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Innovative strategies to improve fertility of buffalo semen

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A Nicola, Francesco ed Emanuele

Se vuoi essere felice ascolta il tuo cuore, perché esso racchiude tutte le risposte che cerchi.

Sergio Bambarén

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Buffalo (Bubalus bubalis) breeding in the world

The world buffalo population amounts to about 190 million of heads (FAO, 2010) distributed as follows: 96.4% in Asia (mainly concentrated in India, China and Pakistan), 2.9% in Africa (with greater consistency in Egypt) and the rest in Europe (especially Italy, Romania, Georgia, Bulgaria and Turkey) and Latin America (Brazil, Venezuela, Colombia and Argentina). In the last years, interest in the world regarding buffalo breeding increased. In tropical Countries climatic and environmental conditions make buffalo an irreplaceable milk producer; in fact, buffalo is the species that best suits to satisfy animal protein demand, as it is easy-fitting, rustic, long-lived and parasite resistant. Buffaloes have a high capacity for adaptation across broad climatic zones. The perfect interaction among reproductive seasonality, environmental conditions and forage availability throughout the year allows the buffalo to be able to compensate the loss of bovine milk recorded during the unfavorable season and to produce animal proteins at competitive costs, exploiting pastures that are typically limiting cattle production (Zicarelli, 1994a, Campanile et al., 2010). The good feed conversion efficiency of buffaloes and the relatively low maintenance requirements are attribute which make them ideal in low-cost production systems (Zicarelli, 1994a).

In the zoological scale the buffalo is in the class Mammalia, order Artiodactyla, Ruminantia subordinate, family Bovidae, sub-family Bovinae, gender Bubalus, species Bubalus bubalis. This species is divided into two groups (Macgregor, 1939): Bubalus bubalis sp. known as “Water or River buffalo” with 50 pairs of chromosomes bred in India and in western countries and Bubalus bubalis var.

kerebau called “Carabao or Swamp buffalo” with 48 pairs of chromosomes, present in the countries of Southeast Asia.

The buffalo bred in Italy is part of a large family of River type. Until few years ago it was called Mediterranean type buffalo, while in 2001 the Ministry of Agriculture and Forestry recognized the "Mediterranean Italian buffalo" breed (Decree Ministerial 201992 of 05/07/2001). This milestone was reached thanks to long isolation and lack of cross-breeding with other strains. Therefore, the buffalo bred in Italy shows a degree of "purity", result of obvious morphological and functional differentiation.

In Italy, buffalo is an important economic resource, especially for the regions traditionally involved in the rearing of this species. The largest concentration of heads can be found in Campania, in the provinces of Caserta and Salerno, where 66% out of 402.659 animals of the Italian total population is bred (Istituto Nazionale di Statistica, 2013). In these areas, buffalo breeders have constantly grown both in professional and managerial terms coming to an efficient intensive or semi-intensive approach. This advance made possible the selection of subjects, with the purpose of increasing productive and genetic value, and transformed a marginal sector into an area with great economic potential.

In our country, the profitability of this species is particularly linked to the “Mozzarella di Bufala Campana” cheese, which gained a very well defined profile in the market, enhanced and protected by a Denomination of Protected Origin (DPO) trademark and, above all, is recognized in an increasing number of countries. The selection is aimed to improve the quantity and the quality of buffalo milk in order to increase the production of mozzarella. Therefore, in Italy, the

commercialization of milk for mozzarella cheese production represents the main income of buffalo breeding.

It is worth to remember that about 98% of the buffaloes in the world is bred in developing Countries that are interested in Italian dairy genetic "material" to crossbreed the local working or unproductive buffaloes with the highly productive Mediterranean buffalo, in order to increase animal proteins and meet human needs. According to our experience, this is also demonstrated by the constant increasing request of the Mediterranean buffalo semen and embryos from many countries. However, the ever-growing trend of milk request and the necessity to cut down fixed production costs make the adoption of an improving production plan essential to farming. In this scenario the competitiveness of buffalo breeding in Italy is necessarily linked to the use of biotechnologies of reproduction that consent to plan selective targets in a shorter time.

Reproductive biotechnologies

Reproductive biotechnologies are undoubtedly one of the most emblematic strategies of applied research in the field of life sciences and animal husbandry. These new technologies have contributed significantly to the evolution of breeding in the last 60 years (Thibier, 2005), laying the basis for a radical transformation of animal husbandry processing and production systems. One of the key features of a modern farm in order to be competitive in the market is certainly to achieve considerable genetic improvement in short time, without neglecting the needs of consumers who are increasingly sensitive to both the quality of product and animal welfare.

In this species the reproductive technologies that offer most of the benefits are: Artificial Insemination (AI), which is a critical tool to enhance the paternal contribution to the genetic improvement, Multiple Ovulation (MO) and Embryo Transfer (ET) programs which increase the intensity of genetic selection, through the maternal lineage, reducing generation intervals and the Ovum Pick-up (OPU) linked to the in vitro embryo production (IVEP), the technology that currently allows to obtain the greatest number of transferable embryos from each donor over a long term (Gasparrini, 2002).

Artificial insemination

Artificial Insemination (AI) can be considered the very first applied reproductive technology, and it was rudimentarily attempted for the first time in 1779 in the dog (Spallanzani, 1784). This technique consisted in the collection of the semen from animals of high genetic merit and its dilution and utilization in recipient females. The application of this technique is worldwide carried out in several animal species, as well as in humans. In particular, in the field of animal science AI provides sanitary, genetic and economic advantages.

From a sanitary point of view it avoids the transmission of some venereal infectious diseases, such as vibrio and trichomonas that are usually spread through natural mating. Furthermore, this technique avoids the transmission and reduces the possibilities of diffusion of other infectious diseases, since the males that are selected for AI undergo severe sanitary controls to verify that they have been in contact with pathogens, such as brucellosis, leucosis, tuberculosis and IBR. It is

worth pointing out that some pathogens can be transmitted through AI, but the collection process allows for the screening of disease agents.

From a genetic point of view, AI allows for evaluation of sires through progeny testing of daughters, whose productive, morphological and reproductive characteristics can be tested. It has been estimated that one bull can mate 20 females in one month by natural insemination and its reproductive activity lasts approximately 12 years: hence, if all the matings resulted in a pregnancy, it has been calculated that one bull would produce less than 3.000 calves in its lifetime. By using AI, one bull can produce up to 200.000 calves, taking into consideration the number of straws that can be obtained from a single ejaculate following dilution and freezing. Further advantages derived from the use of frozen/thawed semen through AI are: the availability of semen even after death of proven sires and the ease of moving semen to promote genetic improvement worldwide. Another important aspect related to AI is the customization of the best sire to the recipient characteristics. This allows correction of some morphological and productive defective traits of recipients that will not be carried over to their progeny. Furthermore, AI can be a flexible tool to obtain calves with different characteristics, following insemination of recipients with semen from different sires.

The economic advantages linked to the utilization of AI are mainly due to a better management of the farm. Bulls are expensive to rear, relatively unproductive, vulnerable to disease or accident and may even prove to be infertile. In addition, they are characterized by higher feed requirement and need special housing and handling equipment. By using AI, farmers can save on space otherwise used by

bulls, for a better farm management, and erase the possibility of any dangerous situation linked to their aggressiveness.

We have to consider though, some possible disadvantages derived from the application of AI. In fact, a long time is needed in order to prove sires through progeny testing, i.e. testing of their daughters for milk production. In the buffalo species, this process requires at least four to five years, since: a little over 10 months is required for pregnancy, at least other 20 months are needed to the progeny to reach puberty, and additional 10 months are required for the subsequent pregnancy of the progeny. Hence, at the end of the first lactation, data will be analyzed, and it is possible that after all this time a bull may show negative effects on milk production and therefore its use would be no longer adopted. Furthermore, the diffusion and use of few proven sires across many farms may cause, in the long run, a considerable increase of the phenomenon of consanguinity and inbreeding. For this reason specific breeding programs must be implemented.

Although buffaloes are probably among the easiest mammals to be trained to serve an artificial vagina, natural mating is still responsible for most of the pregnancies occurring in buffalo herds. In fact, the use of AI in both Swamp and River buffaloes is still marginal for several reasons. The implementation of AI in Swamp buffaloes has its drawbacks in the low number of heads per owner, lack of proper heat detection and the overall poor management. On the contrary, in intensive River buffalo breeding, the use of AI has been kept marginal until a decade ago, due mainly to lack of truly superior progeny tested bulls and to unacceptable low pregnancy rates when using both natural or synchronized oestrus (Zicarelli et al.

1997). Recently though, an improvement of the efficiency in protocols for synchronization of estrus and ovulation has been reported in buffaloes (Neglia et al., 2008), paving the road for a wider implementation of assisted reproduction in this species. The efficiency of AI has doubled (25‑50% pregnancy rate) over a period of approximately 20 years (Zicarelli et al., 1997; Baruselli et al., 2001; Campanile et al., 2011; Neglia et al., 2015).

Currently, at our latitude, AI is strongly influenced by seasonality, as indicated by the high incidence of embryonic loss (was 20-40%) during seasons characterized by a higher number of light hours (Campanile et al., 2005; 2007a; 2007b). Moreover, Rossi et al., (2014) reported a marked seasonal variation in the response of Italian buffaloes to synchronization protocols. Different strategies have been developed to reduce embryonic mortality in buffalo cows that in part is due to reduced progesterone secretion (Campanile et al., 2007b). Therefore, a study was performed during the seasonal transitional period, which corresponds to mid-winter in the Mediterranean region to evaluate the effects of P4 secretion promoters (GnRH agonist, hCG and exogenous P4) administered on day 25 after AI (Campanile et al., 2007b). It was demonstrated that all treatments increased P4 values and reduced embryonic mortality.

In order to spread the worldwide diffusion of AI in buffalo management for a faster enhancement of the genetic potential and its distinctive production a series of aspects need to be considered and further investigated. Among these, it is particularly important to focus on semen cryopreservation procedures and hence, on the quality of frozen-thawed semen, known to be related to poor fertility rates

in buffalo (Muer et al. 1988; Andrabi et al. 2001; Ahmad et al. 2003; Senatore et al. 2004; Kumaresan et al. 2005; 2006; Shukla and Misra 2007).

Multiple ovulation and embryo transfer

In general, MOET schemes are used to increase the intensity of genetic selection, reducing generation intervals (Smith, 1984). The MO consists in an hormonal treatment of the donor, usually starting 9-11 days after the heat (9- 11 d of the cycle), with gonadotropins to induce ovulation of multiple follicles simultaneously (the total dose is divided into 5 days according to a decreasing dose, similar to that used in cattle), combined with induced luteolysis with a prostaglandin analogue on day 4 after initiation of treatment. The heat is longer than normal and the donor is generally subjected to double inseminations. At 6-7 days post insemination the recovery of embryos is performed by trans-cervical insertion of a three way catheter to flush the uterine horns. Embryos are evaluated under a microscope to assess suitability for direct transfer or for cryopreservation. After estrus synchronization of the recipients, the embryos are transferred in surrogate females for the gestation. However, there are many limiting factors using this technique, among which the most important are the need to use subjects in good genital tract conditions, with patent oviducts that should neither be pregnant nor lactating, with regular estrous cycle and after a suitable post-partum period. Using this technique in bovine, the mean embryo recovery per animal is 5, whereas in buffalo there is an average recovery of less than 2 embryos. Several factors may influence the response to the MO in buffalo species, as a high individual variability in the size of the uterine lumen (Zicarelli, 1994a), the age of the donors, the number of daily

milkings (Zicarelli et al., 1993), the calving interval, the period of year (Zicarelli et al., 1994b), and the characteristics of the diet (Di Palo and Cheli, 1995).

Moreover, it has been observed (Zicarelli, 1997) that also season influences the superovulatory response; in particular, the period from November to December-January, favorable to reproductive activity, was the worst in terms of the number of embryos recovered, while the superovulation treatments carried out in February, March and June to September provided higher number of embryos transferred. It was speculated that these unexpected results were accounted for by a sort of natural selection of the best donors occurring during the unfavorable season, as during the breeding season all animals are cycling.

The aim of MOET is to obtain higher number of transferrable embryos per donor, with greater possibility of producing pregnancies. However, because of the low and inconsistent efficiency of MOET treatments in this species (Misra, 1997; Zicarelli et al, 1997b), there is a worldwide increasing interest in large-scale in vitro production of buffalo embryos for faster propagation of superior germplasm and to enhance genetic progress through the maternal contribution. Indeed, OPU linked to IVEP is the reproductive technologies that offer in buffalo most of the advantages in this direction.

