst rates were recorded. The embryos obtained by the end of culture (Day 7) were scored for quality on the basis of morphological criteria (Robertson and Nelson, 2010) and the percentage of transferable embryos, i.e. Grade 1 and 2 tight morulae

and blastocysts (TMBL), and that of Grade 1 and 2 blastocysts (BL) were recorded. Furthermore, the stage of development was assessed and embryos were categorized as slow developing embryos, i.e. tight morulae (TM), early blastocysts (eBl), blastocysts (Bl), and as fast developing embryos, i.e. those that reached the stages of expanded blastocysts (XBl) and hatched blastocysts (HBl) at the end of culture.

Vitrification and Warming

On Day 7 the blastocysts obtained (from eBl to HBl) were vitrified by the Cryotop method, previously described (Kuwayama and Kato, 2000). All equilibration and dilution steps were carried out at 37 °C. Each blastocyst was exposed to VS1 in 200 µL droplet for 3 min, then transferred into a 20 µL droplet of VS2 for 20 to 25 s, and loaded with a glass capillary onto the top of the film strip of each cryotop in 0.1 µL volume. After loading, almost all the solution was removed and the Cryotop was quickly immersed into liquid nitrogen, protected with the cap and stored under liquid nitrogen. For warming, after removal of the cap, the Cryotop strip was immersed directly into 1 mL of the 0.25 M sucrose solution for 1 min and the embryo was transferred into 200 µL droplet of 0.15 M sucrose solution for 5 min. Warmed blastocysts were washed in H199 medium + 10% FCS and cultured for two days. After 24 h and 48 h post-warming culture survival rates were evaluated with morphological criteria, on the basis of the integrity of the embryo membrane and the ZP, and re-expansion of the blastocoel. Furthermore, the percentages of embryos that resumed their development and reached a more advanced developmental stage after culture (development rate), as well as the hatching rates, were recorded.

Differential staining

Only expanded and hatched blastocysts produced in the presence (0.5 µM) or absence of resveratrol on Day 7, were subjected to a DS protocol previously described (Thouas *et al*., 2001) with slight modifications. Briefly, the ZP of viable expanding or expanded blastocysts was removed with 0.2% pronase in Hepes-buffered SOF. After three washes in PBS with 0.1% PVP, blastocysts were first incubated in 500 µL of propidium iodide solution (PBS with 1% Triton X-100 and 100 µg/mL propidium iodide) for 1 min. Blastocysts were then immediately transferred into 500 µL of a fixative solution of 100% ethanol with 25 µg/mL bisbenzimide (Hoechst 33342) for 5 min. The fixed and stained embryos were subsequently transferred into glycerol and mounted onto a glass microscope slide in a drop of glycerol, flattened with a coverslip to a level where all nuclei appeared at the same focal plane, and visualized using a fluorescent microscope (Eclipse E-600 - Nikon) under ultraviolet light with excitation at 460 to 560 nm. A digital image of each embryo (Figure 12) was taken using NIS-Elements-F software and a high-resolution color digital camera (Nikon digital sight DS-Fi 1C) and the numbers of TE (red) and ICM (blue) nuclei were counted.

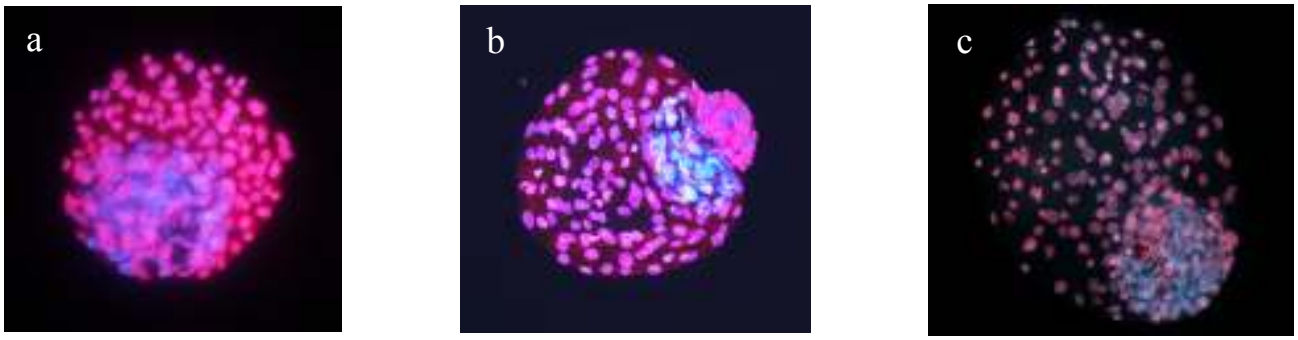


Figure 12. Representative DS pictures of bovine embryos in different developmental stages examined by DAPI filter: XBL (**a**), Hatching BL (**b**) and HBL (**c**).

3.2 STATISTICAL ANALYSIS

Differences among groups in the percentages of blastocysts, as well as in the survival rates after vitrification-warming, were analyzed by Chi square test using SPSS 16.0 statistical software (SPSS Inc., 2007). DS data were analyzed by the Student's *t* test.

3.3 RESULTS

Low concentrations of resveratrol (0.25 and 0.5 μM) during culture did not affect cleavage rate; on the contrary, a decreased cleavage ($P<0.05$) was recorded at the highest concentration (1 μM) compared to the control. Although the percentage of transferable embryos, i.e. tight morulae/blastocysts, was improved ($P<0.05$) when embryos were cultured with 0.25 and 0.5 μM resveratrol, both the percentage of Grade 1 and 2 blastocysts and that of fast developing blastocysts were not affected (Table 2). The latter parameter was reduced ($P<0.05$) in the culture group supplemented with 1 μM resveratrol compared to the other resveratrol-treated groups.

Table 2. Percentages of cleavage, tight morulae-blastocysts (TMBL), Grade 1 and blastocysts (Grade 1,2 BL) and fast developing embryos (fast BL) under different resveratrol concentrations.

Resveratrol (μM)	n. COC	n. (%) Cleaved	n. (%) TMBL	n. (%) Gd 1,2 BL	n. (%) Fast BL
0 (control)	439	315 (71.8)[a]	194 (61.6)[a]	180 (57.1)[a]	74 (23.5)[ab]
0.25	422	281 (66.6)[ab]	187 (66.5)[b]	162 (57.7)[a]	78 (27.8)[a]
0.5	447	304 (68.0)[ab]	213 (70.1)[b]	180 (59.2)[a]	79 (26.0)[a]
1	416	266 (63.9)[b]	146 (54.9)[a]	124 (46.6)[b]	49 (18.4)[b]

[a,b] Values within a column with different superscript indicate significant differences at $P<0.05$

Interestingly, treatment with resveratrol improved the cryotolerance of IVP bovine embryos, as indicated by higher development and hatching rates following post-warming culture. Indeed, despite similar survival rates, 0.5 μM resveratrol increased ($P<0.05$) development rate after 24 h and both development and hatching rate ($P<0.01$) after 48 h culture compared to the control (Table 3).

Table 3. Survival, development and hatching rates after 24 and 48 h culture following vitrification-warming of blastocysts produced in the different culture systems.

Post-warming culture							
Resveratrol (µM)	**n.**	**24 h**			**48 h**		
		n. (%) Survival	**n. (%) Development**	**n. (%) Hatching**	**n. (%) Survival**	**n. (%) Development**	**n. (%) Hatching**
0 (control)	175	163 (93.1)	55 (31.4)^{a}	30 (17.1)^{ab}	146 (83.4)	88 (50.3)^{Aa}	50 (30.9)^{A}
0.25	158	152 (96.2)	52 (32.9)^{a}	21 (13.3)^{a}	131 (82.9)	80 (50.6)^{Aa}	58 (36.7)^{A}
0.5	168	157 (93.5)	75 (44.6)^{b}	42 (25.0)^{b}	151 (89.9)	113 (67.3)^{Bb}	99 (58.9)^{B}
1	112	108 (96.4)	36 (32.1)^{a}	13 (11.6)^{a}	98 (87.5)	60 (53.6)^{a}	40 (35.7)^{A}

^{a,b} Values within a column with different superscript indicate significant differences at $P<0.05$

*The post thaw culture did not contain resveratrol, the resveratrol concentration listed in this table refer to levels added during embryo development.

No differences were recorded in the number of ICM, TE and total cells, as well as in the ratio of ICM:total cells between blastocysts produced in the absence (control) or presence of 0.5 μM resveratrol (Table 4). The blastocysts stained were individually classified into four groups according to the ICM:total cells ratio, as previously described (Koo *et al*., 2002) : group I: <20% ; group II: 20-40%; group III: 40-60%; group IV: > 60%. In both experimental groups the majority of the blastocysts fell into group II (78.3 and 72.3%, respectively in the control and resveratrol groups). In the control the remaining blastocysts were equally distributed into Group I and III (10.9%), whereas in the presence of resveratrol the proportion of blastocysts classified into Group I and III were 4.3% and 23.4%, respectively. No blastocysts fell into Group IV under both culture conditions.

Table 4. Allocation of cells (mean ± SE) in bovine blastocysts cultured in the absence (control) or presence of 0.5 μM resveratrol.

Group	n. blastocyst	ICM	TE	Total cells	ICM:Total cells
Control	46	34.1 ± 1.6	88.1 ± 4.6	122.2 ±5.4	29.1 ± 1.2
Resveratrol	47	36.4 ± 1.4	85.3 ± 4.1	121.7 ± 4.6	31.3 ± 1.3

3.4 DISCUSSION

This study demonstrated that resveratrol supplementation during in vitro culture improves the cryotolerance of in vitro produced bovine embryos. The preliminary dose response trial highlighted a distinct dose-dependent effect of resveratrol, with significant decrease of both cleavage and blastocyst rates at the concentration of 5 μM and no blastocyst production at 10 μM. This suggested to reduce the concentrations (0 to 1 μM) to test in the present experiment. Despite an increase of the transferable embryos (TMBL) obtained with 0.25 and 0.5 μM resveratrol, no improvement was recorded in the blastocyst yields, as well as in the percentage of fast developing blastocysts. This is in contrast with other studies carried out in cattle and swine where resveratrol was added during IVM (Lee *et al*., 2010; Wang *et al*., 2014). It is known, however, that the optimization of the IVM system leads to increased embryo yields, whereas the improvement of culture conditions affects particularly embryo quality (Lonergan *et al*., 2001). The beneficial effect was already evident after 24 h of post-warming culture, when the development rate, i.e. the percentage of embryos reaching a more advanced stage, significantly increased. Furthermore, a significant improvement of both development and hatching rates was recorded after 48 h post-warming culture. Interestingly, the latter parameter showed a 2-fold increase compared to the control; this is important because the ability of an embryo to hatch after cryopreservation is a known reliable indicator of its quality (Semple *et al*., 1995). In fact, hatching may be impaired in cryopreserved embryos, due to the occurrence of zona hardening (Carroll *et al*., 1990; Matson *et al*., 1997).

The beneficial effect on both the proportion of transferable embryos and the cryotolerance was already lost at the concentration of 1 μM. A dose-dependent effect of resveratrol was

previously demonstrated in human cultured cells, with low concentrations promoting cell division and high concentrations exerting anti-proliferation and pro-apoptosis effects (Kuwajerwala *et al*., 2002; Szende *et al*., 2000). A dose-dependent effect was also recorded in porcine parthenogenetic embryos, with increased blastocyst production at low concentrations and toxic effects at high concentrations (Lee *et al*., 2010). In addition, porcine parthenogenetic blastocysts produced in the presence of low levels of resveratrol showed lower expression of Bcl-2 and Caspase-3, suggesting respectively a pro-proliferative and anti-apoptotic effect. Although apoptosis is considered a normal process in pre-implantation embryos to eliminate deviating cells, a high incidence of apoptotic cells is correlated with abnormal morphology of the embryo (Hardy *et al*., 1989). In fact, the percentage of apoptotic cells is significantly higher in the in vitro produced bovine blastocysts than in their in vivo-developed counterparts (Gjørret *et al*., 2003). We speculate that the decreased cleavage and blastocyst rates recorded in the presence of elevated concentrations of resveratrol (from 1 to 10 μM) may be due to the induction of apoptosis, although this was not assessed. However, the beneficial effect on embryo quality indicated by the improved cryotolerance of the IVP embryos does not seem to be related to the pro-proliferation activity demonstrated for resveratrol in other studies (Kuwajerwala *et al*., 2002; Szende *et al*., 2000). In fact, the chronology of embryo development, i.e. the percentage of embryos reaching a more advanced stage by the end of culture, was not improved. Furthermore, neither the total blastocyst cell number nor the relative allocation into the ICM and TE were affected by resveratrol treatment in this study. In addition, the percentage of blastocysts falling into the different ICM: total cells categories was similar. In both culture groups the highest proportion of blastocysts had an ICM:total cells ratio in the

range from 20 to 40%, that is considered normal for IVP embryos (Koo *et al*., 2002; Van Soom *et al*., 1997). It is known that the perfect allocation of the blastocyst cells into ICM and TE is critical, as both cell lineages are vital for embryonic and fetal survival (Hardy *et al*., 1989; Van Soom *et al*., 1997).

It is therefore likely that the beneficial effect of resveratrol on IVP blastocyst cryotolerance is related to its known antioxidant activity. Increased oxidative stress, resulting in pathological levels of ROS, is indeed a major factor impairing mammalian in vitro embryo development (Kitagawa *et al*., 2004; Nasr-Esfahani *et al*., 1990). Following cryopreservation, oocytes and embryos become more sensitive to the oxidative stress, resulting in lipid peroxidation, membrane injury and structural destruction (Tatone *et al*., 2010). It was reported that glutathione (GSH) content sharply decreases after cryopreservation in bovine (George *et al*., 2008) and porcine IVF embryos (Somfai *et al*., 2007*a*), confirming that oxidative stress occurs during the freezing-thawing process. One of the effects of resveratrol is to maintain the redox state of cells and protect them against the harmful effects of oxidative injuries. Resveratrol supplementation during IVM was demonstrated to improve oocyte developmental competence by increasing GSH and decreasing ROS levels in swine and cattle oocytes (Kwak *et al*., 2012; Lee *et al*., 2010; Wang *et al*., 2014). Therefore, it is likely that the improved cryotolerance of IVP bovine embryos here reported is due to the known scavenging properties of resveratrol (Mahal *et al*., 2005). However, the molecular mechanism by which resveratrol exerts its effect on pre-implantation embryos is still not entirely clear. It was demonstrated that the structure of resveratrol is similar to that of the synthetic estrogen diethylstilbestrol (Lobo, 1995), that was shown to bind to human estrogen receptors in binding assay (Gehm *et al*., 1997).

It was also reported that resveratrol during IVM increases progesterone secretion and decreases estrogen secretion by bovine cumulus cells (Wang *et al*., 2014). These findings suggest a relationship between resveratrol and steroids, supporting a hypothesis whereby low concentrations of resveratrol may act as an agonist for specific steroid hormone receptors, during pre-implantation embryo development in vitro.

In conclusion, these results demonstrated that supplementation of culture medium with low levels of resveratrol improves the quality, and hence the resistance to cryopreservation, of IVP bovine embryos, without affecting blastocyst rates and allocation of cells into ICM and TE lineages. Further studies are needed to comprehend the mechanism underlying the beneficial effect and to assess the effect of resveratrol on development to term after transfer of cryopreserved embryos.

CHAPTER 4: Experiment 2

Effect of L-Ergothioneine supplementation during culture on the quality and cryotolerance of bovine in vitro produced embryos

L-Ergothioneine (LE) is a powerful scavenger of OH and an inhibitor of iron or copper ion-dependent generation of OH from H_2O_2 (Akanmu *et al*., 1991). L-Ergothioneine (2-mercaptohistidine trimethylbetaine) is a naturally occurring amino acid that contains an imidazole-2-thione moiety (Figure 13). The thione group exists in a tautomeric thiol-thione equilibrium, with the thione being the predominant form at physiological pH, which distinguishes ergothioneine from other biological thiol compounds.

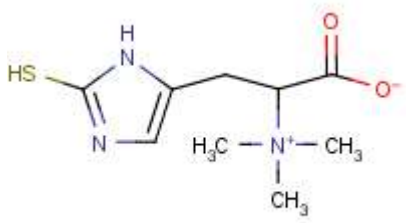


Figure 13. Chemical structure of L-Ergothioneine.

Ergothioneine is synthesized by some bacteria and fungi, but not by animals (Hartman, 1990; Melville *et al*., 1955). Humans acquire ergothioneine from their diet, including mushrooms, oats, corn, and meat (Ey *et al*., 2007). Ergothioneine is found in brain, erythrocytes, liver, kidney, heart, seminal fluid, and ocular tissues of several species (Mitsuyama and May, 1999). However, the biological functions of ergothioneine remain incompletely understood. Ergothioneine has been shown to exert radioprotective effects

(Van den Broeke and Beyersbergen van Henegouwen, 1993); scavenge singlet oxygen, hydroxyl radicals, hypochlorous acid, and peroxyl radicals (Asmus *et al*., 1996; Aruoma *et al*., 1997); inhibit peroxynitrite-dependent nitration of proteins and DNA (Aruoma *et al*., 1999); protect retinal neurons from *N*-methyl- D-aspartate (NMDA)-induced excitotoxicity in vivo (Moncaster *et al*., 2002); and protect against diabetic embryopathy in pregnant rats (Guijarro *et al*., 2002).

A previous study demonstrated that LE inhibits cell death caused by H_2O_2 and DNA oxidation by peroxynitrite in a human neuronal hybridoma cell line in culture and it was suggested that LE could have a role as an antioxidant, *in vivo* non-toxic thiol compound and obtain the oxidative stability in pharmacological preparations (Aruoma *et al*., 1999). Recently, it has been shown that antioxidant activity of LE is the highest, and it eliminates the most active free radicals compared to some classic well-known antioxidants such as glutathione, uric acid and trolox (Franzoni *et al*., 2006).

It has been known that the constant maintenance of the pro-oxidant balance in embryos is difficult and complicated during the addition of antioxidants (Guérin *et al*., 2001); thus, further studies are needed to limit and minimize the oxidative stress during embryo culture. In a recent work in sheep, the effects of LE and L-ascorbic acid (LAA) on the maturation rate of immature oocytes and embryonic development of in vitro matured oocytes were compared. The results showed that, the addition of L-ergothioneine into IVM, IVF and IVC medium has a beneficial effect on in vitro maturation of oocytes and embryonic development, especially from cleavage to morulae stages compared to LAA and Control groups (Öztürkler *et al*., 2010). To our current knowledge, there is no study available in literature on the effects of an antioxidant with strong and unique features, such as LE in

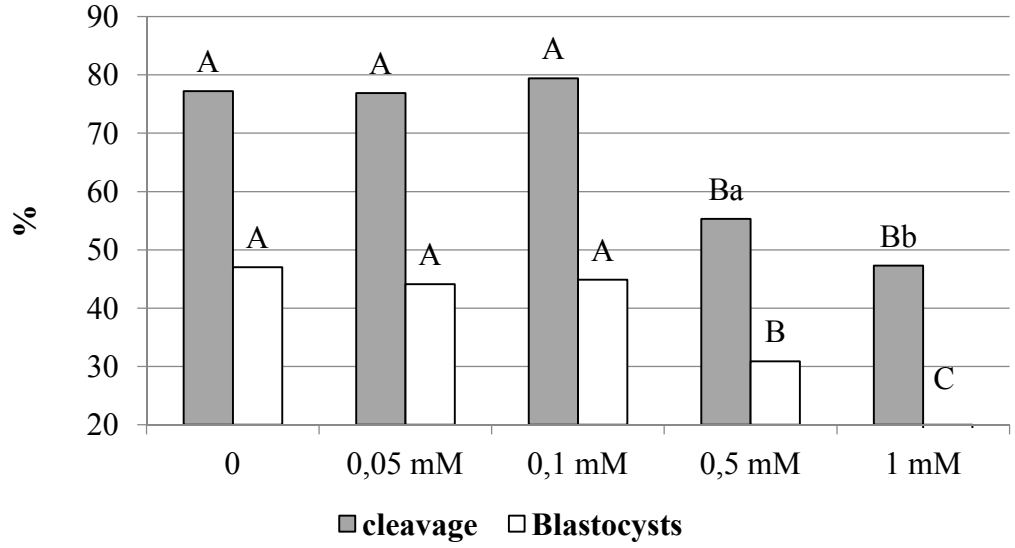
cattle. Therefore, the aim of this work was to evaluate whether supplementation of culture medium with LE improves in vitro blastocyst development and embryo quality in cattle, the latter assessed in terms of cryotolerance, apoptotic cells rate and allocation of blastocyst cells into the inner cell mass (ICM) and trophectoderm (TE) lineages.

4.1 MATERIALS AND METHODS

Experimental Design

A preliminary dose response trial (0 to 1 mM) showed a remarkable decrease of both cleavage and blastocyst development with 0.5 mM LE and an evident toxic effect, with no embryo production, with 1 mM LE (Figure 14).

Figure 14. Cleavage and blastocyst rates with different concentrations of LE during culture



[a,b] Bars with different superscript indicate significant differences at $P<0.01$

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Therefore, the range of concentrations to test was reduced in Experiment 2.1, in which presumptive zygotes were cultured in vitro with 0 (control, n= 282), 0.05 mM (n= 286) and 0.1 mM LE (n= 285), over five replicates. The blastocysts produced (on average 94 per group) at day 7 (end of culture) were vitrified, warmed and cultured for further 2 days, and assessments were carried out both at 24 and 48 h.

In Experiment 2.2, zygotes were cultured in the absence (control, n= 412) and presence of 0.1 mM LE (n= 411), over four replicates and the blastocysts produced on day 8 underwent both DS (n= 47 for both groups) and TUNEL staining to assess apoptosis (n= 65 and 64, respectively).

Considering that differences in the cell number and allocation of blastocysts may appear when the developmental stages are taken into account (Korhonen *et al*., 2010), only good quality XBl and HBL stage embryos (with a clearly visible ICM and blastocoel) were chosen for the evaluation of cell allocation by DS. In addition, for TUNEL staining BL, XBL and HBL were used.

Reagents and media

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich Chemical (Milano, Italy). The In Situ Cell Death Detection Kit (fluorescein) and the RNA-free DNase was obtained from Roche Diagnostics Corporation (Indianapolis, IN, USA).

The aspiration medium was TCM 199 supplemented with 25 mM Hepes, 2 mM sodium bicarbonate, 2 mM sodium pyruvate, 1 mM L-glutamine, 10 µL/mL amphotericin B (H199) supplemented with 2% bovine serum (BS) and 95.6 SI/mL heparin. The IVM medium was TCM 199 supplemented with 15% bovine serum (BS), 0.5 µg/mL FSH, 5 µg/mL LH, 0.8

mM L-glutamine and 50 µg/mL gentamycin. The IVF medium was Tyrode's modified medium (Parrish *et al*., 1986) without glucose and bovine serum albumin (BSA), supplemented with 5.3 SI/mL heparin, 30 µM penicillamine, 15 µM hypotaurine, 1 µM epinephrine and 1% of BS. The IVC medium consisted of Synthetic Oviduct Fluid (SOF) medium (Tervit *et al*., 1972), with 30 µL/mL essential amino acids, 10 µL/mL non-essential amino acids and 5 % BS.

The base medium for the vitrification and warming solutions was H199 with 20% fetal calf serum (FCS). The equilibration medium (VS1) consisted of 7.5% DMSO and 7.5% ethylene glycol (EG) in base medium and the vitrification solution (VS2) consisted of 16.5% DMSO and 16.5% EG with 0.5 M sucrose in the base medium. The warming solutions consisted in 0.25 M and 0.15 M sucrose in the base medium.

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Experiment 2.1: Vitrification and Warming

On Day 7 the blastocysts obtained (from eBl to HBl) were vitrified by the Cryotop method, previously described (Kuwayama and Kato, 2000). All equilibration and dilution steps were carried out at 37 °C. Each blastocyst was exposed to VS1 in 200 µL droplet for 3 min, then

transferred into a 20 µL droplet of VS2 for 20 to 25 s, and loaded with a glass capillary onto the top of the film strip of each cryotop in 0.1 µL volume. After loading, almost all the solution was removed and the Cryotop was quickly immersed into liquid nitrogen, protected with the cap and stored under liquid nitrogen. For warming, after removal of the cap, the Cryotop strip was immersed directly into 1 mL of the 0.25 M sucrose solution for 1 min and the embryo was transferred into 200 µL droplet of 0.15 M sucrose solution for 5 min. Warmed blastocysts were washed in H199 medium + 10% FCS and cultured for two days. After 24 h and 48 h post-warming culture survival rates were evaluated with morphological criteria, on the basis of the integrity of the embryo membrane and the ZP, and re-expansion of the blastocoel. Furthermore, the percentages of embryos that resumed their development and reached a more advanced developmental stage after culture (development rate), as well as the hatching rates, were recorded.

Experiment 2.2: Differential staining

Blastocysts (only XBL and HBL) produced in the presence (0.1 mM) or absence of LE on Day 8, were subjected to a DS protocol previously described (Thouas *et al*., 2001) with slight modifications. Briefly, the ZP of viable expanding or expanded blastocysts was removed with 0.2% pronase in Hepes-buffered SOF. After three washes in PBS with 0.1% PVP, blastocysts were first incubated in 500 µL of propidium iodide solution (PBS with 1% Triton X-100 and 100 µg/mL propidium iodide) for 1 min. Blastocysts were then immediately transferred into 500 µL of a fixative solution of 100% ethanol with 25 µg/mL bisbenzimide (Hoechst 33342) for 5 min. The fixed and stained embryos were subsequently transferred into glycerol and mounted onto a glass microscope slide in a drop of glycerol,

flattened with a coverslip to a level where all nuclei appeared at the same focal plane, and visualized using a fluorescent microscope (Eclipse E-600; Nikon) under ultraviolet light with excitation at 460 to 560 nm. A digital image of each embryo was taken using NIS-Elements-F software and a high-resolution color digital camera (Nikon digital sight DS-Fi 1C) and the numbers of TE (red) and ICM (blue) nuclei were counted.