Ovum-pick up and In vitro embryo production

The technique of Ovum Pick-up (OPU) in buffalo, as in cattle, consists in an ultrasound guided transvaginal procedure aiming to oocytes recovery from a live donor. It is an alternative technique to MOET because it can be performed on a wider typology of donors, such as non-cyclic animals, pregnant cows, subjects with non-patent oviducts or genital tract infections, and animals that are not responsive to hormonal stimulation, the last representing a high proportion in buffalo (Gasparrini, 2010). The application of OPU technology (Pieterse et al., 1991; Boni et al., 1993) is currently carried out in donors of different ages, from 2 months old calves (Armstrong et al., 1992) to elderly subjects, as well as in different physiological conditions (Galli et al., 2001). In fact, OPU does not interfere with the physiological status of the donor and is simply feasible and repeatable (Boni et al., 1997a). Furthermore, it allows the visualization and the aspiration of all follicles with diameter >2mm, which resets the estrous cycle and avoids the dominance of one follicle over the subordinates. It has previously been shown in several species that the optimal interval between aspiration sessions is 3–4 days (Boni, 1997; Galli et al., 2001).

The combined OPU and IVEP technology is currently the most efficient tool for increasing the number of transferable embryos obtainable per donor. In buffalo, this technology is even more competitive in terms of embryo yields compared to MOET (Gasparrini, 2002) both because of the lower response to hormonal stimulation together with the poor embryo recovery (Zicarelli, 1997b), and because of the impossibility to repeat continuously the MOET treatments over a

long-term since multiple ovulations can be induced only in cyclic animals, and it is likely that buffalo cows enter seasonal anestrus. The competitiveness of OPU-IVEP becomes overwhelming when donors are selected on the basis of their folliculogenetic potentials. Earlier OPU trials performed in the Mediterranean Italian Buffalo reported an extreme individual variability in the number of follicles found (Gasparrini, 2002; Neglia et al., 2003; 2011a). Gasparrini et al., (2014) reported that in buffalo the donor influences not only the number of recoverable oocytes but also the developmental competence, i.e. the capacity to develop in vitro up to the blastocyst stage, as shown by great individual variability in the blastocyst rates (4.3–22.8%). Moreover, the number of small follicles registered at the start of OPU may predict the number of superior quality oocytes that can be obtained from an animal during the subsequent 2 months. This is particularly important in buffalo, in which the major limitation is the low number of recoverable oocytes (Gasparrini, 2011). Therefore, for the application of OPU-IVEP technology in the field, it is fundamental to select the best donors on the basis of the follicular population before enrolling them in programs to obtain a number of embryos sufficient to offset the laboratory costs.

However, although the buffalo IVEP system has greatly improved over the years, leading to high blastocyst yields (Gasparrini et al., 2006a; Neglia et al., 2003b) and to the production of offspring (Huang et al., 2005; Hufana-Duran et al., 2004; Neglia et al., 2004; Sà Filho et al., 2005;), this technology is still far from being commercially viable. The low IVEP efficiency recorded in buffalo compared to cattle is in part due to peculiarities of the reproductive physiology of buffalo that are not easily modifiable, such as the low number of oocytes recovered and their

poor quality. In addition, unlike cattle, the reproductive lifespan in buffalo is long (12 years in Italy and even longer in swamp buffalo in China and South East Asia), because, for economic reasons, buffalo cows are usually slaughtered when they are old or when their fertility and productivity are compromised. This results in a further decrease of the number of competent oocytes recoverable in the case of abattoir-derived ovaries as a source of gametes. However, it has been demonstrated over the years that improvements in IVEP are possible through the optimization of each procedural step, especially when taking into account species-specific differences, as shown by the higher blastocyst rates reported over recent years (Gasparrini et al., 2006a).

Recovery and oocyte quality

As mentioned before, a major limiting factor of IVEP in buffalo is the very poor recovery rate of immature oocytes per ovary. When the method of oocyte retrieval employed was the aspiration of 2 to 8 mm follicles, the average recovery of total oocytes per ovary was: 0.7 (Totey et al., 1992), 1.7 (Das et al., 1996), and 2.4 (Kumar et al., 1997). In our experience, controlled follicular aspiration of abattoir-collected ovaries allowed the retrieval of 2.4 good quality oocytes per ovary on average (Gasparrini et al., 2000). The slightly lower oocyte recovery (0.4-1.9) reported in other studies (Das et al., 1996; Kumar et al., 1997; Madan et.al., 1994; Mishra et al., 2008; Samad et al., 1998; Totey et al., 1992) may be due to differences in breed, older age at slaughter, management and nutritional status (Goswami et al., 1992). The recovery rate is therefore much lower than in cattle, in which 10 good quality oocytes are obtained on average per ovary (Gordon, 1994).

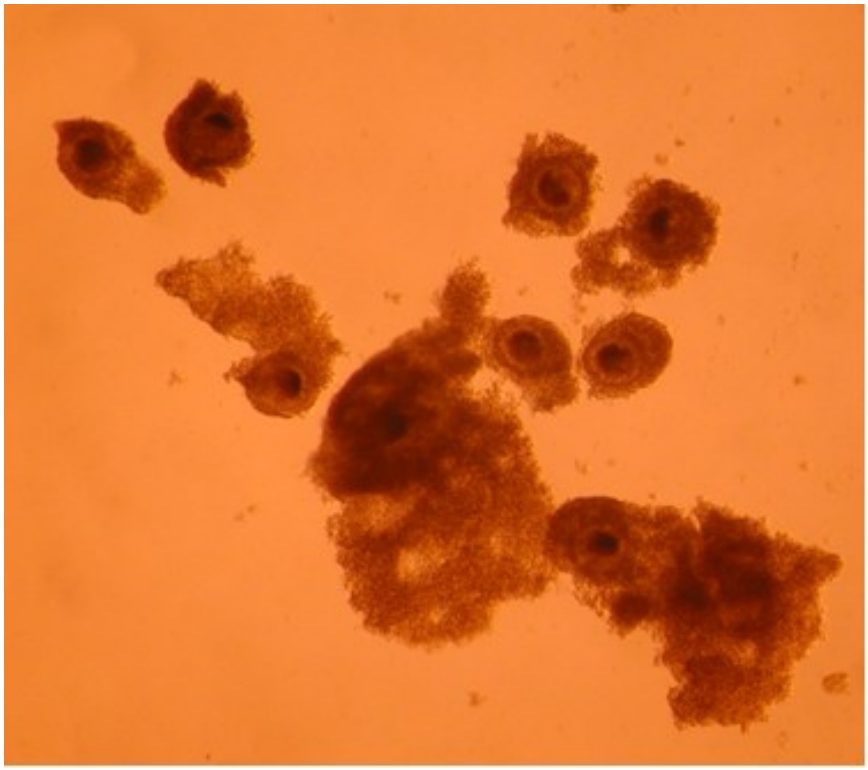
However, this difference in the number of recovered oocytes is expected because of the physiological characteristics of buffalo: lower number of primordial follicles present in the ovary compared to cattle (Misra and Tyagi, 2007), reduced number of antral follicles at all stages of the estrous cycle (Kumar et al., 1997), as well as high incidence of follicular atresia found in slaughtered buffalo ovaries, ranging between 82% (Ocampo, et al. 1994) and 92% (Palta et al., 1998). Nevertheless, for completeness of the topic it is worth to cite that recently Baruselli et al., (2010) reported a number of oocytes recovered per buffalo per OPU session much higher than the average values reported in literature, in a trial in which the interval between sessions was extended to one week (8.5 and 6.1 total and viable oocytes, respectively) and two weeks (10 and 7.2 total and viable oocytes, respectively). The same authors demonstrated that the aspiration at 1, 3 and 5 days after the follicular emergency in heifers gave the same results (Baruselli et al., 2010) but also in this case the average number of follicles, total oocytes recovered and viable oocytes was much higher than in any other report. We speculate that the evident difference in the follicular population may be due to the genetics of the animal, the age (heifers vs adults), the type of breeding and environment, as well as the longer interval between sessions. The latter factor may account for both the greater number of follicles and the lower developmental competence of the oocytes, indicated by the poor blastocyst rate (9 %); indeed, it is known that an extension of the interval increases the number of follicles but also the heterogeneity of the oocyte source, as the phenomena of dominance and atresia in this case occur.

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 (Neglia et al., 2003), with Grade A and B considered suitable for IVEP (Figure 1).

This classification has been recently revised and completed (Di Francesco et al., 2011) as follows:

- Grade A: oocytes with homogeneous cytoplasm and the entire surface surrounded by multiple layers of cumulus cells;
- Grade B: oocytes with homogeneous cytoplasm and with at least 70% of the surface surrounded by multiple layers of cumulus cells;
- Grade C: oocytes with few cumulus cells;
- Grade D: oocytes with obvious signs of cytoplasmic degeneration;
- Naked: oocytes completely free of cumulus cells;
- Expanded: oocytes surrounded by expanded and clustered cumulus cells
- Small: oocytes with a diameter smaller than 120 m;
- Misshapen: oocytes with an abnormal shape;
- ZP: zonae pellucidae.

Figure 1: Grade A and B buffalo oocytes



It is worth pointing out that the percentage of good quality oocytes (Grade A and B) is lower in this species compared to others. An analysis of the data collected over a 4 year period in our lab showed that from a total number of 35.286 abattoir derived oocytes (over 158 replicates) 47.8% were Grade A + B, 6.2 % were Grade C and 46.7 % were unsuitable for IVEP (unpublished data). A recent trial has shown an even lower proportion of good quality oocytes (33.7 % of Grade A+B), together with a higher incidence (37.9 %) of Grade C (Mishra et al, 2008).

Oocyte quality affects early embryonic survival, the establishment and maintenance of pregnancy, fetal development, and even adult disease (Krisher,

2004). The oocyte quality may be affected by several factors, such as the aspiration pressure during collection, the source of gametes, the time between collection and processing, the temperature during transportation, season, etc. In our experience, the oocyte morphology varies with the source of gametes, with an apparent worse quality of OPU-derived oocytes, characterized by fewer layers of granulosa cells, compared to abattoir-derived ones. However, in contrast to what reported in cattle (Lopes et al., 2006), despite their worse morphological appearance, OPU-derived buffalo oocytes have a higher developmental competence, indicated by both increased blastocyst yields (Neglia et al., 2003; Di Francesco et al., 2011) and improved blastocyst cryotolerance compared to abattoir-derived buffalo oocytes (De Rosa et al., 2007).

In vitro maturation (IVM)

An adequate system of in vitro maturation is crucial for in vitro embryo production. During oocyte maturation, nuclear events are accompanied by cytoplasmic and membrane changes that prepare the oocyte for fertilization. Nuclear maturation is completed when oocytes reach metaphase II and can be easily assessed after fixation and staining with aceto-orcein (Gasparrini, 2002). The presence of the chromosomes in MII stage is important at the time of incubation of oocytes with sperm during IVF (Sirard and First 1988). During in vitro maturation, the oocytes must be incubated in a culture medium that allows the accomplishment of the nuclear and cytoplasmic maturation, because the aspirated follicles, either by OPU or aspiration of slaughterhouse ovaries contain immature oocytes.

Buffalo oocytes can be matured in vitro in complex media, such as Tissue Culture Medium 199 (the most widely employed) and Ham's F-10, supplemented with sera, hormones and other additives, such as growth factors and/or follicular fluid (Gasparrini, 2002). Although in vitro maturation can be obtained without the use of hormones, (Madan et al, 1994a) higher maturation and fertilization rates have been recorded when oocytes are matured in the presence of gonadotropins and 17-estradiol (Totey et al., 1992; 1993). It is known that hormones interact with receptors located on the follicular cells, and that the signals are transduced into the oocyte through gap junctions or extracellular mechanisms. It results that the presence of cumulus cells is critical for the acquisition of developmental competence during IVM, as confirmed by the significantly reduced cleavage and embryo development of denuded vs. cumulus-enclosed oocytes following IVF (Pawshe et al., 1993; Gasparrini et al., 2007). This aspect is particularly important in buffalo because of the high proportion of totally or partially denuded oocytes usually recovered in this species.

Moreover, it is known that in vitro mammalian embryo development is negatively affected by the increased oxidative stress occurring under culture conditions. The oxidative damage of cell components via reactive oxygen species interferes with proper cell function. Most mammalian cells possess efficient antioxidant systems such as catalase or superoxide dismutase, as well as thiol compounds that act as metabolic buffers which scavenge active oxygen species (Del Corso et al., 1994). Glutathione (GSH) is a tripeptide thiol compound that has many important functions in intracellular physiology and metabolism. One of the most important roles of GSH is to maintain the redox state in cells, protecting them against

harmful effects caused by oxidative injuries (Gasparrini et al., 2006a). During in vivo maturation of the oocyte the GSH content increases as the time of ovulation is approached (Perreault et al., 1988) creating a reservoir pool which will protect the cell in the later stages of post-fertilization development (Telford et al., 1990). It has also been proven that oocytes are capable of synthesizing GSH during in vitro maturation in several species, such as mouse (Calvin et al.,1986) hamster (Perreault et al.,1988), pig (Yoshida.,1993), cattle (Miyamura et al.,1995), sheep (de Matos et al., 2002), goat (Rodriguez-Gonzalez et al., 2003), and buffalo (Gasparrini et al., 2003). Low molecular weight thiol compounds, such as cysteamine and β-mercaptoethanol, added during bovine in vitro maturation (IVM), improve embryo development and quality, by increasing GSH synthesis (de Matos et al., 1996; de Matos and Furnus, 2000). In buffalo it was previously demonstrated that cysteamine supplementation during IVM improves blastocyst yield (Gasparrini et al., 2000), by increasing intracytoplasmic GSH concentration (Gasparrini et al., 2003), without nevertheless affecting cleavage rate. The addition of cystine, in the presence of cysteamine, to the IVM medium (Gasparrini et al., 2006a) has further increased the GSH reservoir of the oocytes and has significantly improved the proportion of oocytes showing normal synchronous pronuclei post-fertilization (81%), cleavage rate (78%) and blastocyst yield (30%).