Experiment 2.2: TUNEL staining

The blastocysts (BL, XBl and HBL), produced in the presence (0.1 mM) or absence of LE on Day 8 were subjected to a TUNEL staining protocol previously described (Paula-Lopes and Hansen, 2002). The TUNEL assay was used to detect DNA fragmentation associated with late stages of the apoptotic cascade. The enzyme terminal deoxynucleotidyl transferase is a DNA polymerase that catalyzes the transfer of a fluorescein isothiocyanate-conjugated dUTP nucleotide to a free 3' hydroxyl group characteristic of DNA strand breaks. Embryos were removed from SOF culture medium and washed three times in drops of PBS containing 1 mg/mL PVP (PBS-PVP) by transferring the embryos from drop to drop. Embryos were fixed in a 50 μL drop of 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, washed twice in PBS-PVP, and stored in 600 μL of PBS-PVP at 4°C until the time of assay. All steps of the TUNEL assay were conducted using microdrops in a humidified box.

On the day of the TUNEL assay, embryos were transferred to a 50 μL drop of PBS-PVP and then permeabilized in a drop of 0.5% (v/v) Triton X-100 in PBS, containing 0.1% (w/v) sodium citrate for 30 min at room temperature. Controls for the TUNEL assay were incubated in 50 μL of RQ1-RNA free DNase (50 U/mL) at 37°C in the dark for 10 min.

Positive controls and treated embryos were washed in PBS-PVP and incubated with 25 µL of TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase as prepared by and following the guidelines of the manufacturer) for 1 h at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase.

Embryos were then washed three times in PBS-PVP and incubated in a 25 µL drop of Hoechst 33342 (1 µg/mL) for 15 min in the dark. Embryos were washed three times in PBS-PVP to remove excess Hoechst 33342, mounted on 10% (w/v) poly-L-lysine coated slides using 3- to 4 µL drops of glycerol, and coverslips were placed on the slides. Labeling of TUNEL and Hoechst 33342 nuclei was observed using a fluorescent microscope (Eclipse E-600; Nikon). Each embryo was analyzed for total cell number (blue nuclei) and TUNEL-positive blastomeres (green nuclei) with DAPI and FITC filters, respectively, using a 20X and 40X objectives. Digital images were acquired (Figures 15, 16, 17 and 18) using NIS-Elements-F software and a high-resolution color digital camera (Nikon digital sight DS-Fi 1C).

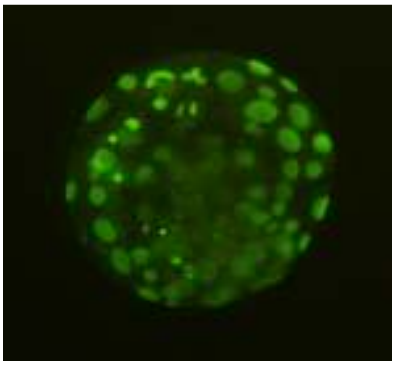


Figure 15. Positive control

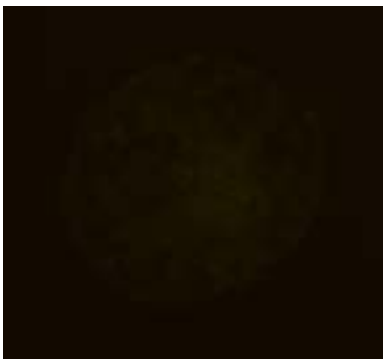


Figure 16. Negative control

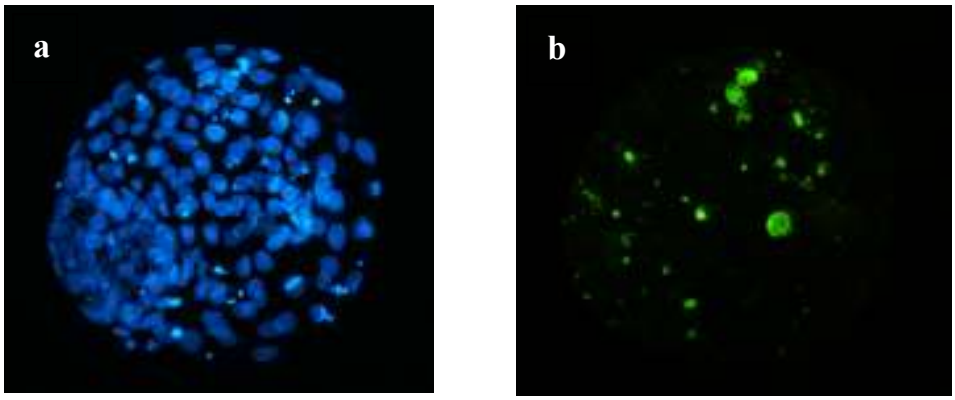


Figure 17. Representative pictures showing total cells staining examined by DAPI filter (**a**) and TUNEL labeling examined by FITC filter (**b**) of the same HBL cultured in the control medium

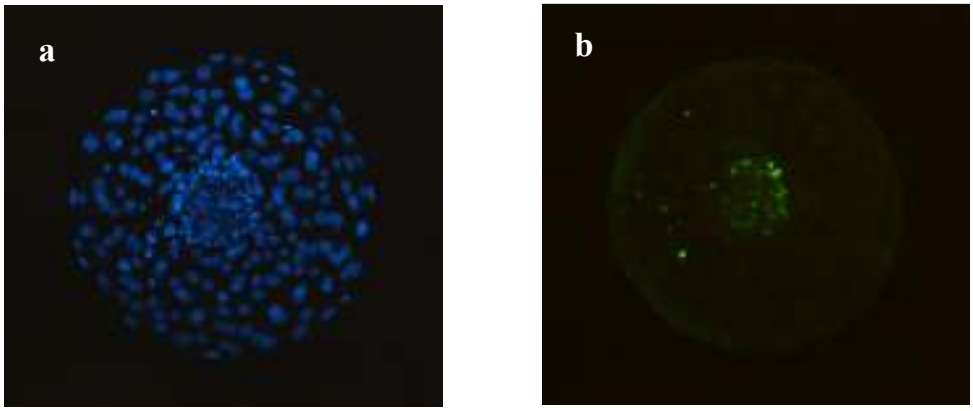


Figure 18. Representative pictures showing total cells staining examined by DAPI filter (**a**) and TUNEL labeling examined by FITC filter (**b**) of the same HBL cultured in the medium enriched with 0.1 mM LE

4.2 STATISTICAL ANALYSIS

Differences among groups in the percentages of blastocysts, as well as in the survival rates after vitrification-warming, were analyzed by Chi square test using SPSS 16.0 statistical software (SPSS Inc., 2007). DS and TUNEL staining data were analyzed by the Student's *t* test.

4.3 RESULTS

As shown in Table 1, the supplementation of LE during culture did not affect cleavage and post-fertilization embryo development, both in terms of total embryo output (TMBL) and superior quality blastocysts (Grade 1,2 BL). The percentage of fast developing embryos was also similar in different groups (Experiment 2.1 - Table 5).

Table 5. Percentages of cleavage, tight morulae-blastocysts (TMBL), Grade 1 and 2 blastocysts (Grade 1,2 BL) and fast developing embryos (fast BL) under different LE concentrations.

LE concentrations	COC n.	Cleaved n. (%)	TMBL n. (%)	Gd 1,2 BL n. (%)	Fast BL n. (%)
0 (control)	282	220 (78.0)	112 (50.9)	102 (46.4)	50 (22.7)
0.05 mM	286	223 (78.0)	109 (48.9)	97 (43.5)	41 (18.4)
0.1 mM	285	229 (80.4)	113 (49.3)	95 (41.5)	54 (23.6)

However, the enrichment of medium with LE improved embryo cryotolerance. In fact, although survival, development and hatching rates after 24 h post-warming culture were not affected, hatching rates following 48 h post-warming culture increased ($P<0.05$) when medium was enriched with 0.1 mM LE (Experiment 2.1 - Table 6).

Table 6. Survival, developmental and hatching rates after 24 and 48 h culture following vitrification-warming of blastocysts produced under different LE concentrations.

Post-warming culture							
LE concentrations	**n.**	**24 h**			**48 h**		
		Survived N (%)	**Developed N (%)**	**Hatched N (%)**	**Survived N (%)**	**Developed N (%)**	**Hatched N (%)**
0 (control)	99	83 (83.8)	47 (47.5)	25 (25.3)	71 (71.7)	62 (62.6)	48 (48.5)[a]
0.05 mM	90	81 (90.0)	48 (53.3)	27 (30.0)	72 (80.0)	57 (63.3)	45 (50.0)[ab]
0.1 mM	94	82 (87.2)	47 (50.0)	32 (34.0)	78 (83.0)	67 (71.3)	60 (63.8)[b]

[a,b] Values within a column with different superscript indicate significant differences at $P<0.05$

Furthermore, the average number of ICM cells, as well as the ratio of ICM:total cells of blastocysts were higher ($P<0.01$ and $P<0.05$, respectively) in the 0.1 mM LE group than in the control, whereas no differences were recorded in the number of TE and total cells (Experiment 2.2 - Table 7).

Table 7. Allocation of cells (Mean ± SE) in bovine blastocysts cultured in the absence (control) or presence of 0.1 mM LE.

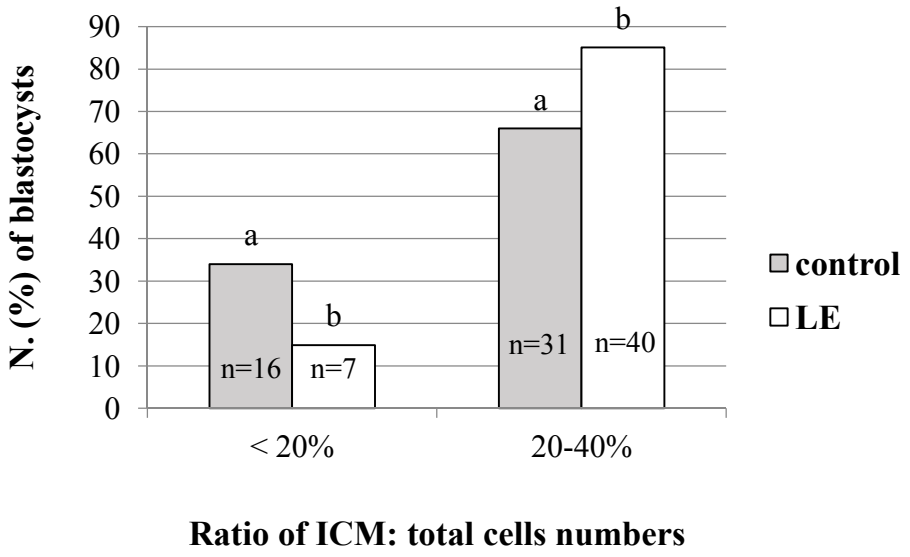
Group	n. blastocyst	ICM	TE	Total	ICM: total cells %
Control	47	37.1 ± 1.2^{A}	126.9 ± 3.5	164.0 ± 4.0	22.8 ± 0.7^{a}
0.1 mM LE	47	42.4 ± 1.6^{B}	129.1 ± 3.5	171.6 ± 4.5	24.7 ± 0.7^{b}

^{a,b} Values within a column with different superscript indicate significant differences at $P<0.05$

^{A,B} Values within a column with different superscript indicate significant differences at $P<0.01$

The blastocysts stained were individually classified into four groups according to the ICM:total cells ratio, as previously described (Koo *et al*., 2002): group I: <20%; group II: 20-40%; group III: 40-60%; group IV: > 60%. As shown in Figure 19 (Experiment 2.2), in the LE group a higher ($P<0.05$) percentage of the blastocysts fell into group II compared to the control (85.1 and 66.0 %, respectively). In contrast, the percentage of blastocysts falling in Group I was lower ($P<0.05$) in the LE group than in the control (14.9 and 34.0 %, respectively). No blastocysts fell into Group III and IV under both culture conditions.

Figure 19. Percentage of blastocysts with different proportion of ICM:total cells ratio in the presence or absence of LE.