Among factors affecting mammalian embryo development in vitro, the duration of IVM also 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).

In vitro fertilization (IVF)

Fertilization has often been considered the most critical step of the IVEP procedures in buffalo, as cleavage rates lower than those obtained in other domestic species have been widely reported (Galli et al., 2001; Gasparrini et al., 2004b; Neglia et al., 2003).

The optimal time for in vitro fertilization coincides with the completion of nuclear maturation of the oocyte, which occurs at different times in different species, ranging from 18-24 hours in cattle (Neglia et al., 2001; Sirard et al., 1989), 36-48 hours in pigs (Prather and Day, 1998). While we have shown large variations in the time of maturation of buffalo oocytes, with the highest percentage of MII oocytes between 16 and 24 hours (Gasparrini et al., 2008; Neglia et al., 2001; Yadav et al., 1997), most authors make in vitro fertilization 24 hours after the start of IVM. It has been recently demonstrated that the duration of IVM affects buffalo oocyte developmental competence, with a progressive decrease of fertilization capability and embryo development as the IVM duration increases from 18 to 30 h (Gasparrini et al., 2008). So the optimal time for fertilization in vitro in buffalo is 18 hours post-IVM and in vitro fertilization should not be done after 24 hours post-IVM.

The most commonly used media for IVF are "Tyrode's Albumin Lactate Pyruvate" (TALP; Boni et al., 1994; Gasparrini et al., 2000; Totey et al., 1992; 1996) or "Brackett Olifant" (Bacci et al., 1991; Chauhan et al., 1997a; Nandi et al., 1998; Madan et al., 1994a; Totey et al., 1992).

The quality of the frozen semen has always been considered the major factor impairing IVF, based on the demonstration of several damages of the male gamete occurring following cryopreservation (Meur et al., 1988), together with the drastic reduction of cleavage rate reported with frozen compared to fresh semen (Totey et al., 1992). Currently, the quality of frozen semen has improved, as indicated by similar fertility parameters, recorded for fresh compared to frozen semen (Wilding et al., 2003), suggesting that other factors may negatively affect fertilization. However, the overall improvement of the quality of cryopreserved sperm has not eliminated another serious impediment, the so-called "bull effect", consisting in the high degree of variation among buffalo bulls in the fertilizing capability in vitro (Totey et al. 1993).

Sperm need to undergo capacitation to acquire the fertilizing ability; this process, which in vivo occurs within the female genital tract, must be induced in vitro. Although several agents have been proven to induce sperm capacitation in vitro, heparin is still the most efficient method in the majority of the domestic species. It is not possible to rule out that the process of capacitation, required by spermatozoa to acquire the fertilizing ability is impaired in the currently used buffalo IVF system. In order to investigate whether the capacitation process in vitro can be improved by agents different than heparin, buffalo sperm have been incubated under different conditions and capacitation has been indirectly assessed by evaluating the capability of sperm to acrosome react following incubation with lysophosphatidilcholine, a fusogenic lipid, known to induce acrosome reaction in capacitated sperm without affecting motility (Boccia et al. 2005; 2006a; 2007). It has been demonstrated that progesterone induces buffalo sperm capacitation in

vitro and may be considered as an alternative capacitating agent for buffalo IVF (Boccia et al., 2006a). Furthermore, it has been shown (Boccia et al., 2007) that sperm treatment with sodium nitroprusside, a well-known generator of nitric oxide in vitro, improves the efficiency of buffalo sperm capacitation in vitro compared to heparin, when the incubation is extended to 2 or 3 h (60.1 vs 44.1 % respectively at 2 h; 68.8 vs 36.6 % respectively at 3h). The most promising results have been obtained by incubating sperm with some biological fluids, such as buffalo estrus serum (BES) and the follicular fluid (FF) recovered from a pool of dominant follicles (Boccia et al., 2005). In fact, sperm treatment with both BES and FF has resulted in a significantly higher incidence of AR sperm than with heparin treatment (84.3, 94.5 vs 50.1 % respectively), regardless of the incubation times. It is likely that factors derived by BES and FF, present in the oviduct environment around fertilization, play a critical role in processing the male gamete in vivo. These results strongly suggest to investigate the effects of BES and FF also on the fertilizing capability of buffalo spermatozoa. Recently it was also reported that treatment with osteopontin in the presence of heparin improved sperm in vitro capacitation in buffalo species (Boccia et al., 2013). Indeed, the addition of OPN to the IVF medium had a positive influence on total penetration, synchronous pronuclei formation and IVEP efficiency as shown by the improvement of blastocyst yields (Boccia et al., 2013).

A positive effect of cumulus cells at the time of IVF has been observed in buffalo, as in cattle, (Zhang et al.,1995), as demonstrated by the higher cleavage rate and embryo development obtained with cumulus-enclosed oocytes vs oocytes that were freed of their cumulus investment (Gasparrini et al., 2007).

Another factor that may affect embryo development is the duration of gamete co-incubation during IVF. 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. It has been recently demonstrated that the optimal sperm-oocyte co-incubation time for maximizing the blastocyst yield in buffalo is 16 h (Gasparrini, 2007). Shortening the gamete co-incubation length to 8 h resulted in a significant reduction of oocyte cleavage, contrary to what reported in cattle. In fact, Kochhar et al., (2003) reported that 6 h gamete co-incubation length allowed the highest proportion of cleaved oocytes to reach the blastocyst stage. On the contrary, extending gamete co-incubation to 20 h was deleterious because, despite similar cleavage rates, the blastocyst production was reduced both when calculated in relation to COCs and to cleaved embryos (Gasparrini, 2007). Furthermore, increasing the sperm-oocyte incubation time to 20 h was found to be correlated to a higher incidence of polyspermy (Gasparrini, 2007; Sumantri, et al., 1997).

The efficiency of in vitro fertilization is also influenced by the proportion of sperm-oocyte incubation. The best results were obtained with a concentration of 2 x 10^6 sperm per ml. This will get satisfactory rates of cleavage (65%), without increasing the incidence of polyspermy that is known to be higher in the presence of a high concentration of sperm (Gasparrini et al., 2002).

In vitro culture (IVC)

After about 24 hours of IVF, the presumptive zygotes should be removed from fertilization medium and transferred to a specific culture medium. The development of in vitro culture systems for buffalo embryos has imitated that for other ruminant species. Buffalo embryos have been co-cultured with cumulus and oviductal cells (Madan et al., 1994; Totey et al., 1992) or with established cell lines such as BRL (Boni et al., 1999). Although many authors still prefer the co-culture system for embryo production in this species, the utilization of defined media for embryo culture has become necessary to comprehend the requirements of buffalo embryos in vitro which, in turn, would allow the formulation of an optimal species-specific culture system.

A defined medium, such as the Synthetic Oviduct Fluid (SOF) has been utilized for embryo culture in this species since 1999 (Boni et al., 1999). Subsequently, buffalo zygotes/embryos have been successfully cultured either in SOF or in another defined cell-free system, known as Potassium Simplex Optimized Medium (KSOM) with similar embryo development (Caracciolo di Brienza et al., 2001). The great improvement of blastocyst yields (35-40%) achieved in the following years is, according to our experience, due to the optimization of the IVM and, in part of the IVF systems rather than to modifications applied to the IVC system. In fact, despite attempts to modify its original composition, at present the original version of SOF remains the most suitable medium for embryo culture in buffalo. It has been demonstrated (Monaco et al., 2006) that, in contrast to sheep and cattle, the presence of glucose is absolutely required for in vitro culture of buffalo

embryos, particularly during the early embryonic development (up to Day 4). In order to reduce the accumulation of free radicals, ammonium and other catabolites that may affect embryo development, it has been suggested to use the easy expedient to change the medium more times during culture. However, no significant differences in buffalo embryo development have been recorded by changing the IVC medium 3 (Day 1, 3 and 5) or 2 (Day 1 and 5) times during culture, with a tendency of improvement in the latter case (Boccia et al., 2006b).

Buffalo embryos in vitro develop approximately 12-24 h earlier than cattle embryos (Galli et al., 2001) and this pattern of development reflects that observed in vivo, with most of the blastocysts collected by uterine flushing in the hatched stage at 6.5 days after the onset of estrus (Drost and Elsden, 1985). On Day 6 (Day 0 = IVF) it is possible to find embryos in advanced stages of development, including hatched blastocysts but most embryos reach the blastocyst stage on Day 7. A small proportion of embryos are delayed, reaching the blastocyst stage on Day 8 but their quality and viability is poor, as demonstrated by their lower resistance to cryopreservation (Gasparrini et al., 2001). The evaluation of cleavage in buffalo, is usually performed on day 5 of culture (day 0 =IVF day), when the embryos are transferred to fresh medium for further 2 days. At 7 days embryo yield is evaluated, and embryos are scored for quality and for stage of development, i.e. compact morulae, early blastocysts, blastocysts, expanded blastocysts and hatched blastocysts.

Reproductive seasonality

The majority of the authors report that buffalo is characterized by delayed puberty, prolonged post-partum ovarian inactivity, long intercalving intervals and a tendency for seasonality (Madan, 1988; Misra and Tyagy, 2007).

Reproductive seasonality in mammals is the consequence of numerous complex elements based on the underlying physiological mechanisms of the adaptation of animals to local environment. Although domestication processes generally reduced seasonality of reproduction compared to what is observed in their wild counterparts, a majority of animal-derived products remain accessible only seasonally (Chemineau et al., 2008). Indeed, also domestic animals commonly show seasonal variations in their reproductive activity. In fact, this characteristic is still present in some genetic types of bovine extensively bred, such as Podolica, Sarda, Maremmana, Bos Indicus and the Highland bovine, but also in the horse, sheep, goat and buffalo (Zicarelli, 1997c).

Buffalo shows a pattern of reproductive efficiency closely related to the environmental and climatic conditions of the area of origin that is the Indo Valley (currently territory of Pakistan and India). Indeed, the buffalo is a short-day breeder but in equatorial zones, where the light/dark ratio varies little during the year, can show estrous cycles throughout the year provided that nutrition is adequate to maintain reproductive function (Seren et al., 1995). Buffaloes become increasingly seasonally polyestrous with increasing distance from the equator (Baruselli et al., 2001a; Campanile et al. 2010; Zicarelli, 1997) and females that calve during the non-breeding season have an extended postpartum anestrous

period, with a proportion not resuming ovulation until the following breeding season (Zicarelli, 2007a). In Italy (latitude between 47°05'29 N and 35°29'24 N), because of the advanced model of farming, in which forage is always available, buffalo is a seasonally polyestrous species, in which the increase of ovarian cyclic activity and, subsequently, fertility coincides with decreasing day-light hours. The improvement of reproductive efficiency with negative photoperiod demonstrates that moving into new areas of domestication and breeding has not modified the sensitivity of the hypothalamic hypophyseal axis to a decreasing light/dark ratio.

Interestingly, despite differences in environmental temperature, rain fall and forage availability, the common feature is that buffalo reproductive activity benefits from a negative photoperiod (Zicarelli, 2007). The influence of photoperiod means that, without intervention, buffaloes have seasonal cycles in conception, calving and milk production (Campanile et al., 2010). The need to make calving and weaning coincides with both the most suitable season, in order to satisfy the heat and nutritive requirements of the offspring, and with the period in which the causal agents of infections and infestations express less pathogenic effect (Zicarelli, 1994a), represents one of the causes of this adaptation process. The individuals born in more favorable conditions have given rise to natural selection of subjects with a seasonal reproductive behavior better suited to the survival of the species, whose reproductive features were probably determined by stimuli incorporated in the central nervous system during gestation or during the first days of life (Zicarelli 1997).

This reproductive pattern depends on the duration and the intensity of the light source, which is captured by the retina (retinal photoneurons) and then

subsequently passes through different neural connections: first, it is developed by the suprachiasmatic nucleus (biological clock which regulates the endogenous circadian rhythm). From this level, the information reaches the superior cervical ganglion and then the pineal gland which is the main regulatory organ in the seasonality of breeding. It has no efferent projections, and therefore it affects neuroendocrine function by humoral means (Cardinali, 1984) producing indoleamins, of which melatonin is the most important. Melatonin is produced and secreted during the night (dark). As days become shorter, the exposure to melatonin increases; this hormone, through a complex action on the hypothalamus-pituitary- gonads axis, simulates the condition of the beginning of estrus (Lincoln, 1992) via exerting a stimulating effect on GnRH secretion by the hypothalamus in short-day breeders. Plasma melatonin concentrations, two hours after sunset, result higher in buffaloes that are more sensitive to photoperiod (Parmeggiani & Di Palo 1994). Furthermore, this feature does not change by moving these animals in places where other females show low plasma melatonin concentrations and less sensitivity towards light stimulation (Di Palo et al. 1997). The importance of this finding is related to the high repeatability (0.733) of plasma melatonin levels (Di Palo et al. 1993), suggesting that it is hereditable and that may be incorporated into genetic selection programs for buffalo (Zicarelli, 1994b).

As it has been previously affirmed, in Italy the main economic reason for buffalo breeding is given by the production of mozzarella cheese, exclusively produced with fresh buffalo milk (Zicarelli, 1992). It has to be observed that in our country the seasonality of the species is a big issue, since it implies that the greater milk production does not coincide with the increased market demand for mozzarella

cheese. To solve this obstacle, and then to produce milk in synchrony with the market requirements, a special procedure was defined and developed , the out of breeding mating season (OBMS; Zicarelli, 2010). It consists in the interruption of sexual promiscuity in the herd between September and December during the first year of application, and from September to March in the following steps of the technique. It allows modifying calving intervals in order to obtain a better distribution of them throughout the year. Currently, the OBMS technique is utilized in more than 60% of the farms in Campania region offering unquestionable economic advantages.

Nevertheless, the efficiency of reproductive technologies is generally reduced during the unfavorable season. Recently, a retrospective study carried out on abattoir-derived ovaries over a long term has showed that season affects the oocyte developmental competence. In fact, although the follicular population and the number of oocytes recovered per ovary is not influenced, a significant increase of both cleavage (71.7 vs 58%) and blastocyst (26.5 vs 18.8%) rates is observed during autumn months compared to spring months, indicating a seasonal effect on oocyte developmental competence, that reflects the in vivo reproductive pattern (Di Francesco et al., 2011).The worsening of oocyte competence during the unfavorable season was also observed in an OPU trial carried out in different seasons; in this case the embryo yields doubled during autumn months (Di Francesco et al., 2012).

Although the majority of studies focused on the female seasonality, it is worth noting that the season may also affect male reproductive activity. The effects are clear when the animals are maintained in natural mating condition, since a higher

pregnancy rate is observed, at our latitudes, between January and April in buffaloes inseminated by AI compared with those naturally mated. This may occur because the AI avoids the negative effect of the bull (Pelagalli et al., 2009).

The function of the male gonads is also influenced by the light and dark hour fluctuation of the day; in fact, the rhythmus of melatonin incretion regulates hypothalamic-hypophyseal activity, gonadal function and finally sperm composition and quality (Zicarelli, 1997). Interestingly, both weight and size of scrotal circumference together with epididymal weight, are slightly but not significantly reduced in the course of the non-mating season when compared to the mating season (Ibrahim, 1985). Even more remarkable is the finding related to the histological evaluation of seminiferous tubules which showed a maintained spermatogenesis during the non-mating season, and a significantly higher epithelial lining, indicative of a possible enhancement of function due to the pressure of sperm accumulation, which is typical of this part of the epididymis (Arrighi et al., 2010). These findings suggest the potential of buffalo bulls to breed throughout the year, although reproductive function is somewhat reduced during the non-mating season, as confirmed by a large variability in semen quality reported among Nili-Ravi, Murrah and Mediterranean Italian buffaloes (Kumar et al., 1993; Presicce et al., 2003; Saeed et al., 1990). These considerable differences may be explained by the lack of long time selection for semen freezability in this species. In addition to semen quality and its freezability, photoperiod has also been reported to affect sexual activity and bull libido (Sansone et al., 2000). Furthermore, to underline the sensitivity to seasonality of the buffalo bull, it has been reported a neuro-endocrine interaction between androgen hormones and the

autonomic nerve supply in the regulation of male buffalo reproductive functions. In fact, during the mating period, a dense noradrenergic innervation can be observed to supply the vas deferens as well as the accessory sex glands, whereas during the non-mating period the noadrenergic nerves are dramatically and significantly reduced (Mirabella et al., 2007). With regard to buffalo bred at Italian latitudes, it is known that the reproductive seasonality in males is characterized by the reduction of the libido (Sansone et al., 2000). It often happens that, in increasing day length months bulls stop mounting and, hence, do not produce ejaculates. This has not made easy to allow the evaluation of semen in relation to the season. Therefore, it is not possible to rule out that ejaculates collected immediately before and/or after this period of silence of the reproductive activity, are sub fertile.

As reported above, the improvement of fertility is a major contribution that the scientific research may provide to buffalo breeding. Several studies have been performed in order to improve the reproductive management of the female (Baruselli et al., 2001a, Campanile et al., 2010; Neglia et al., 2015; Rossi et al., 2014), whereas few experiences were carried out regarding the male effect on reproductive efficiency. The selection of the males for breeding in many domestic species is performed on clinical and laboratory evaluation that allow examining the function of the male genital tract and, therefore, to exclude subjects clearly characterized by hypofertility. However, laboratory test routinely utilized in sire centers are not able to reveal the level of fertility of the semen, in particular after AI. It is known that an accurate estimation of the fertility of an ejaculate may be obtained by carrying out several tests in vitro (Petrunkina et al., 2007).

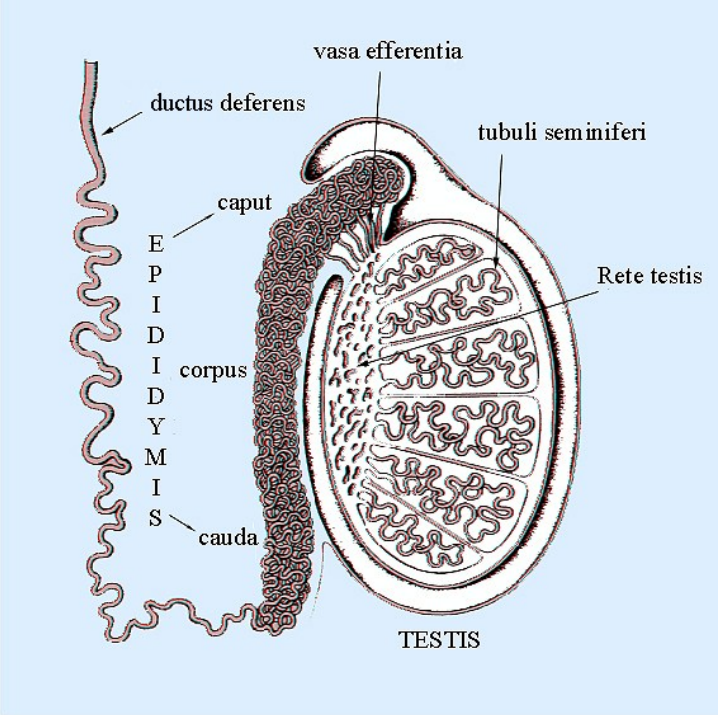
Furthermore, it is possible that semen, judged suitable on the basis of fertility parameters tested in the laboratory, gives poor results after IVF.

It is worth to point out that the diffusion in the field of AI and IVEP technology in buffalo and the possibility for our country to be competitive in the international market as "genetic producer" are strongly penalized by the quality of frozen-thawed semen. Therefore, as the aim of the thesis was to develop strategies to improve semen quality and cryotolerance, it is worth providing an updated dissertation on the topics of sperm physiology and cryopreservation, with a particular emphasis on buffalo species.

Semen physiology

Spermatozoa are produced in the testis, outside the abdominal cavity in a pouch of skin called the scrotum. The scrotum protects the testicle against extremes of temperature (Setchell, 1998). This is essential for normal sperm formation, which occurs only at a temperature several degrees below normal body temperature. Each testis is constituted of seminiferous tubules containing spermatogonia, which are responsible for the production and the differentiation of spermatocytes into spermatids and finally into spermatozoa. This process takes several days and is called spermatogenesis. After formation, spermatozoa are transported into larger tubules to form the rete testis at the center of the testis. Arising from the rete testis are 12 or more out-going ducts, the vasa efferentia, which emerge from the testis and enter the epididymis (Figure 2).

Figure 2: Testis and epididymis of the bull



The epididymis is a compact, flat, elongated structure closely attached to one side of the testicle. In the bovine it may cover a total length of 40 meters (Hoskins et al., 1978). The epididymis is anatomically divided into three major portions: caput (head), corpus (body) and cauda (tail) (Figure 2) and by histological criteria, several further subdivisions may be recognized (Cosentino and Cockett, 1986). Spermatozoa are transported further along the ductus deferens. The ductus deferens is a muscular tube, which propels the spermatozoa from the cauda epididymidis to the urethra at the time of ejaculation. The accessory genital glands (seminal vesicles, prostate and bulboutheral glands) are found in the region where the left and right ductus deferens unites into the urethra. These glands produce the secretions that make up most of the liquid portion of the semen of the bull. At ejaculation, the opening between the bladder and the beginning of the urethra

closes. At the same time the spermatozoa and the accessory glands secretion mix together and enter the urethra to be forced out of the external opening at the apex of the penis. The volume of semen and the number of spermatozoa ejaculated by different bulls varies considerably. However, most bulls will ejaculate 3 to 5 ml of semen containing 1 billion spermatozoa per ml, or 3 to 5 billion spermatozoa per ejaculate. Specific characteristics of spermatozoa confer to them the peculiarity of being a "terminal cell".

The spermatozoon is a haploid cell, almost devoid of cytoplasm and other cellular organelles, except for the nucleus, the acrosome, and a series of mitochondria in an end-to-end helical arrangement located at the anterior region of the flagellum (Eddy and O'Brien, 1994). In the nucleus, the chromosomes are highly condensed and thus inhibit any transcriptional activity to replace proteins. The acrosome allows the spermatozoon to interact with and penetrate the oocyte at fertilization. The mitochondria provide ATP, which is mostly used to maintain motility. Spermatozoa lack the intracellular transport and storage systems of the endoplasmic reticulum and Golgi apparatus. To maintain functional cell membranes and to undergo maturational changes that confer to the sperm motility and ability to fertilize, sperm rely on the absorption of molecules from the surrounding environment (Amann et al., 1993; Yanagimachi, 1994).

Semen cryopreservation

Cryopreservation is a non-physiological method that involves a high level of adaptation of biological cells to osmotic and thermic shocks that occur during

dilution, cooling–freezing and thawing procedures (Holt 2000a, b; Watson et al., 1992). Immediately after collection, the semen is diluted in an appropriate medium, often based on egg yolk or milk, although there is a market for novel extenders containing no animal-derived components. Traditionally, sperm are diluted at a temperature close to body temperature (30 to 39°C) then cooled to 5°C at which point a cryoprotectant (i.e. glycerol) is added (Polge et al., 1949). For most domestic species, cooling from body temperature to near freezing represents a major stress to the sperm plasma membrane resulting in the rearrangement and destabilization of membrane components, and calcium influx (Collin et al., 2000; de Leeuw et al., 1993; Maxwell and Johnson, 1997; Noiles et al., 1995). White (1993) speculated that this increased permeability is due to irreversible structural changes to the membrane architecture caused by cooling below the thermotropic phase transition temperature of membrane phospholipids. At the transition temperature, there is an abrupt change from the gel to the liquid-crystal phase of membrane phospholipids, and this would lead to irreversible structural changes to the membrane architecture and accounts for this increased permeability. Since sperm membranes are composed of many phospholipids in a species-specific manner (White, 1993), with each phospholipid having a precise phase transition temperature, the degree of structural damage depends on the temperature and the lipid composition of the membrane.

The plasma membrane is the primary site of injury to cryopreserved sperm and the principal damage occurs during freezing and thawing (Hammerstedt et al., 1990; Parks and Graham, 1992). As sperm are frozen, ice crystals form in the extracellular medium, increasing the osmolality of the unfrozen water.

Intracellular water diffuses out of the sperm in response to this osmotic gradient, thus dehydrating the cell and plasma membrane. At thawing, the phenomenon is repeated in reverse as the extracellular ice crystals melt and water diffuses into the sperm. Ultrastructural deformation of the plasma membrane occurs as a consequence of osmotic stress and drastic change in volume during freezing and thawing. The inclusion of a cryoprotectant, commonly glycerol, for mammalian sperm, is essential for cell survival during cryopreservation. The efficacy of glycerol is partially due to its osmotic effects that dramatically increase the osmolality of the medium, which retards ice formation (Hammerstedt et al., 1990). The osmotic effects of glycerol are marked, but transitory, and such rapid change in cell volume does not appear to be detrimental to sperm function (Liu and Foote, 1998). Furthermore, glycerol acts directly on the sperm plasma membrane, possibly to reduce some of the phase transitions, and to increase membrane fluidity during cooling (Noiles et al., 1995).