[a,b] Bars with different superscript indicate significant differences at $P<0.05$

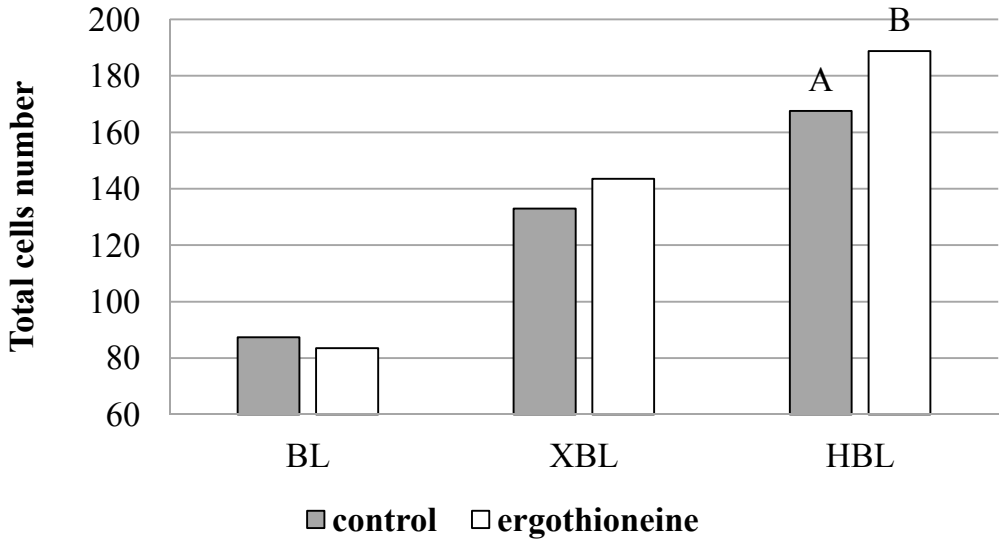
Both the average number and the proportion of apoptotic cells in blastocysts were reduced ($P<0.01$) in the presence of 0.1 mM LE (Experiment 2.2 - Table 8), whereas the total cell number was unaffected. However, when these parameters were analyzed in relation to the stage of development, the total cell number was higher ($P<0.01$) in the LE group but only in case of HBL (Figure 20). Within each developmental stage (BL, XBl and HBL) the incidence of apoptotic cells out of the total decreased ($P<0.01$) in the enriched group (Figure 21).

Table 8. Cell allocation on day 8 bovine blastocysts by TUNEL staining in the absence (control) or presence of 0.1 mM LE.

Groups	n.	Total cells	Apoptotic cells	% apoptotic cells	Apoptotic/total cells ratio
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Control	65	126.6 ± 5.2	9.1 ± 0.3^{A}	8.1 ± 0.5^{A}	0.08 ± 0.006^{A}
0.1 mM LE	64	133.5 ± 6.1	$4.3 \pm 0.^{2B}$	3.6 ± 0.3^{B}	0.03 ± 0.005^{B}

^{A,B} Values within a column with different superscript indicate significant differences at $P<0.01$

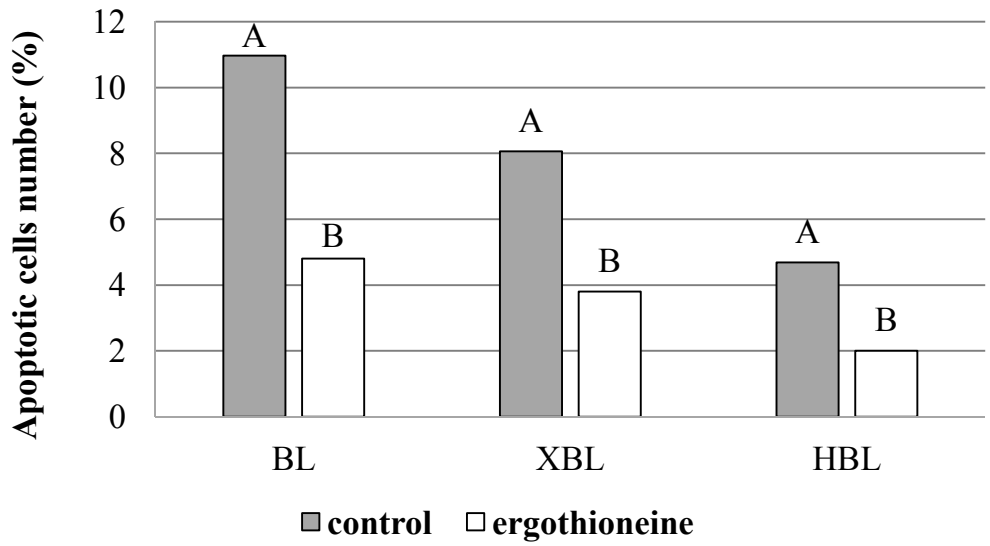
Figure 20. Total cell numbers of day 8 blastocysts cultured with or without LE according to the developmental stage.



^{A,B} Bars with different superscript indicate significant differences at $P<0.01$

* The number of BL, XBL and HBL stained were respectively 25, 19 and 21 in the control and 25, 20 and 19 in the LE group.

Figure 21. The incidence of apoptotic cells of day 8 blastocysts cultured with or without LE according to the developmental stage.

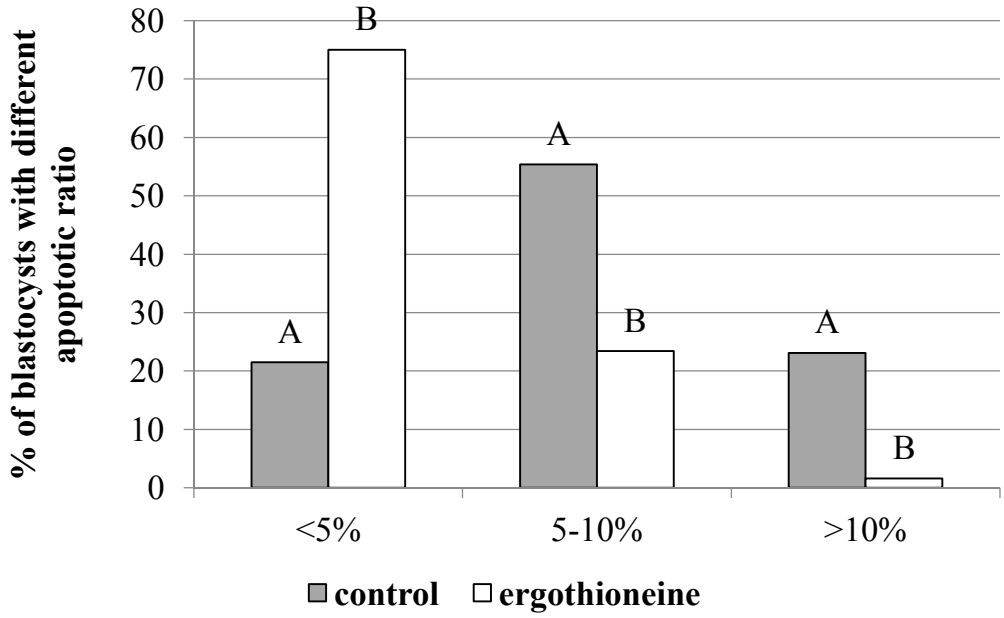


[A,B] Bars with different superscript indicate significant differences at $P<0.01$

* The number of BL, XBL and HBL stained were respectively 25, 19 and 21 in the control and 25, 20 and 19 in the LE group.

Furthermore, in the enriched group a higher ($P<0.01$) percentage of blastocysts had <5% of apoptotic cells, whereas a lower ($P<0.01$) percentage of blastocysts exhibited 5-10% and >5% apoptotic cells compared to the control (Figure 22).

Figure 22. Percentage of blastocysts with low (<5%), average (5-10%) and high (>10%) levels of apoptosis in the control and LE groups.



[A,B] Bars with different superscript indicate significant differences at $P<0.01$

4.4 DISCUSSION

The results of the present study demonstrated that enrichment of culture medium with low doses of the natural antioxidant LE, improves embryo quality, as indicated by increased cryotolerance, reduced incidence of apoptotic cells and higher percentage of blastocysts with the most physiological ICM:total cells ratio.

The preliminary dose response trial showed a negative effect on both cleavage and blastocyst rates with 0.5 mM LE and an evident toxic effect, with further reduction of cleavage and no blastocyst production at the concentration of 1 mM. This is in contrast with

previous tissue culture studies reporting that ergothioneine was not toxic to cells with an LD50 of 50 mM (OXIS Technical Document, 1997). Also, the concentration that was embryotoxic in the present study (1 mM) is close to the concentration that was estimated to be present in human and mammalian tissues (1-2 mM). It has been reported that ergothioneine is present in millimolar concentrations in specific tissues such as erythrocytes, kidney, seminal fluid and liver (Grigat *et al*., 2007). However, according to the OXIS technical Document (1997) the circulating levels of ergothioneine are estimated to be in the high micromolar range.

After the preliminary trial Experiment 2.1 was carried out with 0 (control), 0.5 and 0.1 mM LE, and showed that the supplementation of culture medium with the antioxidant did not affect cleavage rate and embryonic development. This finding is in disagreement with the results recorded in the only work present in literature in the sheep, in which 10 mM LE supplemented both during IVM and IVC increased maturation rates, cleavage and development to morulae (Öztürkler *el al*., 2010). However, in that work both cleavage and embryo development were very poor (on average respectively 26 and 4%) and it is known that it is easier to detect a beneficial effect in a suboptimal system. What is more surprising is that the concentration that was beneficial in the sheep is 10 times higher than the concentration that was toxic on bovine embryos in the present study. Due to the paucity of information available we can only speculate that this is related to species-specific differences. The most important issue arising from the results of the study is the improved embryo quality obtained in culture medium enriched with 0.1 mM LE. Indeed, we followed the strategy to enrich IVC medium with a natural antioxidant just in order to improve embryo quality, that is known to be mainly affected by culture environment (Lonergan *et*

al., 2001). Although embryo output was not affected, it was demonstrated that 0.1 mM LE increased embryo cryotolerance that is a highly reliable marker of embryo quality (Imai *et al*., 2002; Lonergan *et al*., 2003*a*; 2003*b*; Rizos *et al*., 2003). The beneficial effect was evident after 48 h of post-warming culture, when a significant increase of hatching rate was recorded. The increased hatching rate after culture of vitrified-warmed blastocysts is a strong indicator of viability because cryopreservation results in zona hardening that interferes with the process of hatching (Carroll *et al*., 1990; Matson *et al*., 1997). Our work was the first to evaluate the effect of LE on embryo cryotolerance and hence we cannot compare our results with others'; the only work available in literature, in fact, has been limited to assess the effect of LE on ovine in vitro embryo development in quantitative terms. However, previous studies demonstrated that supplementation with antioxidants during culture improves embryo quality and cryotolerance (Hosseini *et al*., 2009; Takahashi, 2012). In the Experiment 1 of this thesis we also recorded a significant improvement of cryotolerance when culture medium was enriched with resveratrol. The improved cryotolerance of bovine embryos obtained with LE during culture is likely due to its high antioxidant capacity, as oxidative stress occurs during the freezing-thawing process, leading to lipid peroxidation, membrane injury and structural damage (Somfai *et al*., 2007*b*). This may be due to the decrease of GSH levels following cryopreservation in bovine embryos (George *et al*., 2008). Therefore, LE addition during culture increases the capability of bovine embryos to hatch after cryopreservation, that is the most important parameter to assess the embryo resistance to cryopreservation (Semple *et al*., 1995).

In addition to embryo cryotolerance, total cell count and particularly cell allocation to the inner cell mass (ICM) and the trophectoderm (TE) lineages are commonly used as embryo

quality parameters. It is well documented that the perfect allocation of the blastocyst cells into ICM and TE is critical for embryonic survival (Koo *et al*., 2002; Knijn *et al*., 2003) and post-implantation development (Iwasaki *et al*., 1990), as both cell lineages are essential (Hardy *et al*., 1989; Van Soom *et al*., 1997). In the present study blastocysts produced in the medium enriched with LE showed an increase in the average number of ICM cells compared to the control, whereas total cell count and TE cells were unaffected. This resulted in a slightly higher ICM:total cell ratio in the enriched group. It is known that ICM cells contribute to all embryonic tissues and part of the extraembryonic membranes, whereas the TE cells mainly form the outer layer of placenta (Fouladi-Nashta, 2005). A well defined ICM is known to be essential to support development after embryo transfer. In fact, it has been reported that the number of ICM cells in IVP bovine blastocysts is lower than that of in vivo produced blastocysts (Iwasaki *et al*., 1990; Du *et al*., 1996). High total cell count and normal ICM proportion (20-40%) are known to indicate good embryo quality and survival after transfer (Iwasaki *et al*., 1990; Cho *et al*., 2002; Russell *et al*., 2006). In this work the majority of blastocysts had an ICM proportion falling within the optimal range (20-40%) and the remaining blastocysts showed an ICM:total cells ratio lower than 20%. Interestingly, differences were found between groups. Indeed, when embryos were cultured in the presence of LE the percentage of blastocysts with the most appropriate ICM:total cells ratio (20-40%) significantly increased, confirming a beneficial effect on embryo quality.