Moreover, during cryopreservation, the generation of reactive oxygen species (ROS) by several potential sources occurs. Several reports indicate significant physiologic roles of ROS during normal sperm function, including hyperactivation, capacitation and the acrosome reaction, and zona binding (Kodama et al., 1996; de Lamirande et al., 1997). Conversely, when the balance between ROS production and detoxification by antioxidants is disrupted, an excess of ROS creates oxidative stress. ROS such as H_2O_2 are known to arrest motility and block oxidative metabolism in sperm (Tosic, 1947). ROS also decrease oocyte penetration by sperm and block sperm-egg fusion in the mouse via a mechanism involving oxidation of sperm -SH groups (Mammoto et al., 1996). Sperm DNA

damage by ROS has been reported too, which has serious consequences for post fertilization development (Aitken et al., 1998). The generation of ROS by several potential sources occurs during cryopreservation. Increased ROS production by both human sperm and seminal leukocytes during cooling to 48°C was reported by Wang et al., (1997). In somatic cells, ROS leakage from the mitochondrial respiratory chain has been evaluated at 2 to 5% (Boveris, 1977), and this is probably a major source of ROS produced by sperm. Contaminating leukocytes in the semen can produce large amounts of ROS if stimulated (Krausz et al., 1992). Aitken et al., (1997) hypothesized that an NADH/NADPH oxidase could exist on sperm as occurs on many other cell types. Specifically, the activation of an aromatic amino acid oxidase following the death of ram and bull sperm has been identified as a major source of ROS production in the semen of these animals (Tosic, 1947; Upreti et al., 1998). The release of this oxidase from dead sperm in egg yolk extender reduces the motility and viability of the remaining living bull sperm (Shannon and Curson, 1972).

Recent studies indicate that cryopreservation decreases the antioxidant defenses of whole semen. Loss of superoxide dismutase activity occurs in cryopreserved human and bull semen when compared with fresh semen (Lasso et al., 1994). Moreover, cryopreservation reduced bull sperm glutathione levels by 78% (Bilodeau et al., 2000).

Capacitation-like changes due to cryopreservation

Watson (1995) suggested that cryopreservation-induced modifications to sperm membranes make them more reactive to their environment after thawing such that cryopreserved sperm are in a partially capacitated state. Indeed, numerous groups have reported that cooled or cryopreserved sperm exhibit capacitation-like behavior. It was observed that freezing and thawing reduced the time required for sperm to penetrate zona-free hamster eggs (Byers et al., 1989). Pérez et al., (1996) showed that cryopreserved ram sperm undergo capacitation more quickly than fresh controls as assessed by reactivity to calcium ionophore and the chlortetracycline (CTC) fluorescent assay. CTC fluorescence also revealed a greater proportion of pattern B (‘‘capacitated’’; Ward and Storey, 1984) sperm due to cooling and immediately after thawing in mice (Fuller and Whittingham, 1996), bull (Cormier et al., 1997), ram (Gillian et al., 1997), and boar (Maxwell and Johnson, 1997). Furthermore, sperm were confirmed to be functionally capacitated by cooling or cryopreservation, as reflected by their ability to fertilize homologous oocytes in vitro without any pre-treatment normally required by uncooled sperm (Cormier et al., 1997; Fuller and Whittingham, 1997).

The mechanism by which cryocapacitation occurs is not fully understood and is complicated by the fact that the onset of normal capacitation has yet to be elucidated. Capacitation of mammalian sperm is associated with reorganization of the head plasma membranes due to phospholipid redistribution and cholesterol removal (Langlais and Roberts, 1985; Lin and Kan, 1996). Watson (1995) speculated that cryopreservation introduces a sub-lethal modification of the sperm

membranes, which makes them more sensitive to the environment after thawing. As outlined above, cooling and cryopreservation also modify sperm membrane architecture and behavior. Similarities in the membrane changes associated with capacitation and cooling or cryopreservation are evident, based on the increased incidence of sperm displaying the CTC pattern B after either of these phenomena. Furthermore, based on flow cytometric analysis of CTC stained sperm, Maxwell and Johnson (1997) suggested that pattern B reflects membrane destabilization and calcium influx to the cell. Thus, the CTC pattern B fluorescence induced by cooling and cryopreservation appears to be related to sperm capacitation in the functional sense, as these treatments favor sperm fertilization in vitro. In bovine sperm, an increase in intracellular calcium accompanies heparin-induced capacitation in vitro (Parrish et al, 1999), although its mechanism of entry is unclear. As previously described, restructured membranes and altered lipid-protein associations are thought to favor calcium influx during cryopreservation. Visconti et al., (1998) speculated that during capacitation, membrane modifications stimulate adenylyl cyclase to initiate cyclic adenosine monophosphate–mediated tyrosine phosphorylation of sperm proteins. Furthermore, elevated sperm calcium levels are thought to trigger an intracellular signaling cascade that has recently been associated with capacitation. To test whether cryopreservation induces the signal transduction events associated with capacitation, the presence of phosphotyrosine-containing proteins was evaluated in bull sperm immediately after thawing. In support of this hypothesis, a series of tyrosine phosphorylated proteins was evident in cryopreserved sperm immediately after thawing (Bailey and Bérubé, 1998). In contrast, fresh bovine sperm require incubation for at least 4

hours with heparin for these phosphorylated proteins to be visualized (Galantino-Homer et al., 1997).

Indeed, a study on protein tyrosine phosphorylation revealed that fresh buffalo spermatozoa undergo spontaneous capacitation-associated tyrosine phosphorylated protein at 0 h of incubation compared to 4 h in cattle (Roy and Atreja, 2009).

Moreover, in vitro capacitation and tyrosine phosphorylation of sperm proteins are correlated with superoxide anion generation in human sperm (de Lamirande et al., 1998). Conversely, both superoxide dismutase and catalase inhibit capacitation and tyrosine phosphorylation (Leclerc et al., 1997). It is tempting to speculate that cryocapacitation is at least partly induced by ROS activity generated during sperm processing.

Evaluation and quality of buffalo semen

Collection

In buffalo, semen is usually collected by using an artificial vagina (AV), whereas electro-ejaculation is very rarely used (Nordin et al., 1990). However, the use of AV, in this species, is mainly restricted to those bulls kept at the AI centers, since they need to be well trained to mount a restrained cow or teaser, training that often takes a long time (Bhosrekar et al., 1992). Semen collection using AV is therefore difficult, if not impossible, under field conditions. The ideal method to assess the fertility of a breeding male, other than its ability to produce pregnancy, is by semen examination. Standards for semen evaluation can be established, and any deviations can be recognized and correlated with fertility. Minimal standards for a

classification of “probably fertile” specimen of buffalo semen are: a) over 500.000/mm^3 spermatozoa; b) more than 60% of motile sperm with forward progression and c) more than 70% of the spermatozoa conform to normal morphology. Semen evaluation should be rapid and effective so that carefully collected samples can be processed to preserve initial quality and fertility. No single test has been developed as accurate predictor of the fertility of individual ejaculates, but the combination of several tests could allow selecting a potential higher fertility ejaculate. In particular, special tests applied soon after semen collection may predict if the sample is adequate for freezing and for artificial insemination or if they can be selected to apply joined tests for utilization in in vitro fertilization (Barnabe, 1997). Spermatozoa are highly specialized cells that are well designed to accomplish a single objective: fertilization of an oocyte. To achieve fertilization, semen must have enough spermatozoa with progressive motility to gain access to the egg. During their transport in the male and female reproductive tracts, spermatozoa encounter different environments in which they must survive and develop the capacity of progressive motility as the morphology and metabolic function of mature spermatozoa. Accurate determination of the number of spermatozoa per ml of semen is extremely important and when combined with the volume of the ejaculate, it is possible to predict how many females can be inseminated, each with the optimal number of sperm cells.

Semen evaluation and characteristics

Immediately after semen collection the physical characteristics of the ejaculate are recorded, including volume, color, consistency and pH, followed by assessment of motility, concentration, morphology and the other parameters that relate to semen quality.

The color depends upon variation in sperm cell concentration, thus certain degrees of color or density reflect different concentration of sperm cells in the ejaculate, which is affected, among other factors, by frequency of ejaculation. Generally the normal color of buffalo semen is variable from white, milky to white or creamy (Vale, 1997). Volume of buffalo ejaculate is measured immediately following semen collection through graduate collection tubes and it is less than in cattle. The volume is variable according to the breed and the age. Young bulls at puberty start with a semen volume around 1 ml per collection that increases until 3 ml after they reach sexual maturity, while old bulls can ejaculate 6 ml or more per collection. Attention must be paid in case of testicular degeneration caused by heat stress, when not only the volume but also the other semen attributes tend to be altered.

Concentration is the sperm density commonly estimated per mm^3 of the semen volume. Normal buffalo sperm concentration shows a wide range of variation (600.000 to 1.200.000 cells per mm^3 i.e. 800.000 per mm^3 on average) and this parameter is highly sensitive to seasonal and nutritional factors. First ejaculate contains higher number of spermatozoa per ml compared to second ones. There are many different methods to assess sperm concentration; however, the two most used are the haemocytomer and photo-electric colorimeter methods.

Buffalo spermatozoa have distinct morphological features. They are shorter than that of Bos Taurus and measure about 62 microns. The mean head length, maximum head breadth and breadth at the base of the head are reported to be 7.20, 4.45, 2.40 microns, respectively. Head area is between 24.3 and 26.6 sq. microns and head shape index 1.62. Evaluation of sperm morphology in buffalo bulls is usually done using phase contrast light microscopy to examine wet smears of unstained buffered formalin solution-fixed spermatozoa (Gopalakrishna & Rao, 1978; Jainudeen et al., 1982; Kunavongkrit & Bodhipaksha, 1978; Mathias & Yusuf, 1985), or light microscopy for smears stained either with eosin nigrosin (Ahmad, et al., 1987; Jainudeen, et al., 1982) or with carbol-fuchsin-eosin (Kunavongkrit & Bodhipaksha, 1978). Transmission (TEM) and scanning (SEM) electron microscopy have also been used to describe sperm abnormalities in riverine buffalo bulls (Azmi et al., 1990; Bawa et al., 1993; Tripathi et al., 1975) but not in Swamp buffaloes. The percentage of abnormal sperm in the buffalo ejaculate is an important point to be observed if the aim is to freeze the semen. Morphological study of spermatozoa is done through preparation of smears from freshly collected semen. The smears should be stained through the use of suitable staining techniques. The various types of structural abnormalities associated with head, middle piece, and tail are recorded. Those abnormalities may either be hereditary or linked to developmental and environmental aspects and can be classified as in cattle according to their source and nature.

Freshly collected buffalo semen usually displays a wide range of pH (6.5 to 7.2). Although buffalo ejaculate has a lower concentration of sugars, the formation of lactic acid can decrease the pH of semen. In addition, the seasonal and climatic

factors have a strong effect on the morphological and chemical seminal characteristics. In temperate regions, it has been reported that better quality semen is produced during the winter and spring while it has been shown deterioration in semen quality during summer and autumn. On the other hand, in tropical regions the quality of semen is found to be good during the rainy season. Moreover, in warm and humid tropical Amazon region, the best time to work with semen freezing is between January and June. Buffaloes are sensitive to heat stress, thus decline in semen quality is a common finding during the hot season of the year. The best manner to overcome the problem of semen quality deterioration due to heat stress during summer is to sprinkle the animals with water during the hotter part of the day or allow the animals to wallow, protect the animals from radiation exchange and hot wind, and keep the animals in ventilated paddock. Free access of the animal to water and shadow is very important, since buffalo have a poorly-developed thermo-regulatory system, causing them to suffer of heat stress during summer.

Sperm motility can be examined by using wet smears immediately after semen collection. A small drop of semen is placed on a clean and dry glass slide maintained at 37°C and then covered with a cover slide, to be examined by light microscopy (preferably with phase-contrast optics) at 40 or 100 magnification. Initial motility varies with the frequency and season of collection. Usually when the routine semen collection is done twice a week, first and second ejaculates show good motility, with more than 60% of sperm cells with normal progressive rectilinear motility. It is also worth to evaluate the vigor or individual motility, which is scanned for the field with the highest number of motile sperm cells. One

sperm must be followed until it disappears from the area. This operation should be repeated many times. A good ejaculate must have more than 60% of individual motility.

Cryopreservation of buffalo semen

The first successful freezing of buffalo semen was reported by Roy et al., (1956) and Basirov (1964) was the first to report the pregnancy with frozen–thawed buffalo spermatozoa. Buffalo spermatozoa are more susceptible to hazards during freezing and thawing than cattle spermatozoa, thus resulting in lower fertilizing potential (Raizada et al., 1990; Andrabi et al., 2008). Freezing–thawing of buffalo spermatozoa causes considerable damage to motility apparatus, plasma membrane, and acrosomal cap (Rasul et al., 2000) and leakage of intracellular enzymes (Dhami and Kodagali, 1990). Recently, it was reported a high percentage of sperm capacitation-like changes induced by cryopreservation in buffalo (Elkhawagah et al., 2014). Moreover, a significant seasonal variation was observed in sperm kinematics and hypoosmotic swelling (HOS) reactivity in Murrah buffalo (Mandal et al., 2003). In particular, sperm dynamics were higher during summer and rainy season and significantly lower in winter season. Seasonal changes did not appear to cause deleterious changes in sperm quality in swamp buffalo AI-sires in tropical Thailand (Koonjaenak at al., 2007a). However, another study reported that post-thaw plasma membrane stability and plasma membrane integrity, assessed by flowcytometry, improved in sperm samples processed during winter (November-February) than in samples processed during the other seasons of the year, i.e. the rainy season (July-October) and summer (March-June; Koonjaenak et al., 2007b).