Beside cell distribution in ICM and TE lineages, apoptotic cell ratio is another important embryo quality marker, providing an additional criterion to morphological evaluation of embryo to effectively predict embryo viability (Pomar *et al*., 2005). In the present study we

demonstrated that LE during culture significantly reduces both the average number and the incidence of apoptotic cells in the blastocysts. The incidence of apoptotic cells in blastocysts produced in the presence or absence of LE was assessed by TUNEL labeling. Apoptosis is considered the default pathway of defective cells in embryos (Raff *et al*., 1993) and predominantly occurs in the ICM in both mouse (Hardy, 1997) and bovine embryos (Byrne *et al*., 1999). During embryonic development, apoptosis plays a role in cell population control and maintenance of cellular quality in the ICM lineage, removing abnormal or defective cells that possess damaged DNA or chromosomal abnormalities (Brison, 2000). Although apoptosis is a normal process in pre-implantation embryos to eliminate deviating cells, a high incidence of apoptotic cells indicates an abnormal morphology of the embryo (Hardy *et al*., 1989). In fact, the incidence of apoptotic cells is higher in vitro produced embryos than in vivo produced embryos (Bergeron *et al*., 1998). In the present study both the number and the incidence of apoptotic cells decreased in the LE-enriched group within each developmental stage, i.e. blastocysts, expanded blastocysts and hatched blastocysts. Furthermore, when only HBL were considered the total cell count also increased in the LE group compared to the control. This finding is only in apparent contrast with the data obtained with the DS previously discussed. In fact, embryos that underwent DS were only expanded and hatched blastocysts and numbers were too low to allow an analysis within the stage. What is overwhelming is the difference between groups in the distribution of blastocysts with low, average and high proportion of apoptotic cells. The enrichment of IVC medium with LE significantly increased the proportion of blastocysts with low apoptotic cells compared to the control (75 vs 22%). It follows that in the LE group the percentages of blastocysts showing both average and high apoptotic index were significantly reduced. In

particular, only 1.6% of the blastocysts showed a high apoptotic index vs 23.1 % of the control. It is known that high levels of ROS have deleterious effects on cellular function by inducing oxidative damage to the intracellular components and by triggering apoptosis (Guérin *et al*., 2001; Yang *et al*., 1998). The accumulation of superoxide radicals and a decline in SOD levels are involved in impaired embryo development and apoptotic cell death, whereas antioxidants can inhibit apoptosis (Guérin *et al*., 2001). Increased H_2O_2 levels were found to be associated to apoptosis also in human fragmented embryos (Yang *et al*., 1998).

In conclusion, the results of this study taken together demonstrated that enrichment of bovine IVC medium with low doses of LE improves the overall embryo quality, that is the most important factor affecting post-implantation development after embryo transfer. The beneficial effect on embryo quality is highlighted by the improved cryotolerance following culture, and by the increased proportion of blastocysts with a correct ICM:total cells ratio and with a low percentage of apoptotic cells.

CHAPTER 5: Experiment 3

Effect of Crocetin supplementation during culture on the quality and cryotolerance of bovine in vitro produced embryos

Saffron (*Crocus sativus* Linnaeus) from iridaceous genus is a stable plant with a height about 30 cm with long and green leaves. Saffron flowers are purple, well ordered with a long tube that ends to 3 petals and sepals. The interested segment of saffron is its stigma. Saffron is considered as an important herb in medical, cosmetics and hygienic industries; it has a sweet smell and a biting taste which has been in use from long times before as a food additive and coloring, flavoring agent (Abdullaev, 1993). Commercial saffron is the dried tripartite stigma of the *Crocus sativus* L. flowers. It is cultivated in many countries such as Azerbaijan, France, Greece, India, Iran, Italy, Spain, Turkey, Egypt, and Mexico (Abe and Saito, 2000; Lage and Cantrell, 2009).

Saffron is one of the most used pigments. It consists of mixtures of pigments and other unidentified substances. Nevertheless, volatile factors (safranal), bitter principles (picrocrocin) and dye materials, such as crocetin and its glycoside crocin (Ríos *et al*., 1996), were classified agents of the therapeutic effects of saffron. Regarding therapeutic characteristics, saffron is beneficial for curing nervous pains, insomnia, paroxysm, asthma, rheumatism, gingivitis, cough, gastric disorders, sleeplessness, uterus chronic hemorrhage, femininity disorders, scarlet fever, influenza, cardiovascular disorders and brain damages (Bisset and Wichtl, 2001; Fatehi *et al*., 2003; Gainer and Jones, 1975; Modaresi *et al*., 2008; Mohajeri *et al*., 2008; Wuthrich *et al*., 1997). In traditional medicine, saffron has been utilized with various applications, such as sexual potential stimulant (Abe and Saito, 2000),

anti-spasm (Hosseinzadeh and Talebzadeh, 2005), anti-depression (Moshiri *et al*., 2006), sedative and anti-inflammation (Modaresi *et al*., 2008), anti-tumoral (Abdullaev *et al*., 2002; Escribano *et al*., 1996) anti-flatulence, regulating menstruation and increasing factor of body transpiration (Abdullaev and Espinosa-Aguirre, 2004; Akhondzadeh *et al.,* 2004; Hosseinzadeh and Younesi, 2002; Ochiai *et al*., 2007).

In addition, saffron extract effect on declining blood pressure and its loosening influences on smooth muscles on rats and guinea pigs has been examined (Fatehi *et al*., 2003). Other studies have demonstrated that saffron extract reduces ischemia and blood re-establishment in kidney and skeleton muscles in rats (Hosseinzadeh *et al*., 2007). Saffron potential anti-arrhythmias role has been reported in curing supraventricular tachycardia arrhythmias in rabbit (Khori *et al*., 2007). Saffron extract anti-diabetic effects and its efficiency in reduction of blood sugar and fat amount in rats have also been proven (Mohajeri *et al*., 2008). Further studies have displayed that saffron extract has anti-mutation (Fernandez, 2004) properties and prevents the nucleic acid synthesis in malignant cells in humans (Nair *et al*., 1992; 1995). Also, radical scavenger effects of saffron, as well as learning and memory-improving properties (Abe *et al*., 1999; Zhang *et al*., 1994) and promotion of the diffusion of oxygen in different tissues (Palozza and Krinsky, 1992), have also been reported.

The carotenoids components of saffron, known as biological antioxidants, play important roles in human health via the protection of cells and tissues from damaging effects of free radicals and singlet oxygen (Edge *et al.,* 1997). Saffron ability to protect cells from oxidative stress by scavenging free radicals comes from the two main chemical components of saffron: crocin and crocetin (Bors *et al.,* 1982). Crocetin is a unique carotenoid with short

carbon chain length (C_{20} apocarotenoid) and with two carboxyl groups at both ends of the carbon chain (Figure 23; >98%, HPLC). Crocetin has been shown to have multiple pharmacological actions, including the inhibition of tumor formation (Wang *et al*., 1996), anti-hyperlipidemia (Lee *et al*., 2005), anti-atherosclerosis (Zheng *et al*., 2005), protection against hepatic damage (Wang *et al*., 1991).

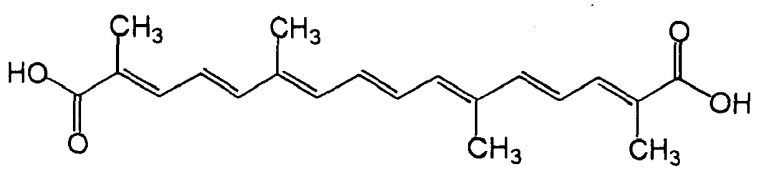


Figure 23. Chemical structure of crocetin.

Previously, it has been suggested that crocetin has excellent antioxidant effect (Tseng *et al*., 1995; Gong *et al*., 2001). In fact, it was demonstrated that crocetin could prevent the adhesion of leukocyte to bovine endothelial cells (BEC) by down-regulating the expression of intercellular adhesion molecular-1 protein (ICAM-1), and the possible mechanisms might be related to its antioxidant activity, which is through up-regulation of the activity of antioxidant enzymes and protection for mitochondrion (Xiang *et al*., 2006).

Furthermore, Meng and Cui (2008) proved that crocetin inhibits high glucose-induced apoptosis, at least partly, via phosphatidylinositol 3' kinase (Pl3K) and/or endothelial nitric oxide synthase (eNOS) pathway in human umbilical vein endothelial cells (HUVECs), and may exert a beneficial effect in preventing diabetes-associated cardiovascular complications. Recently, Mardani *et al*. (2014) revealed that saffron, administrated via oral-gastric catheter, does not influence rat sperm concentration, but significantly improves

motility and normal sperm morphology, suggesting that saffron can protect sperm against DNA damage and chromatin anomalies. In addition, it was shown that the supplementation of low concentrations of natural saffron aqueous extract (SAE) during IVM resulted in both higher rates of maturation and blastocyst formation of mouse oocytes (Tavana *et al.*, 2012); the improved rate of *in vitro* embryo development may be due to antioxidative effects of SAE components to which oocytes are exposed during IVM procedure.

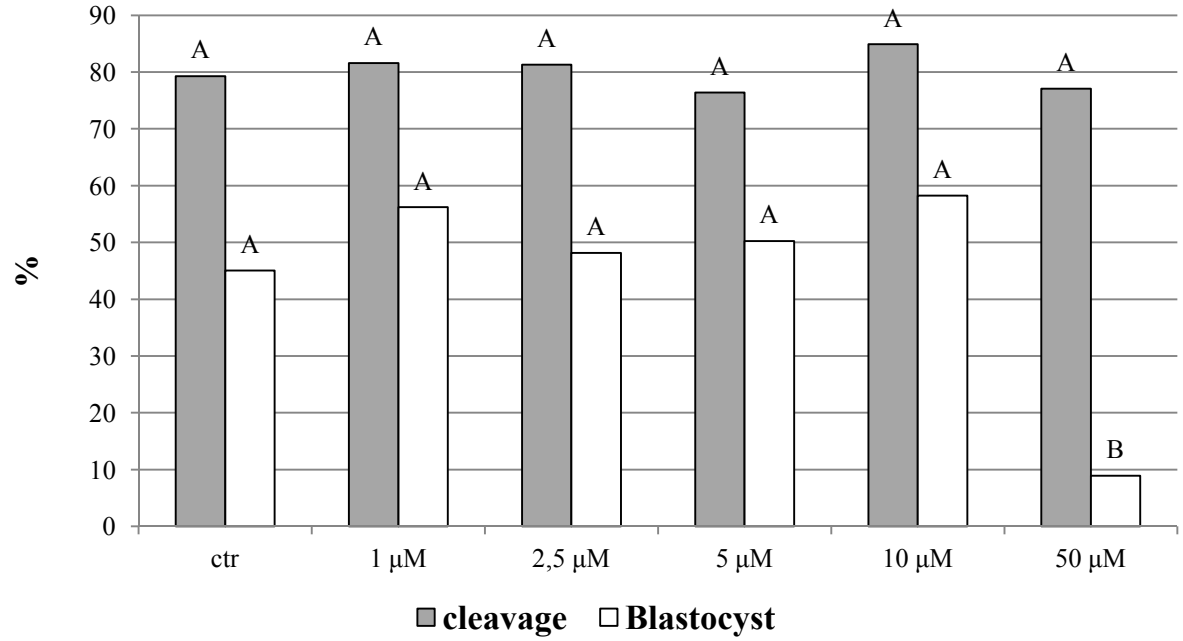
To date there are no studies available on the effects of both saffron extract and its active components, such as crocin and crocetin, on in vitro embryonic development in cattle. Therefore, the aim of this work was to evaluate whether supplementation of culture medium with crocetin improves in vitro blastocyst development and embryo quality in cattle, the latter assessed in terms of cryotolerance, apoptotic cells rate and allocation of blastocyst cells into the inner cell mass (ICM) and trophectoderm (TE) lineages.

5.1 MATERIALS AND METHODS

Experimental Design

A preliminary dose response trial (0 to 50 μM) showed no significant difference of both cleavage and blastocyst development between the control and crocetin treated groups up to the concentration of 10 μM, with an evident toxic effect at the highest concentration tested (50 μM), indicated by a substantial decreased ($P<0.01$) embryo production (Figure 24). Also, the preliminary assessment of embryo cryotolerance showed a negative effect with 10 μM crocetin.

Figure 24. Cleavage and blastocyst rates with different concentrations of crocetin during culture



[A,B] Bars with different superscript indicate significant differences at $P<0.01$

Therefore, the range of concentrations to test was reduced in Experiment 3.1, in which presumptive zygotes were cultured in vitro with 0 (control, n= 208), 1 µM (n= 208), 2.5 µM (n= 208), and 5µM crocetin (n= 208), over four replicates. The blastocysts produced (on average 73 per group) at day 7 (end of culture) were vitrified, warmed and cultured for further 2 days, and assessments were carried out both at 24 and 48 h.

In Experiment 3.2, zygotes were cultured in the absence (control, n= 217) and presence of 1 µM crocetin (n= 216), over three replicates and the blastocysts produced on day 8 underwent both DS (n= 40 for each group) and TUNEL staining to assess apoptosis (n= 39 and 41, respectively).

Considering that differences in the cell number and allocation of blastocysts may appear when the developmental stages are taken into account (Korhonen *et al*., 2010), only good quality XBl and HBL stage embryos (with a clearly visible ICM and blastocoel) were chosen for the evaluation of cell allocation by DF staining. In addition, for TUNEL staining BL, XBL and HBL were used.

Reagents and media

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich Chemical (Milano, Italy). The In Situ Cell Death Detection Kit (fluorescein) and the RNA-free DNase was obtained from Roche Diagnostics Corporation (Indianapolis, IN, USA).