Furthermore, it was demonstrated that frozen–thawed swamp buffalo sperm chromatin integrity is not seriously affected by the seasonal variations in temperature and humidity seen in tropical Thailand (Koonjaenak et al., 2007d). In the Mediterranean Italian buffalo bulls, Di Francesco (2011) reported a seasonal effect on the post-thaw motility, which increased during autumn.

Due to the poor fertility rate with frozen–thawed semen, AI in buffalo remains still unpopular (Muer et al., 1988; Andrabi et al., 2001; Ahmad et al., 2003; Senatore et al., 2004; Kumaresan et al., 2005, 2006; Shukla and Misra, 2007). However, few scattered reports indicate a pregnancy rate of approximately 60% (Tomar and Singh, 1970; Akhter et al., 2007, Campanile et al., 2013). Villa and Fabbri (1993) conducted two AI trials in farms located in three Italian provinces using semen frozen in Laiciphos® (IMV) extender and doses of insemination containing 8×10^6 live spermatozoa. The high variation in the conception rates (30.5–57.1%) found in buffalo cows inseminated by the same method in all farms indicated a strong influence of environmental factors. According to Vale (1997), a pregnancy rate higher than 50% can be regarded as a good result after insemination with frozen–thawed buffalo semen.

It is worth noting that the quality of the frozen semen is considered a major factor impairing also in vitro fertilization, based on the demonstration of several damages of the male gamete occurring following cryopreservation (Meur et al., 1988), together with the drastic reduction of cleavage rate reported with frozen compared to fresh semen (Totey et al., 1992). Although the quality of frozen semen has improved over the years (Wilding et al., 2003), it is widely recognized that there is still room for further improvements.

Strategies to improve semen quality and cryotolerance

Protection of the sperm surface and DNA by antioxidants

Uncontrolled overproduction of ROS and the resulting oxidative stress has become a serious problem in matters related to male fertility (Agarwal et al., 1994). High concentrations of ROS produced by spermatozoa (Aitken and Clarkson, 1987; Alvarez et al., 1987) or by the combinations of xanthine plus xanthine oxidase (Aitken et al., 1993) or of iron (Fe^{2+}) plus ascorbic acid (Aitken et al., 1989) induce the formation of toxic lipid peroxides (Jones et al., 1979; Windsor et al., 1993) and compromise sperm viability. Lower concentrations of ROS cause a reversible loss of sperm motility that is due to intracellular ATP depletion and insufficient axonemal protein phosphorylation (de Lamirande and Gagnon, 1992a;b), causing a decrease in sperm viability and an increase in middle piece morphology defects, with deleterious effects on sperm capacitation and acrosome reaction, ultimately leading to infertility.

Studies have shown that antioxidants protect spermatozoa from ROS produced by leukocytes, prevent DNA fragmentation, improve semen quality, reduce cryodamage to spermatozoa, block premature sperm maturation and provide an overall stimulation to the sperm cells (Agarwal et al., 2007). Spermatozoa and the seminal plasma contain several antioxidants, which provide protection against the toxic effects of free radicals (Alvarez & Storey, 1983; Jeulin et al., 1989; Nissen & Kreysel, 1983). However, following the freeze–thawing process, this antioxidant system fails in protecting spermatozoa against oxidative damage and the toxic effects of free radicals (Bilodeau et al., 2000; Chatterjee et al., 2001). Due to these

reasons, with an aim to reduce oxidative damage and the toxic effects of free radicals during the freeze-thawing of sperm, semen extenders have been supplemented with various antioxidant compounds, and thereby, post-thaw sperm parameters improved (Bucak et al., 2010; Uysal & Bucak, 2007).

Earlier studies revealed that controlling oxidation by exogenous antioxidants in the extender, such as catalase, greatly helped to maintain sperm quality (Foote, 1967). Moreover, inclusion of natural antioxidants had a protective effect on metabolic activity and cellular viability of cryopreserved bovine sperm (Beconi et al., 1991; 1993). However, the addition of cysteine or EDTA (sperm membrane stabilizer and capacitation inhibitor) in semen diluents was not effective to reduce enzyme leakage (lactate dehydrogenase) from buffalo spermatozoa during freezing (Dhami and Sahni, 1993). In another study the inclusion of ascorbic acid (2.5 mM) in the semen diluent increased post-thaw motility and survivability (Singh et al., 1996).The antioxidant effect of ascorbate is related to direct vitamin E regeneration by reducing the tocopheroxyl radical in the one-electron redox cycle (Dalvit et al., 1998; Packer et al., 1979). Later on, Kolev (1997) evaluated the effect of vitamins A, D and E in extender on motility, survivability and acrosomal integrity of cryopreserved buffalo bull spermatozoa, showing a beneficial effect with 0.3 mg/mL vitamin E. It is well-known that α-tocopherol inhibits lipid peroxidation (LPO) in biological membranes, acting as a scavenger of lipid peroxyl and alkoxyl radicals, thus preventing oxidative damage in cryopreserved bovine semen (Beconi et al., 1991). Fabbrocini et al., (2000) demonstrated that the addition of sodium pyruvate (1.25 mM) to the extender for freezing buffalo spermatozoa, improves post-thaw progressive motility and viability. The

beneficial effect of pyruvate and a-ketoacids is attributed to its antioxidant property. It was also reported that the addition of n-propyl gallate (15 μM) was beneficial on post-thaw motility and viability of buffalo spermatozoa (Shukla and Misra, 2005). It is known that n-propyl gallate protects against oxidation by hydrogen peroxide and oxygen-free radicals, in a catalytic manner by converting hydrogen peroxide into water and oxygen.

Recently, taurine and trehalose were supplemented to freezing extender of bull (Chen et al., 1993; Sariozkan et al., 2009), buffalo (Kumar et al., 2013), boar (Hu et al., 2009), ram (Bucak et al., 2007), goat (Atessahin et al., 2008), dog (Martins-Bessa et al., 2009) semen to improve post-thaw sperm characteristics. Trehalose and taurine act as nonenzymatic scavengers playing an important role in the protection of spermatozoa against ROS (Bansal and Bilaspuri, 2011). Moreover, Bukac et al., (2014) reported that lycopene and resveratrol-supplemented extenders improved sperm motility, mitochondrial activity and DNA integrity of bovine sperm.

Therefore, all these studies suggested that the strategy to include antioxidants in the composition of buffalo semen extender may be successfully pursued and hence, provided the rationale of part of this work. Among different candidate molecules with antioxidant properties our attention was drawn to L-carnitine and resveratrol for reasons reported below.

Carnitine is a vitamin-like compound, present in the form of L and D isomers; however, only the L isomer of carnitine is biologically active, while the D isomer may even be noxious for the organism (Szilagyi, 1998). L-Carnitine (β-OH-γ-N-trimethylaminobutyric acid) is a water-soluble vitamin like amino acid that occurs

naturally in microorganisms, plants, and animals (Bremer, 1983; Vav and Wanders, 2002). Its concentration in animals varies widely according to species, tissue type, and nutritional status of the animal (Rabie and Szilagyi, 1998). L-Carnitine is biosynthesized in the kidneys and liver from lysine and methionine in the presence of ferrous ions and three vitamins including ascorbate, niacin, and pyridoxine (Bieber, 1988; Leibetseder, 1995; Rebouche, 1991).

L-Carnitine is a powerful antioxidant able to reduce the availability of lipids for peroxidation by transporting fatty acids into the mitochondria for β-oxidation to generate ATP energy (Matalliotakis et al., 2000; Rani and Panneerselvam, 2002). This reduces the amount of lipids available for peroxidation (Kalaiselvi and Panneerselvam, 1998). Furthermore, L-carnitine, through its antioxidant properties, has been shown to increase the activity and levels of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase in the plasma (Neuman et al., 2002). L-Carnitine is also known to fulfill important roles in mammalian sperm maturation and metabolism when spermatozoa pass through the epididymis (Yakushiji et al., 2006). In fact L-carnitine is found in mammalian epididymis and spermatozoa. Epididymal cells and spermatozoa derive energy from carnitine that is present in epididymal fluid (Ford et al., 1990). A commercial study has also reported that L-carnitine can increase the number of viable spermatozoa and the number of AI doses (Yeste et al., 2010). Conversely, Kozink et al., (2004), who studied the effects of L-carnitine supplement on boar semen characteristics and the maintenance of sperm motility during 7-day liquid storage, observed beneficial effects of such supplements only when the boars were submitted to an intensive collection period in the first 3 days of the study, whereas

no significant effects on semen characteristics either in young or in mature boars were reported at the normal collection rate. Carnitine has been claimed to be involved in the acquisition of sperm motility, and in human beings, the uptake of carnitine into spermatozoa and its conversion into acetylcarnitine is considered evidence of good epididymal function (Stradaioli et al., 2004). It is taken from the blood stream and then released in epididymal lumen by active transporters (Enomoto et al., 2002), which are regulated by androgens and depletion of epididymal carnitine causes a reduction in fertilizing capacity of hamsters spermatozoa (Lewin et al., 1997; Stradaioli et al., 2004). Carnitine and acetylcarnitine modulate many sperm metabolic functions (Jeulin and Lewin, 1996) such as the β-oxidation of fatty acids, the intramitochondrial acetyl CoA/free CoA ratio, which provide readily available acetyl groups and increase the utilization of pyruvate and lactate as energetic substrates in mature spermatozoa (Stradaioli et al., 2004). In humans, rams, and stallions, seminal carnitine is correlated with spermatozoa count and progressive motility (Brooks, 1979; Stradaioli et al., 2000; Zöpfgen et al., 2000). It has also been demonstrated in the rooster that L-carnitine was able to increase sperm concentration and to reduce lipid peroxidation (Neuman et al., 2002); while in boars it improved the seminal characteristics (Baumgartner, 1998).

Resveratrol (3,5,40-trihydroxystilbene), a nonflavonoid polyphenol found in grapes, berries, peanuts, as well as in red wine (Savouret and Quesne, 2002) plays an important role as an antioxidant and acts as an effective scavenger of free radicals (superoxide anion, hydroxyl radical and metal-induced radicals) (Leonard et al., 2003; Willcox et al., 2004). Besides known cardio protective effects,

resveratrol exhibits anticancer properties: it suppress cell proliferation, has a growth inhibitory effect, potentiate apoptotic effects of cytokines, chemotherapeutic agents and ionizing radiation as reviewed by Aggarwal et al., (2004).

Regarding male fertility, recent in vivo studies in animal models demonstrated that resveratrol administration enhances sperm production in rats by stimulating the hypothalamic–pituitary– gonadal axis without inducing adverse effects (Juan et al., 2005). It may have also a positive effect by triggering penile erection and by enhancing blood testosterone levels, testicular sperm count and epididymal sperm motility, as demonstrated in rabbits (Shin et al., 2008). A protective effect of resveratrol against oxidative damage but not against the loss of motility induced by the cryopreservation of human semen has recently been observed as well (Garcez et al., 2010). Tvrdá et al., (2012) demonstrated that the addition of resveratrol could be beneficial for the overall stimulation of spermatozoa activity and protection against possible in vitro oxidative stress development. Resveratrol also exhibits a protective effect against lipid peroxidation and DNA damage caused by free radicals in sperm cells (Branco et al., 2010; Collodel et al., 2011; Revel et al., 2001). Moreover, Bucak et al., (2014) reported that the supplementation of resveratrol to the semen extender produced a higher mitochondrial activity and a lower percentage of sperm with damaged DNA.