The aspiration medium was TCM 199 supplemented with 25 mM Hepes, 2 mM sodium bicarbonate, 2 mM sodium pyruvate, 1 mM L-glutamine, 10 µL/mL amphotericin B (H199) supplemented with 2% bovine serum (BS) and 95.6 SI/mL heparin. The IVM medium was TCM 199 supplemented with 15% bovine serum (BS), 0.5 µg/mL FSH, 5 µg/mL LH, 0.8 mM L-glutamine and 50 µg/mL gentamycin. The IVF medium was Tyrode's modified medium (Parrish *et al*., 1986) without glucose and bovine serum albumin (BSA), supplemented with 5.3 SI/mL heparin, 30 µM penicillamine, 15 µM hypotaurine, 1 µM epinephrine and 1% of BS. The IVC medium consisted of Synthetic Oviduct Fluid (SOF) medium (Tervit *et al*., 1972), with 30 µL/mL essential amino acids, 10 µL/mL non-essential amino acids and 5 % BS.

The base medium for the vitrification and warming solutions was H199 with 20% fetal calf serum (FCS). The equilibration medium (VS1) consisted of 7.5% DMSO and 7.5% ethylene glycol (EG) in base medium and the vitrification solution (VS2) consisted of 16.5% DMSO

and 16.5% EG with 0.5 M sucrose in the base medium. The warming solutions consisted in 0.25 M and 0.15 M sucrose in the base medium.

In vitro embryo production

Abattoir-derived oocytes were matured and fertilized in vitro according to our standard procedure (Rubessa *et al*., 2011). Briefly, bovine ovaries were recovered from a local abattoir and transported to the laboratory, in physiological saline at 30 to 35 °C. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 2 to 8 mm in diameter and only those with uniform cytoplasm and multilayered cumulus cells were selected, washed twice in the aspiration medium and once in the IVM medium. Groups of 25 COCs were matured in 400 µL of IVM medium, covered with mineral oil, in four well plates (NuncTM, Roskilde, Denmark), for 22 h at 39 °C, and 5% CO_2 in air. In vitro matured COCs were washed and transferred, 25 per well, into 300 µL of IVF medium covered with mineral oil. Frozen sperm from a bull previously tested for IVF were thawed at 37 °C for 40 s and selected by centrifugation (25 min at 300 X g) on a Percoll discontinuous gradient (45 and 80%). The pellet was reconstituted into 2 mL of IVF medium and centrifuged twice, at 160 and 108 X g for 10 min. The pellet was diluted with IVF medium and added in the fertilization wells at the concentration of 1 x 10^6 sperm/mL. Gametes were co-incubated for 20 h at 39 °C, in 5% CO_2 in air, after which presumptive zygotes were vortexed for 2 min to remove cumulus cells in Hepes-TCM with 5% BS, washed twice in the same medium and randomly distributed (30 to 50 per well, into 400 µL of IVC medium) into different culture groups differing on the basis of LE concentrations. Zygotes were incubated in a humidified mixture of 5% CO_2, 7% O_2 and 88% N_2 in air at a temperature of 39 °C. On days 7 and 8,

Experiment 3.1 and 3.2 respectively, cleavage and blastocyst rates were recorded. The embryos obtained by the end of culture were scored for quality on the basis of morphological criteria (Robertson and Nelson, 2010) and the percentage of transferable embryos, i.e. Grade 1 and 2 tight morulae and blastocysts (TMBL), and that of Grade 1 and 2 blastocysts (BL) were recorded. Furthermore, the stage of development was assessed and embryos were categorized as slow developing embryos, i.e. tight morulae (TM), early blastocysts (eBl), blastocysts (Bl), and as fast developing embryos, i.e. those that reached the stages of expanded blastocysts (XBl) and hatched blastocysts (HBl) at the end of culture.

Experiment 3.1: Vitrification and Warming

On Day 7 the blastocysts obtained (from eBl to HBl) were vitrified by the Cryotop method, previously described (Kuwayama and Kato, 2000). All equilibration and dilution steps were carried out at 37 °C. Each blastocyst was exposed to VS1 in 200 µL droplet for 3 min, then transferred into a 20 µL droplet of VS2 for 20 to 25 s, and loaded with a glass capillary onto the top of the film strip of each cryotop in 0.1 µL volume. After loading, almost all the solution was removed and the Cryotop was quickly immersed into liquid nitrogen, protected with the cap and stored under liquid nitrogen. For warming, after removal of the cap, the Cryotop strip was immersed directly into 1 mL of the 0.25 M sucrose solution for 1 min and the embryo was transferred into 200 µL droplet of 0.15 M sucrose solution for 5 min. Warmed blastocysts were washed in H199 medium + 10% FCS and cultured for two days. After 24 h and 48 h post-warming culture survival rates were evaluated with morphological criteria, on the basis of the integrity of the embryo membrane and the ZP, and re-expansion

of the blastocoel. Furthermore, the percentages of embryos that resumed their development and reached a more advanced developmental stage after culture (development rate), as well as the hatching rates, were recorded.

Experiment 3.2: Differential staining

Blastocysts (only XBL and HBL) produced in the presence (1 μM) or absence of crocetin on Day 8, were subjected to a DS protocol previously described (Thouas *et al*., 2001) with slight modifications. Briefly, the ZP of viable expanding or expanded blastocysts was removed with 0.2% pronase in Hepes-buffered SOF. After three washes in PBS with 0.1% PVP, blastocysts were first incubated in 500 μL of propidium iodide solution (PBS with 1% Triton X-100 and 100 μg/mL propidium iodide) for 1 min. Blastocysts were then immediately transferred into 500 μL of a fixative solution of 100% ethanol with 25 μg/mL bisbenzimide (Hoechst 33342) for 5 min. The fixed and stained embryos were subsequently transferred into glycerol and mounted onto a glass microscope slide in a drop of glycerol, flattened with a coverslip to a level where all nuclei appeared at the same focal plane, and visualized using a fluorescent microscope (Eclipse E-600; Nikon) under ultraviolet light with excitation at 460 to 560 nm. A digital image of each embryo was taken using NIS-Elements-F software and a high-resolution color digital camera (Nikon digital sight DS-Fi 1C) and the numbers of TE (red) and ICM (blue) nuclei were counted.

Experiment 3.2: TUNEL staining

The blastocysts (BL, XBl and HBL), produced in the presence (1 μM) or absence of crocetin on Day 8 were subjected to a TUNEL staining protocol previously described (Paula-Lopes and Hansen, 2002).

The TUNEL assay was used to detect DNA fragmentation associated with late stages of the apoptotic cascade. The enzyme terminal deoxynucleotidyl transferase is a DNA polymerase that catalyzes the transfer of a fluorescein isothiocyanate-conjugated dUTP nucleotide to a free 3' hydroxyl group characteristic of DNA strand breaks. Embryos were removed from SOF culture medium and washed three times in drops of PBS containing 1 mg/mL PVP (PBS-PVP) by transferring the embryos from drop to drop. Embryos were fixed in a 50 μL drop of 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, washed twice in PBS-PVP, and stored in 600 μL of PBS-PVP at 4°C until the time of assay. All steps of the TUNEL assay were conducted using microdrops in a humidified box.

On the day of the TUNEL assay, embryos were transferred to a 50 μL drop of PBS-PVP and then permeabilized in a drop of 0.5% (v/v) Triton X-100 in PBS, containing 0.1% (w/v) sodium citrate for 30 min at room temperature. Controls for the TUNEL assay were incubated in 50 μL of RQ1-RNA free DNase (50 U/mL) at 37°C in the dark for 10 min. Positive controls and treated embryos were washed in PBS-PVP and incubated with 25 μL of TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase as prepared by and following the guidelines of the manufacturer) for 1 h at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Embryos were then washed three times in PBS-PVP and incubated in a 25 μL drop of Hoechst 33342 (1 μg/mL)

for 15 min in the dark. Embryos were washed three times in PBS-PVP to remove excess Hoechst 33342, mounted on 10% (w/v) poly-L-lysine coated slides using 3- to 4 µL drops of glycerol, and coverslips were placed on the slides. Labeling of TUNEL and Hoechst 33342 nuclei was observed using a fluorescent microscope (Eclipse E-600; Nikon). Each embryo was analyzed for total cell number (blue nuclei) and TUNEL-positive blastomeres (green nuclei) with DAPI and FITC filters, respectively, using a 20X and 40X objectives. Digital images were acquired (Figures 25, 26, 27 and 28) using NIS-Elements-F software and a high-resolution color digital camera (Nikon digital sight DS-Fi 1C).

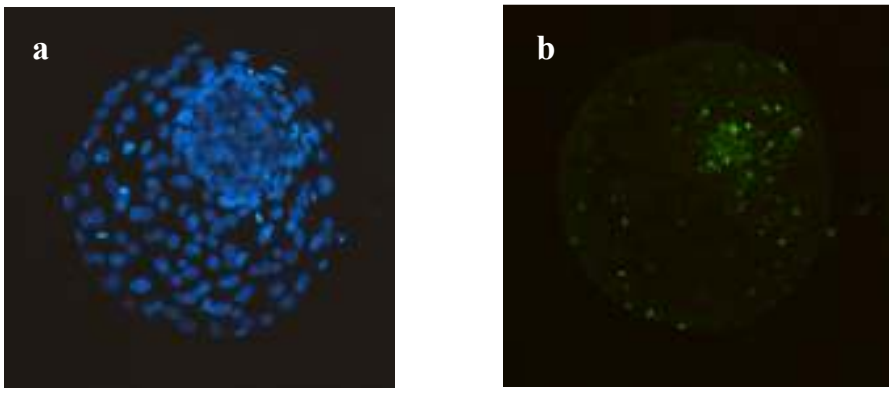


Figure 25. Representative pictures showing total cells staining examined by DAPI filter (**a**) and TUNEL labeling examined by FITC filter (**b**) of the same HBL cultured in the control medium

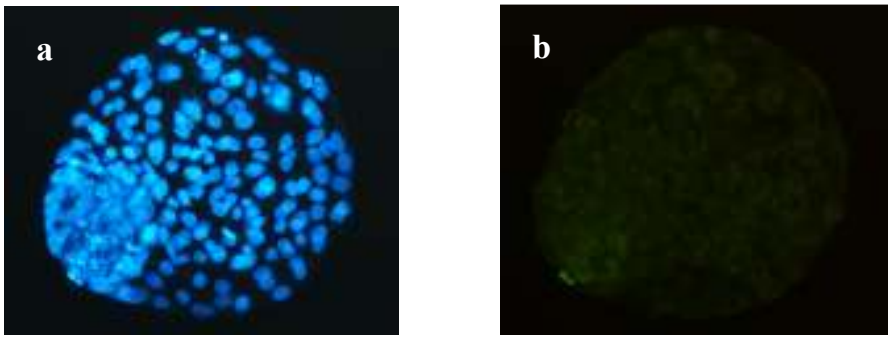


Figure 26. Representative pictures showing total cells staining examined by DAPI filter (**a**) and TUNEL labeling examined by FITC filter (**b**) of the same HBL cultured in the medium enriched with 1 µM crocetin

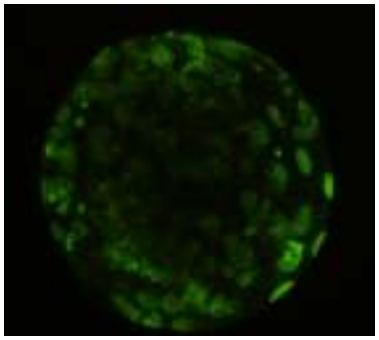


Figure 27. Positive control

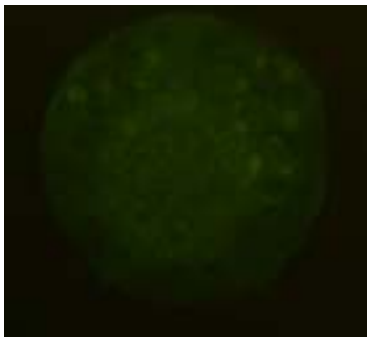


Figure 28. Negative control

5.2 STATISTICAL ANALYSIS

Differences among groups in the percentages of blastocysts, as well as in the survival rates after vitrification-warming, were analyzed by Chi square test using SPSS 16.0 statistical software (SPSS Inc., 2007). DS and TUNEL staining data were analyzed by the Student's *t* test.

5.3 RESULTS

As shown in Table 1, the supplementation of crocetin during culture did not affect cleavage; however, post-fertilization embryo development was higher in the 1 μM crocetin group compared to the others, both in terms of total embryo output (TMBL; $P<0.01$) and superior quality blastocysts (Grade 1,2 BL; $P<0.05$). Moreover, the percentage of fast developing embryos (Figure 29) was also increased in 1μM ($P<0.01$) and in 5 μM ($P<0.05$) crocetin groups compared to the control (Experiment 3.1 - Table 9).