Stabilization of the sperm surface with cholesterol and other lipids

Sperm sensitivity to cold shock damage is determined by membrane phospholipid composition as well as the membrane cholesterol: phospholipid (C/P) ratio (Holt, 2000a). Sperm possessing high C/P ratio (rabbit and human sperm) are more resistant to the cold shock damage than sperm which have low cholesterol: phospholipid ratios (boars, stallions, rams, and bulls; Pamornsakda et al., 2011). The integrity of the plasma membrane is important for the spermatozoa to withstand harmful effects of the cryopreservation process. Cholesterol efflux from the plasma membrane is a modification thought to be essential for fertilization (Parinaud et al., 2000), but that also increase the fragility of the sperm surface, rendering it more sensitive to handling imposed deterioration (Van Gestel et al., 2005b). Similar to capacitated spermatozoa, frozen–thawed spermatozoa also show some signs of membrane lipid modification such as higher membrane fluidity, partial phospholipid scrambling (Thomas et al., 2006) and loss of polyunsaturated fatty acids and cholesterol (Chakrabarty et al., 2007; Maldjian et al., 2005). It has long been known that specific vesicle additives can stabilize spermatozoa and protect them from freeze–thaw damage (Holt & North 1988; Watson, 1981). The addition of certain lipid mixtures, including cholesterol (Moore et al., 2005), appeared to be the best way to prevent deterioration, and more recently, cholesterol-loaded cyclodextrins (CLC; molecules with specific affinity for free sterols; Shadan et al., 2004) have been used (Moce et al., 2010, Serin et al., 2011). Cholesterol is a hydrophobic molecule and is not soluble in aqueous semen diluents. Cyclodextrins have been used to insert or remove

cholesterol from synthetic and cell membranes. Cyclodextrins are cyclic oligosaccharides obtained by the enzymatic degradation of starch, and they possess an external hydrophilic face and an internal hydrophobic core (Dobziuk, 2006) that can encapsulate hydrophobic compounds such as cholesterol. Such approaches led to increased cellular levels of cholesterol by monomeric transfer of these lipids from their carriers and spermatozoa pre-loaded with cholesterol are more stable during freeze–thawing than those that have not been pre-loaded (Aksoy et al., 2010; Moraes et al., 2010; Moce & Graham, 2006; Oliveira et al., 2010). The responsiveness of the surviving cells to in vitro capacitating treatments was questioned but studies in the bull (Purdy and Graham, 2004a) and stallion (Spizziri et al., 2010) indicate that CLC-treated spermatozoa remain fertile.

Aim of the work

Considering the aforementioned introduction, this thesis has been articulated in two parts. The Experiment 1 was carried out to investigate the effect of season on fresh and frozen-thawed semen at our latitude. In particular the influence of photoperiod on volume, concentration, motility, and viability and capacitation status of buffalo sperm was assessed. Furthermore the influence of season was also investigated on the fertilizing ability of buffalo frozen-thawed sperm, after heterologous IVF.

In the second part of the thesis, aim of the work was to develop strategies to prevent or reverse semen cryocapacitation damages during cooling and cryopreservation. Therefore, with an aim to reduce oxidative damage and the toxic effects of free radicals during the freeze-thawing of sperm, semen extenders have been supplemented with two different antioxidant compounds, carnitine (Experiment 2) and resveratrol (Experiment 3) before cryopreservation. In particular the effect of carnitine supplementation was evaluated on semen fertility parameters, such as viability, motility, capacitation status, and on in vitro fertility (Experiment 2); while the effect of resveratrol addition was also investigated on in vivo fertility after AI (Experiment 3).

Finally, since many studies have shown that spermatozoa capacitation is accompanied by a change in the lipid composition of the plasma membrane involving a decrease in the membrane cholesterol: phospholipid (C/P) ratio, known to be lower in buffalo sperm (Rajoriya et al., 2014), the aim of Experiment 4 was to analyze the effects of CLC supplementation on semen fertility

parameters, such as viability, motility, capacitation status on in vivo fertility after AI.

Experiment 1- Experimental design

The aim of this study was to evaluate the effect of season on buffalo male fertility. Four healthy Italian Mediterranean buffalo (Bubalus bubalis) bulls were selected for the trial and four ejaculates were collected from each bull during 2 seasons with different day length: spring and autumn.

Each ejaculate was split in 2 aliquots, one aliquot was used for fresh semen assessments while the other one was diluted at 37°C with an animal protein free extender, BioXcell (IMV-technologies, France), to a final concentration of 30×10^6 spermatozoa per mL and frozen according to standard procedures.

Sperm viability, motility and capacitation status, the latter evaluated both by CTC and the immune-localization of tyrosine phosphorylated protein, were assessed as described below both in fresh semen samples and after thawing of frozen semen samples. Moreover, the seasonal influence on the fertilizing ability of buffalo frozen-thawed sperm, after heterologous IVF was assessed by heterologous IVF. In order to do so, frozen-thawed sperm that were collected in spring and autumn were used to inseminate in vitro matured abattoir-derived bovine oocytes (n = 408 vs 496, respectively, over 16 replicates/season).

Materials and methods

Unless otherwise stated, reagents were purchased from Sigma-Aldrich® (Milano, Italy).

Semen collection and cryopreservation

The study was carried out after approval of Animal Ethics Committee of the Institute. Four healthy Italian Mediterranean buffalo (Bubalus bubalis) bulls (4–6 years age) maintained at an authorized National Semen Collection Center (Centro Tori Chiacchierini, Civitella D'Arna, Italy) under uniform management conditions were used in the study. A total of sixteen semen ejaculates per season (spring vs autumn), collected using artificial vagina method and having more than 70% progressive motility were utilized. Immediately after collection, the volume (mL) was assessed using a graduated tube, while the concentration (10^9/mL) was estimated using a spectrophotometer (LKB Biochrom Ltd Novaspec II, Cambridge, England). The percentage of motile sperm was assessed by a visual evaluation of fresh semen using a phase-contrast microscope (Olympus, Tokyo, Japan) at 40x magnification, with the sample kept at constant temperature. Subsequently, each ejaculate was split in two aliquots one for fresh analyses and one diluted as described in the experimental design. The diluted semen was packaged in 0.5 ml French straws and subjected to a combined cooling with equilibration period of 3 h at 5°C. The straws were kept in automatic programmable biological cell freezer (IMV technology, France) until temperature

of straws reached −145°C. Then straws were plunged into liquid nitrogen (−196°C) for storage.

Sperm washing and processing

Freshly ejaculated and cryopreserved spermatozoa after thawing (37°C/40sec), were washed in Modified Sperm Tyrode's albumin lactate pyruvate medium (Sperm-Talp) according to Parrish et al., (1986). For washing 0.5 ml of freshly ejaculated or frozen-thawed semen were diluted in two mL of Sperm-Talp, mixed gently and centrifuged at 300g for 10 min. Supernatant was discarded, and sperm concentration was adjusted to 20x 10^6 cells/ml in Sperm-Talp.

Evaluation of sperm motility

Sperm motility was assessed in cryopreserved spermatozoa by phase contrast microscopy (Nikon Diaphot 300) at 40 x magnification on a clean and dry glass slide maintained on thermo-regulated stage at 37°C. On the basis of the mass activity, semen motility was evaluated as previously described by Vale (1997) with slight modifications, i.e. graded into scores from 1 to 10.

Trypan blue/Giemsa technique

The viability was assessed by Trypan blue/Giemsa technique as reported by Boccia et al., (2007). The viability staining consisted of 0.27% Trypan blue (0.4% solution diluted 2:1 with buffered saline). The fixative was composed of 86mL 1N HCl plus 14mL 37% formaldehyde solution and 0.2g neutral red. The acrosome

staining was a 7.5% Giemsa stock solution in distilled water prepared freshly before use.

On a clean slide, 5 µl of semen and 5 µl Trypan blue were mixed, spread and left to dry in vertical position then, the slides were plunged in the fixative solution for 2 min, rinsed in distilled water and left to dry. The slides were put in jars with Giemsa (7.5%) and left overnight (16-20 h) at room temperature. The day after, the slides were rinsed again in distilled water and left to dry in vertical position. After drying, they were coverslipped with Entellan and the sperm cells were microscopically evaluated at 40x magnification. Based on staining characteristics of sperm cells we differentiated as live only the sperm displaying both head and tail viable (pink colored) and as dead those with either the head or the tail unviable(black-dark violet colored).

Localization of tyrosine phosphorylated protein assay

Localization of phosphotyrosine containing protein was detected using an indirect immunoflourescence assay as described by Tardif et al., (2001). Aliquots of 20 million spermatozoa were centrifuged as described above, fixed in 2% (v/v) formaldehyde for 1 h at 4 °C, and centrifuged again at 300 g for 10 min. The sperm pellets were incubated overnight at 4 °C in modified phosphate buffer saline (mPBS: 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 5.5 mM glucose and 1.0 mM pyruvate, pH 7.4) containing 2% (w/v) BSA. After centrifugation (250 g, 10 min, 25 °C), the sperm pellets were resuspended in mPBS. Twenty µl of sperm suspension were smeared onto a slide, air dried and permeabilized with absolute ethanol for 5 min. The permeabilized spermatozoa

were incubated with anti-phosphotyrosine primary antibody produced in rabbit (diluted 1:10 in tris buffered saline-TBS: 20 mM Tris-HCl, 0.8% NaCl, pH 7.6) for 1 h at room temperature. Excess antibodies were removed by plunging 4–5 times in TBS. The slides were incubated with secondary antibody, FITC conjugated goat anti-rabbit IgG (diluted 1:10 in TBS) for 1h in the dark at room temperature.

Excess antibodies were removed by plunging the slides 4–5 times in TBS and the slides were mounted with 90% (v/v) glycerol. Green fluorescence was observed by epifluorescent microscope using FITC filter (Diakon 300). A total of 100 spermatozoa were screened per slide and classified according to one of the three fluorescence patterns described by Cormier and Bailey (2003) and illustrated in Figure 3:

1) Pattern a—uniform fluorescence over the entire acrosome (low capacitation level)

2) Pattern E—a short line or triangle of fluorescence in the equatorial segment; (medium capacitation level)

3) Pattern EA—fluorescence at both equatorial and anterior acrosomal regions, (high capacitation level).

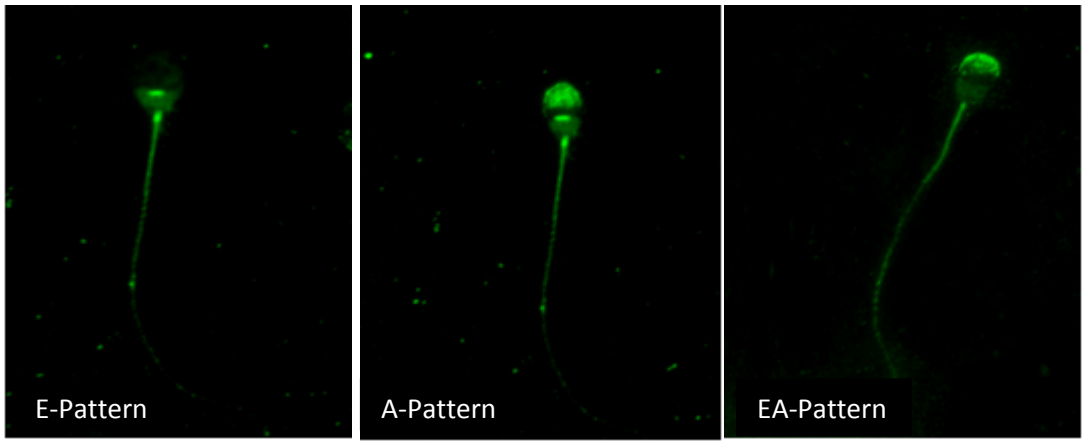


Figure 3: Different categories of buffalo frozen/thawed sperm immune-localized tyrosine phosphorylated protein. E-Pattern: a short line or triangle of fluorescence in the equatorial segment; A-Pattern: uniform fluorescence over the entire acrosome and EA-Pattern: fluorescence at both equatorial and anterior acrosomal regions.

Chlortetracycline (CTC) fluorescent assay

The capacitation status of fresh and frozen-thawed buffalo spermatozoa was assessed by CTC fluorescent staining as assessed by Fraser et al., (1995).

The CTC solution (750 mM CTC, 5 mM cysteine in 130 mM NaCl, and 20 mM Tris acid, pH 7.4) was prepared freshly, pH adjusted to 7.8, and stored at 4 °C under dark condition. Fifteen microliters of sperm suspension were mixed with an equal volume of CTC solution on a glass slide at room temperature, to which 1.5 µl of glutaraldehyde (12.5% in 20 mM Tris-HCl, pH 7.4) was added. A drop of 0.22 M 1, 4-diaza-bicyclo (2, 2, and 2) octane dissolved in glycerol: phosphate-buffered saline (9:1) was added to retard the fading of CTC fluorescence. The sperm suspension was covered with coverslip and stored at 4 °C overnight in the dark. Chlortetracycline fluorescence was observed under microscope equipped with phase contrast and epifluorescent optics at 100x magnification (Nikon

Diaphot 300). At least 100 spermatozoa per slide were analyzed and classified into one of three CTC staining patterns as described by Fraser et al., (1995) and shown in Figure 4:

1) Uniform bright fluorescence over the whole head (uncapacitated spermatozoa, pattern F);

2) fluorescence-free band in the post-acrosomal region (capacitated spermatozoa, pattern B);

3) Dull fluorescence over the whole head except for a thin punctuate band of Fluorescence along the equatorial segment (acrosome reacted spermatozoa, pattern AR).