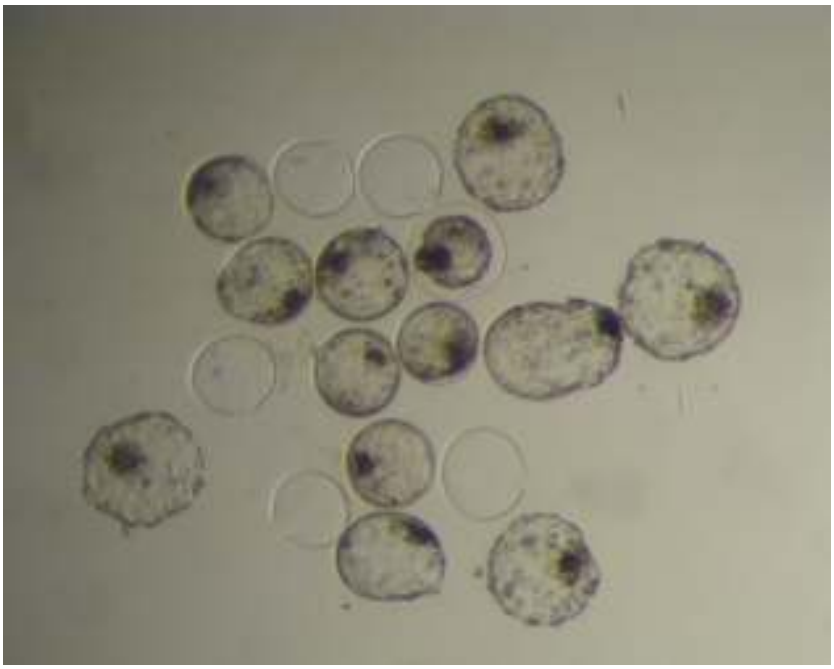


Figure 29. Fast developing embryos

Table 9. Percentages of cleavage, tight morulae-blastocysts (TMBL), Grade 1 and 2 blastocysts (Grade 1,2 BL) and fast developing embryos (fast BL) under different crocetin concentrations.

Crocetin concentrations	**COC n.**	**Cleaved n. (%)**	**TMBL n. (%)**	**Gd 1,2 BL n. (%)**	**Fast BL n. (%)**
0 (control)	208	153 (73.6)	66 (43.1)^{A}	62 (40.5)^{a}	27 (17.7)^{Aa}
1 µM	208	155 (74.5)	94 (60.7)^{B}	84 (54.2)^{b}	53 (34.2)^{B}
2.5 µM	208	170 (81.7)	83 (48.9)^{A}	77 (45.3)^{a}	41 (24.1)^{Aa}
5 µM	208	157 (75.5)	82 (52.2)^{A}	76 (48.4)^{a}	43 (27.4)^{Ab}

^{a,b} Bars with different superscript are significantly different; $P<0.05$
^{A,B} Bars with different superscript are significantly different; $P<0.01$

In addition, the enrichment of medium with crocetin improved embryo cryotolerance. In fact, although survival, development and hatching rates after 24 h post-warming culture were not affected, hatching rates following 48 h post-warming culture increased ($P<0.05$) when medium was enriched with 1 μM crocetin concentration (Experiment 3.1 - Table 10).

Table 10. Survival, developmental and hatching rates after 24 and 48 h culture following vitrification-warming of blastocysts produced under different crocetin concentrations.

Post-warming culture							
Crocetin concentrations	**n.**	**24 h**			**48 h**		
		Survived N (%)	**Developed N (%)**	**Hatched N (%)**	**Survived N (%)**	**Developed N (%)**	**Hatched N (%)**
0 (control)	62	54 (87.1)	32 (51.6)	14 (22.6)	41 (66.1)	37 (59.7)	26 (41.9)[a]
1 µM	79	59 (74.7)	41 (51.9)	22 (27.8)	58 (73.4)	55 (69.6)	48 (60.8)[b]
2.5 µM	75	38 (50.7)	20 (26.7)	11 (14.7)	30 (40.0)	24 (32.0)	18 (24.0)[a]
5 µM	74	42 (56.8)	28 (37.8)	3 (4.6)	36 (48.6)	34 (45.9)	23 (31.0)[a]

[a,b] Values within a column with different superscript indicate significant differences at $P<0.05$

As shown in Table 3, no differences were recorded in the average number of ICM, TE and total cells, as well as in the ratio of ICM:total cells between 1 μM crocetin and control groups (Experiment 3.2 - Table 11).

Table 11. Allocation of cells (Mean ± SE) in bovine blastocysts cultured in the absence (control) or presence of 1 μM crocetin.

Group	n. blastocyst	ICM	TE	Total	ICM: total cells %
Control	40	29.2 ± 1.7	122.6 ± 2.8	151.8 ± 3.7	19.0 ± 0.8
1 μM Crocetin	40	30.5 ± 1.4	126.6 ± 3.8	157.0 ± 4.5	19.4 ± 0.7

The blastocysts stained were individually classified into four groups according to the ICM:total cells ratio, as previously described (Koo *et al*., 2002): group I: <20%; group II: 20-40%; group III: 40-60%; group IV: > 60%. No differences between groups were observed in the percentages of blastocysts falling into group I (67.5 and 65 %, in crocetin and control groups, respectively) and into group II (32.5 and 35 %, in crocetin and control groups respectively). No blastocysts fell into Group III and IV under both culture conditions.

On the other hand, when embryos were cultured in the presence of 1 μM crocetin (Experiment 3.2) the total blastocyst cell number increased ($P<0.05$) and both the average number and the percentage of apoptotic cells in blastocysts decreased ($P<0.01$), as shown in Table 12.

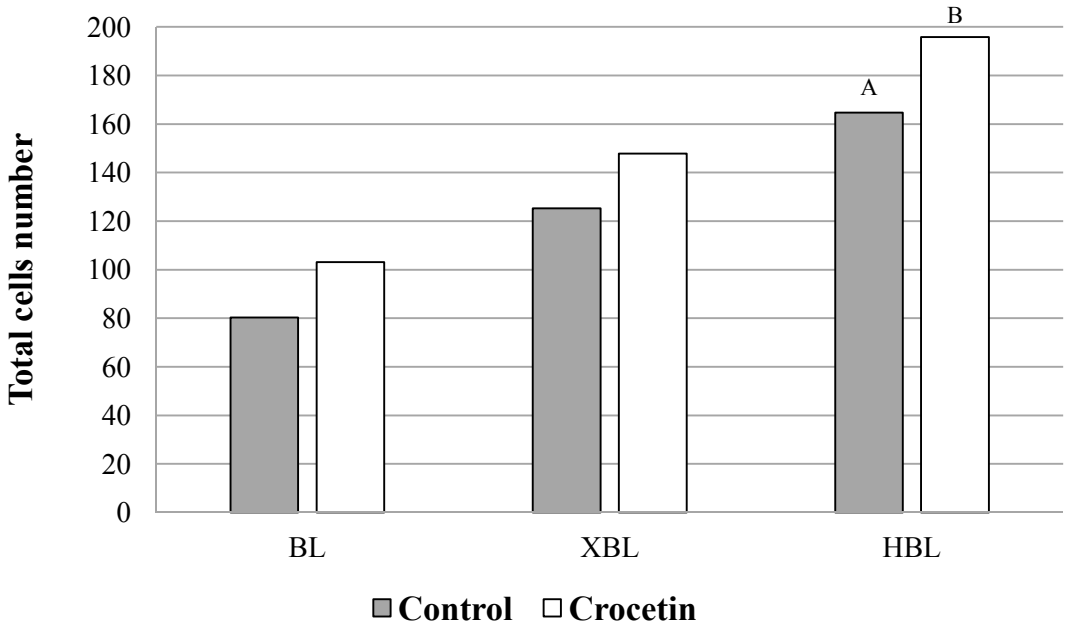
Table 12. Cell allocation on day 8 bovine blastocysts by TUNEL staining in the absence (control) or presence of 1 μM crocetin.

Groups	**n.**	**Total cells**	**Apoptotic cells**	**% apoptotic cells**
		Mean ± SE	Mean ± SE	Mean ± SE
Control	35	141.1 ± 7.1^{a}	10.9 ± 0.5^{A}	8.6 ± 0.7^{A}
1 μM Crocetin	37	162.8 ± 8.0^{b}	7.9 ± 0.3^{B}	5.3 ± 0.3^{B}

^{A,B} Values with different superscript are significantly different; $P<0.01$
^{a,b} Values with different superscript are significantly different; $P<0.05$

In addition, when these parameters were analyzed in relation to the stage of development, the total cell number was higher ($P<0.01$) in the crocetin group but only in case of HBL (Figure 30). Within each developmental stage (BL, XBl and HBL) the incidence of apoptotic cells out of the total decreased ($P<0.01$) in the enriched group (Figure 31).

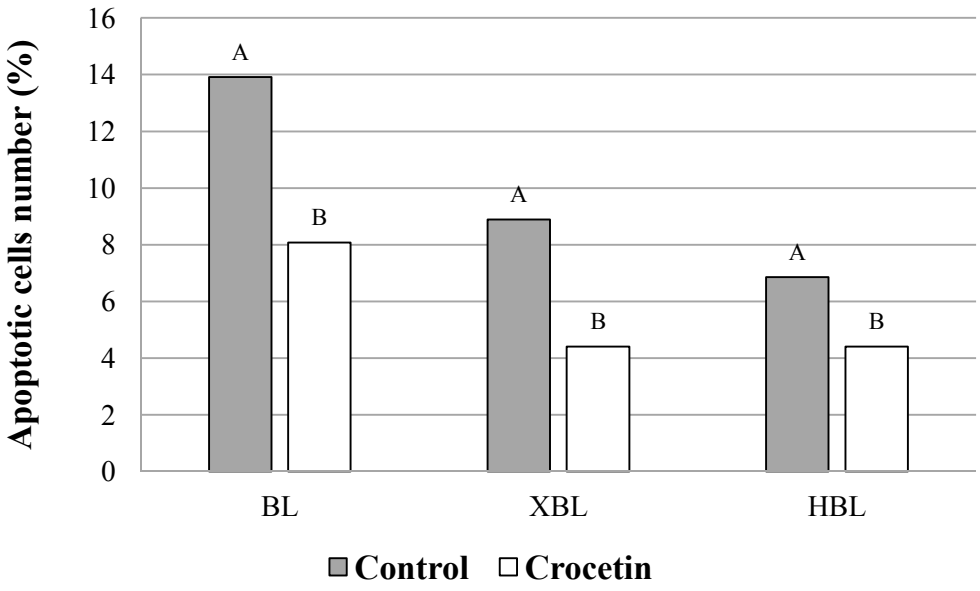
Figure 30. Total cell numbers of day 8 blastocysts cultured with or without crocetin according to the developmental stage.



[A,B] Bars with different superscript are significantly different; $P<0.01$

* The number of BL, XBL and HBL stained were respectively 7, 6 and 22 in the control and 8, 7 and 21 in the crocetin group.

Figure 31. The incidence of apoptotic cells of day 8 blastocysts cultured with or without crocetin according to the developmental stage.

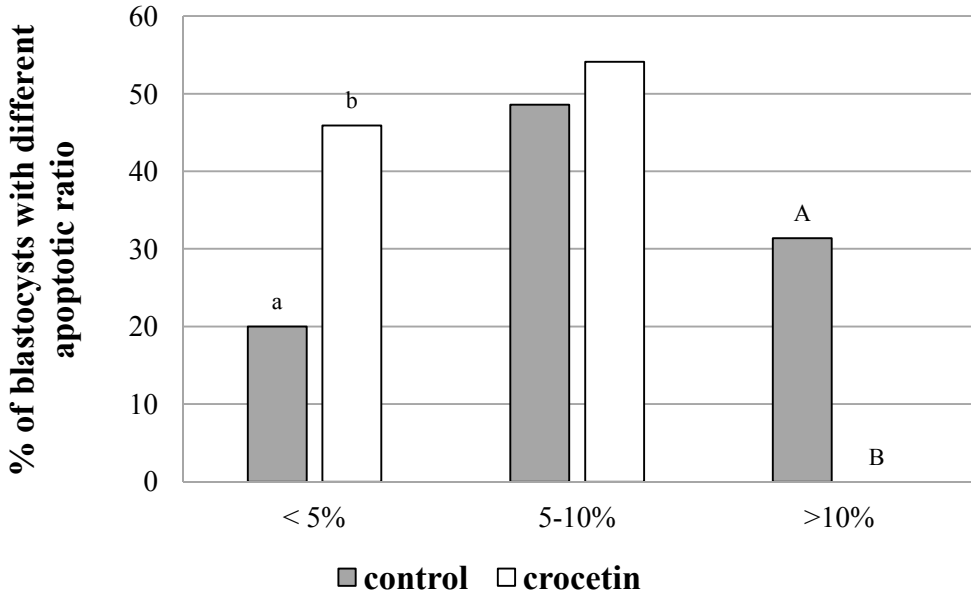


[A,B] Bars with different superscript are significantly different; $P<0.01$

* The number of BL, XBL and HBL stained were respectively 7, 6 and 22 in the control and 8, 7 and 21 in the LE group.

Furthermore, in the crocetin-enriched group a higher ($P<0.05$) percentage of blastocysts had <5% of apoptotic cells compared to the control; in particular, no blastocysts exhibited >10% apoptotic cells in the enriched group vs about 30% in the control ($P<0.01$; Figure 32).

Figure 32. Percentage of blastocysts with low (<5%), average (5-10%) and high (>10%) levels of apoptosis in the control and crocetin groups



[A,B] Bars with different superscript are significantly different; $P<0.01$

[a,b] Values with different superscript are significantly different; $P<0.05$

5.4 DISCUSSION

The aim of this work was to evaluate whether supplementing in vitro culture medium with crocetin, one of the active biological components of saffron, improves bovine embryo development and quality. The results of the present study demonstrated that enrichment of culture medium with low doses of the natural antioxidant crocetin (1 μM), improves both embryo output and quality, as indicated by increased blastocyst yields, developmental chronology and cryotolerance and by reduced incidence of apoptotic cells.

The preliminary dose response trial showed an evident toxic effect only for 50 μM concentration of crocetin, that was associated to a substantial decreased embryo production. This is in contrast with previous frog (*Xenopus*) embryo culture studies reporting that crocetin was not toxic to embryo development, until a concentration of 100 μM (Martin *et al*., 2002). On the other hand, previous studies reported that high concentrations of crocetin tested by the DPPH antioxidant activity assay, exhibited lower antioxidant effects (Kanakis *et al*., 2009).

However, the results of the preliminary trial suggested to reduce the range of concentrations to test in Experiment 3.1, where the dose-response study was carried out with 0 (control), 1, 2.5 and 5 μM crocetin. The fact that cleavage rate was not affected by the treatment was expected, as the enrichment was carried out only during culture with the aim to improve particularly embryo quality, that is known to be mainly affected by culture environment (Lonergan *et al*., 2001). Nevertheless, in contrast with the results of the previous experiments of this thesis, in which other antioxidant compounds were tested, crocetin supplementation at the concentration of 1 μM positively influenced post-fertilization embryo development, as indicated by higher total embryo output, superior quality blastocysts and faster developing embryos. The increased embryo production recorded in the presence of crocetin was relevant, as the treatment provided about 17% more transferable embryos (TMBL) and 14% more superior quality blastocysts (G 1 & 2 BL) than the control culture group. No other studies have been published on the effect of crocetin on embryo development; however, an improved embryo development was recorded in mice when IVM medium was supplemented with low doses of saffron extracts (Tavana *et al*., 2012).

In addition, a clear indication of an improvement of embryo quality in culture medium enriched with 1 μM crocetin derives from the 2-fold increase in the percentage of fast developing embryos, i.e. the percentages of blastocysts reaching the most advanced stages of development such as XBl and HBl by the end of culture (Day 7), that was observed compared to the control. In fact, one the most important criterion to determine the quality of embryos to be transferred is the timing of development to specific morphological stages (Leibfried-Rutledge *et al.,* 1987; McKiernan and Bavister, 1994; Bavister, 1995; Van Soom *et al.,* 1997).

It has also been demonstrated that embryos with a faster kinetics of development survive cryopreservation better than those with slower developmental speed (Nedambale *et al*., 2004). In agreement with this result, embryo cryotolerance was also significantly increased when 1 μM crocetin was present during culture. It is well known that the major factor affecting the cryotolerance of *in vitro* produced embryos is their quality (Han *et al*., 1994) that has been proven to be determined by the culture system. It results that embryo resistance to cryopreservation is a highly reliable marker of embryo quality (Imai *et al*., 2002; Lonergan *et al*., 2003*a*; 2003*b*; Rizos *et al*., 2003). Also in this experiment the beneficial effect on embryo cryotolerance was evident after 48 h of post-warming culture, when a significant increase of hatching rate was recorded. This is known to be the most reliable parameter to assess embryo cryotolerance, as cryopreservation hardens the ZP, consequently preventing blastocyst hatching process (Carroll *et al*., 1990; Matson *et al*., 1997), necessary for the appropriate elongation of the conceptus after ET. This was the first work that evaluated the effect of crocetin on embryo cryotolerance. The only work available in literature, in fact, has been limited to assess the effect of crocetin on frog in vitro embryo

development in morphological terms (Martin *et al*., 2002). In particular, it was demonstrated that concentrations much higher than our effective dose may have theratogenic effects.

However, previous studies demonstrated that supplementation with antioxidants during culture improves embryo quality and cryotolerance (Hosseini *et al*., 2009; Takahashi, 2012). In the Experiments 1 and 2 of this thesis we also recorded a significant improvement of cryotolerance when culture medium was enriched with either resveratrol or L-ergothioneine. The improved cryotolerance of bovine embryos obtained with crocetin during culture is likely due to its high antioxidant capacity, as oxidative stress occurs during the freezing-thawing process, leading to lipid peroxidation, membrane injury and structural damage (Somfai *et al*., 2007*b*). This may be deducted from the work performed by Xiang *et al.* (2006), in which it was demonstrated that 1 μM crocetin concentration prevents the adhesion of leukocyte to bovine endothelial cells (BEC) culture, by down-regulating the expression of intercellular adhesion molecular-1 protein (ICAM-1), increasing the activity of SOD and significantly inhibiting the generation of malonicdialdehyde (MDA), a products of lipid peroxidation and a classic indicator of oxidative stress.

In addition to cryotolerance, we also assessed embryo quality by morphological parameters, such as the total cell count and particularly the cell allocation to the ICM and the TE, as well as the apoptotic cell ratio, an additional criterion proven to effectively predict embryo viability (Pomar *et al*., 2005). In fact, it is well documented that both cell lineages are essential for embryonic survival (Koo *et al*., 2002; Knijn *et al*., 2003) and post-implantation development (Iwasaki *et al*., 1990). The distribution of blastocyst cells in the ICM and TE lineages was not affected by the crocetin treatment and most of the embryos had an ICM:total cells ratio lower than 40% in both groups. On the other hand, crocetin during

culture significantly reduced both the average number and the incidence of apoptotic cells in the blastocysts. Although apoptosis physiologically occurs during pre-implantation embryo development, to control cell population by eliminating defective cells (Brison, 2000), a high incidence of apoptotic cells is correlated to abnormal morphology of the embryo (Hardy *et al*., 1989). In fact, in vitro produced embryos are known to exhibit a higher incidence of apoptosis than their in vivo counterparts (Bergeron *et al*., 1998). In the present study both the number and the incidence of apoptotic cells decreased in the crocetin-enriched group within each developmental stage, i.e. blastocysts, expanded blastocysts and hatched blastocysts. Furthermore, when only HBL were considered the total cell count also increased in the crocetin group compared to the control. A similar result was found in Experiment 2, in which the difference in cell number was also evident only at the hatched stage. Therefore, also in this case the finding is only in apparent contrast with the data obtained with the DS. As previously stated, embryos that were examined by DS were only expanded and hatched blastocysts, and the low numbers did not allow to analyze this parameter within the stage. What is overwhelming is the difference between groups in the percentage of blastocysts with low, average and high proportion of apoptotic cells. The enrichment of IVC medium with crocetin significantly increased the proportion of blastocysts with low apoptotic cells (<5%) compared to the control (46 vs 20%). In addition, no blastocysts showed a high apoptotic index in the enriched group vs 31.4 % of the control. The reduced incidence of apoptosis recorded in the presence of crocetin is also likely due to its antioxidant properties. It is known that oxidative stress that normally occurs during in vitro culture triggers apoptosis (Guérin *et al*., 2001; Yang *et al*., 1998). Impaired embryo development and apoptotic cell death have been previously associated with increased levels

of reactive oxygen species and decreased levels of SOD (Guérin *et al*., 2001). It has also reported that antioxidants during culture inhibit apoptosis (Guérin *et al*., 2001). These results are in agreement with those obtained in Experiment 2, in which apoptosis was reduced by enriching culture medium with LE.

In conclusion, the results of this study taken together demonstrated that enrichment of bovine IVC medium with low doses of crocetin improves blastocyst production and the overall embryo quality, that is the most important factor affecting post-implantation development after embryo transfer. The beneficial effect on embryo quality is highlighted by the enhanced post-fertilization embryo development, especially in term of superior quality blastocysts and fast developing embryos, as well as by the improved cryotolerance following culture, and by the increased proportion of blastocysts with a low percentage of apoptotic cells.

CONCLUSIONS

Currently, the ovum pick-up and IVEP technology is the best tool available to improve the maternal contribution to the genetic improvement. There has been an increasing interest in the large scale embryo production through IVEP around the world; in some countries, such as Brazil, the majority of the bovine embryos produced for commercial purposes comes from IVEP. However, despite numerous efforts carried out over the years, a critical issue is still given by the poor quality of the IVP embryos and an optimal medium for IVC has still not been formulated. This hampers the benefit/cost ratio of the technology, due to the lower pregnancy rates recorded after ET of cryopreserved IVP embryos. A substantial amount of evidence exists to demonstrate that the culture conditions to which the embryo is exposed, particularly in the post-fertilization period, can have perturbing effects on the pattern of gene expression and on the embryo quality, with potentially important long-term consequences (Lonergan, 2007).

Several factors are known to affect the in vitro embryo development, such as ammonia, oxygen radicals, growth factors, etc. Among these, the increased oxidative stress is a major factor impairing mammalian in vitro embryo development. It has been demonstrated that oxidative stress results in several intracellular damages, lipid peroxidation leading to reduced cryotolerance, apoptosis and abnormal allocation of cells into ICM and TE lineages (Somfai *et al*., 2007*b*, Guérin *et al*., 2001; Yang *et al*., 2008). Therefore, in order to protect the embryos from increased oxidative stress, and the consequent damages caused by suboptimal culture conditions, in the last years there has been an increased interest in strategies based on the supplementation of antioxidant compounds during in vitro embryo

culture. To reduce the toxicity of ROS for improving embryo development, antioxidants supplementation is effective to regulate intra- and extra-embryonic environments; in fact, maintaining of intra- and extra-cellular redox balance is very important to provide suitable environments for metabolic and gene expressions followed by embryo development (Takahashi, 2012). Currently, addition of several enzymatic and non-enzymatic, as well as protein and non-protein antioxidants seem to be a very effective method to improve embryo development by scavenging ROS and serving embryos a low oxidative stress conditions.

This was the rationale of the thesis that focused on evaluating the effects of different natural antioxidants during bovine in vitro embryo culture on embryo development and quality.

The choice of the candidate molecules fell on Resveratrol (Experiment 1), L-Ergothioneine (Experiment 2) and Crocetin (Experiment 3) because beneficial properties and positive effects in other species and environmental conditions were previously reported. The results of this thesis demonstrated that improving IVC medium environment, by natural antioxidant compounds supplementation, enhances the overall embryo quality, affecting the efficiency of embryo development, cryotolerance, cell differentiation and apoptotic rate.

More specifically, resveratrol improved the cryotolerance of in vitro produced bovine embryos, as shown by a significant improvement of both development and hatching rates recorded after 48 h post-warming culture (Experiment 1). Hatching rate showed a 2-fold increase compared to the control and blastocysts exhibited a correct allocation of cells into the ICM and TE.

Similarly, in Experiment 2 LE during culture increased embryo cryotolerance, especially after 48 h of post-warming culture, when a significant increase of hatching rate was observed. Blastocysts produced in the medium enriched with LE showed an increase in the

average number of ICM cells compared to the control, and an increased percentage of blastocysts with the most appropriate ICM:total cells ratio (20-40%), confirming a beneficial effect on embryo quality. Moreover, both the number and the incidence of apoptotic cells decreased in the LE-enriched group within each developmental blastocyst stage.

In contrast to previous antioxidant compounds, in Experiment 3 crocetin supplementation at the concentration of 1 μM increased blastocyst yields in term of higher total embryo output, superior quality blastocysts and fast developing embryos. In addition, crocetin improved the cryotolerance of IVP embryos compared to the control, as indicated by higher hatching rates recorded after 48 h post-warming culture. Blastocysts produced in the absence (control) and presence of crocetin had a similar number of ICM, TE and total cells, as well a similar ratio of ICM:total cells. On the other hand, crocetin during culture significantly reduced both the average number and the incidence of apoptotic cells in the blastocysts. In particular, the enrichment of IVC medium with crocetin significantly increased the proportion of blastocysts with low apoptotic cells, and eliminated blastocysts showing a high apoptotic index in.

Handling of mammalian oocytes and embryos *in vitro* is an effective technique to obtain offspring by embryo transfer. The improved embryo quality obtained with all the antioxidants tested is a very important finding, as better embryo quality may may lead to increased pregnancy rate after ET of IVP embryos. Indeed, increasing the number of good quality embryos is the most practical and clinically useful approach in embryo transfer of mammalian pre-implantation embryos.

In conclusion, optimization of IVC systems by the formulation of antioxidant-enriched culture media is critical to increase embryo quality, viability and resistance to freezing, likely promoting a reduction of birth defects and of embryonic and neonatal mortality incidence. These results lay the basis for future perspective studies. In particular, it is worth investigating whether the inclusion of resveratrol, LE and crocetin also during post-warming embryo culture would further improve embryo quality. It is likely, indeed that a surplus source of exogenous antioxidant may prevent DNA fragmentation during the critical period of cryopreservation and post-warming embryo culture. In addition, further studies are needed to investigate the exact mechanisms by which these antioxidants protect the cells. Finally, these promising results strongly encourage to evaluate the post implantation embryo developmental competence after ET of IVP embryos cultured in enriched media.

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