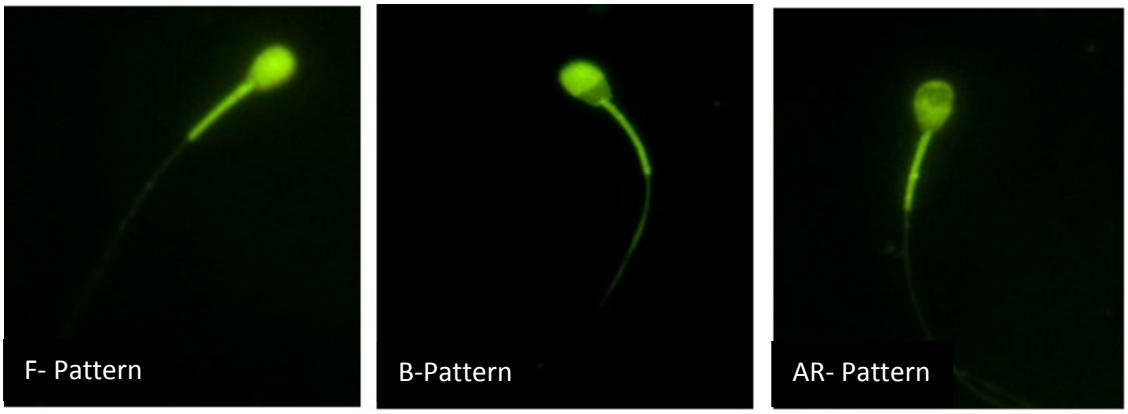


Figure (4): Different categories of CTC stained frozen/thawed buffalo sperm. F-pattern; non-capacitated spermatozoa with bright fluorescence over the entire sperm head, B- pattern; capacitated spermatozoa with a fluorescence-free band in the post-acrosomal region and AR-pattern; acrosome reacted spermatozoa with no acrosome and only a thin band of fluorescence along the equatorial segment.

Sperm fertilizing ability

Bovine ovaries were collected from a local abattoir, transported to the laboratory within 3–4 h from slaughter at approximately 35°C in physiological saline supplemented with 150 mg/L kanamycin. Cumulus–oocyte complexes (COCs) were recovered by aspiration of 2–8mm follicles using an 18-G needle under vacuum (40–50 mmHg). Only COCs with uniform cytoplasm and multilayered cumulus cells were selected for in vitro maturation (IVM). The selected oocytes were washed twice in Hepes-TCM medium and matured in 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 for 22 h at 39 °C, and 5% CO_2 in air. In vitro matured COCs were then fertilized in TALP buffered with 25 mM sodium bicarbonate and supplemented with 0.2 mM penicillamine, 0.1 mM hypotaurine, and 10 µg/mL heparin (IVF medium). Frozen-thawed sperm were selected by centrifugation (25 min at 300 × 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 then at 108 × g for 10 min each. The pellet was diluted with IVF medium and added in the fertilization wells at the concentration of 2×10^6 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, and incubated in a humidified mixture of 5% CO^2, 7% O_2 and 88% N_2 in air at a temperature of 39 °C. After 24 h of culture, the cleavage rate was assessed and confirmed by fixation of zygotes with absolute ethanol overnight and staining with DAPI for nuclei examination under epifluorescence

microscope (Nikon Diaphot 300) after zona removal by pronase (2 mg/mL) digestion. The penetration, normal fertilization and polyspermy rates were assessed.

Statistical analysis

Differences between seasons in volume, concentration, viability, motility, as well as in the percentages of sperm showing different CTC and tyrosine phosphorylation patterns, of fresh semen were analyzed by the Student T test. The same analysis was carried out to evaluate differences between seasons in viability, motility and CTC and tyrosine phosphorylation patterns of frozen-thawed semen. The percentages of cleavage, total, normospermic and polyspermic penetration were analyzed by Chi Square test.

Results

A comparison of fertility parameters between fresh and frozen buffalo semen is shown in Table 1. Regardless of the season, freezing of semen decreased ($P<0.01$) both sperm motility and viability. Furthermore, in frozen semen an increase of capacitation-like changes was detected, as indicated by the differences in both tyrosine phosphorylation and CTC patterns (Table 1). In particular, freezing decreased ($P<0.01$) pattern A (low capacitation level) and increased ($P<0.01$) pattern EA (high capacitation level). Moreover, the percentages of sperm displaying pattern F (non-capacitated sperm) decreased ($P<0.01$) and the percentages of sperm showing pattern B (capacitated) and AR (acrosome reacted) increased ($P<0.01$) after freezing.

Table 1. Tyrosine phosphorylation and CTC patterns, motility and viability, expressed as mean ± SE, of buffalo fresh and frozen sperm, over 32 replicates

Groups	Tyrosine phosphorylation patterns						CTC patterns		
	Motility	NF	A	E	EA	Viability	F	B	AR
Fresh	82.5 ± 0.8^{A}	0.0 ± 0.0^{A}	92.2 ± 0.9^{A}	0.0 ± 0.0^{A}	7.8 ± 0.9^{A}	92.5 ± 1.0^{A}	86.1 ± 0.7^{A}	13.8 ± 0.7^{A}	0.2 ± 0.76^{A}
Frozen	47.8 ± 2.9^{B}	3.0 ± 0.6^{B}	60.1 ± 2.9^{B}	2.1 ± 0.6^{B}	34.8 ± 3.0^{B}	78.7 ± 1.8^{B}	32.6 ± 1.6^{B}	63.0 ± 1.3^{B}	4.4 ± 0.7^{B}

A, B Values with different superscripts are significantly different; $P<0.01$

Fertility parameters

Both the volume of the ejaculate and the sperm concentration were not influenced by season (5.8±0.3vs6.2±0.6 and 1027.6±5.1vs 946.3±6.7 in spring and autumn, respectively). Moreover viability, i.e. the percentage of live sperm cells, evaluated by Trypan blue/Giemsa technique, did not differ in spring compared to autumn (75.9±2.4 vs 81.5 ±2.3, respectively). The motility of fresh semen was also not affected by the season (83.1±0.8 vs 81.9±1.4 in spring and autumn, respectively). In contrast, the motility of frozen thawed-semen increased during autumn compared to spring (54.4±2.7 and 41.2±4.7, respectively; $P<0.05$).

Capacitation status (localization of tyrosine phosphorylated proteins)

In fresh samples, sperm showing pattern E and no fluorescence were not observed in both seasons. The percentages of sperm exhibiting pattern A increased during autumn compared to spring (94.9±1.2 vs 89.5±0.9 respectively; $P<0.01$). On the contrary, the percentages of sperm exhibiting pattern EA decreased in autumn compared to spring (5.1±1.2 vs 10.5±0.9 in spring and autumn, respectively; $P<0.01$). As regard the frozen-thawed semen, pattern A and EA were not affected by season (60.6±4.0 vs 59.6±4.3 and 31.5±4.3 vs 38.1±2.2 in spring and autumn, respectively). Interestingly, sperm displaying pattern E (medium capacitation level) decreased ($P<0.05$) during the favorable

season compared to the unfavorable one (0.7±0.4 vs 3.4±1.0 in autumn and spring, respectively). Unexpectedly, an increased percentage of no fluorescent sperm was found in spring (4.4±0.9 vs 1.6±0.8 in spring and autumn, respectively $P<0.05$). However, the latter categories had little impact on the total sperm population.

Viability and capacitation status (CTC assay)

The combined CTC/Hoechst technique also allowed to assess viability that in fresh semen was very high and not affected by seasonal variation (93.6±0.8 vs 91.4±1.9 % in spring and autumn, respectively). Moreover, in fresh semen no differences in the proportion of sperm displaying the three CTC fluorescent patterns were detected between spring and autumn (Pattern F: 85.6±0.9 vs 86.5±0.9; Pattern B: 14.3±0.9vs 13.25±1.0; Pattern AR: 0.12±0.9 vs 0.25±0.11, respectively).

Conversely, in frozen-thawed semen, CTC fluorescent patterns were strongly affected by season as reported in Table 2. In particular, the percentage of sperm showing Pattern B (capacitated spermatozoa) decreased ($P<0.05$) and that of Pattern F sperm (non-capacitated spermatozoa) increased ($P<0.01$) in autumn compared to spring. Furthermore, the incidence of acrosome reacted sperm (AR-pattern) was lower in autumn than in spring ($P=0.054$). However, sperm viability was similar in the two seasons, as shown in Table 2.

Table 2. Percentages of F-pattern, B-pattern, AR-pattern and viability in buffalo frozen-thawed semen during two different seasons.

Season	N.	F- pattern	B- pattern	AR- pattern	Viability
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Spring	1600	28.1 ± 2.2^{A}	66.2 ± 2.0^{a}	5.8 ± 0.9^{a}	75.9 ± 2.4
Autumn	1600	37.1 ± 1.8^{B}	59.9 ± 1.4^{b}	3.1 ± 0.9^{b}	81.5 ± 2.3

$^{A, B}$ Values with different superscripts within columns are different; P< 0.01

$^{a, b}$ Values with different superscripts within columns are different; P< 0.05

Sperm fertilizing ability

As reported in Table 3, cleavage, total penetration and normospermic penetration rates increased (P<0.01) during autumn compared to spring, whereas the polyspermic rate was similar in the two seasons.

Table 3. Percentages of cleavage, total and normospermic penetration and polispermy in different seasons.

Season	N.	Cleavage	Total penetration	Normospermic penetration	Polyspermy
		n (%)	n (%)	n (%)	n (%)
Spring	496	$176\ (35.5)^{A}$	$236\ (47.6)^{A}$	$212\ (42.7)^{A}$	24 (4.8)
Autumn	408	$240\ (58.8)^{B}$	$264\ (64.7)^{B}$	$252\ (61.8)^{B}$	12 (2.9)

$^{A, B}$ Values with different superscripts within columns are different; P< 0.01

Experiment 2- Experimental design

The results obtained in the first experiment suggested to limit the collection of the ejaculates for Experiment 2 during the favorable season, to reduce the variability related to the seasonal effect and use a superior quality semen in order to assess the effectiveness of the substances tested, before planning strategies to improve semen quality in the unfavorable season.

Therefore the aim of this Experiment was to evaluate whether supplementing the semen extender with carnitine, a vitamin-like compound, with antioxidant properties, improves the freezability of buffalo spermatozoa. Four healthy Italian Mediterranean buffalo (Bubalus bubalis) bulls were selected for the trial and four ejaculates per bull were collected. Each ejaculate was split in 3 aliquots that were diluted at 37°C with an animal protein free extender, BioXcell (IMV-technologies, France), containing 0 (control group), 2.5 and 7.5 mM Carnitine to a final concentration of 30×10^6 spermatozoa per mL. The aliquots were frozen according to standard procedures.

After thawing sperm viability, motility and capacitation (the latter evaluated both by CTC and the immune-localization of tyrosine phosphorylated protein) were assessed as described before. Moreover, sperm in vitro fertilizing capability of control and Carnitine-treated group was assessed by evaluating cleavage, penetration, and polyspermy rates after heterologous IVF. Therefore, frozen-thawed sperm were used for IVF of in vitro matured abattoir-derived bovine oocytes (n= 429, n= 430, n= 403, control group, 2.5 and 7.5 mM Carnitine respectively; over 10 replicates/group).

Materials and methods

The materials and methods related to the evaluation of semen parameters such as viability, motility and capacitation and those related to in vitro fertilizing ability are the same described in detail in Experiment 1.

Statistical analysis

Sperm parameters, CTC patterns and tyrosine phosphorylation data were analyzed after angular transformation of the percentages by a linear mixed model with the bull as repeated effect. The Bonferroni method was used to evaluate the differences among groups. In case variances were not homogeneous, the non-parametric Kruskal-Wallis was used.

The percentages of cleavage, total, normospermic and polyspermic penetration were analyzed by Chi Square test.

Results

Fertility parameters

No differences among groups were found in sperm motility (44.4±3.5, 53.1±3.9 and 52.5±3.6 control, 2.5mM and 7.5mM carnitine, respectively). Similarly viability, evaluated by Trypan blue/Giemsa technique, was not affected by the treatments (80.7±2.4; 84.2±2.1; 84.6±2.0 control, 2.5mM and 7.5mM carnitine, respectively).

Capacitation status (localization of tyrosine phosphorylated proteins)

Supplementation of carnitine to the freezing extender decreased the level of tyrosine phosphorylation in buffalo spermatozoa as indicated by the low level of immunofluorescence at acrosomal region and equatorial plate (pattern EA). The pattern EA decreased in both treated groups compared to the control but the effect was greater ($P<0.01$) with the higher carnitine concentration (Table 4). Furthermore, when the extender was supplemented with 7.5 mM carnitine an increase ($P<0.05$) of pattern A was observed compared to the control. Interestingly, the percentage of sperm exhibiting no fluorescence also increased ($P<0.01$) when sperm were treated with 7.5 mM carnitine. No differences in sperm displaying pattern E, however, were detected among groups.

Table 4. Tyrosine phosphorylated proteins patterns of buffalo frozen-thawed semen in different Carnitine concentrations

Carnitine concentrations	N.	NF- pattern Mean ± SE	A- pattern Mean ± SE	E- pattern Mean ± SE	EA- pattern Mean ± SE
Control	1600	2.8 ± 1.0^{A}	65.8 ± 3.6^{a}	1.1 ± 0.8	30.3 ± 3.8^{Aa}
2.5mM	1600	5.1 ± 1.9^{A}	75.8 ± 2.7	0.4 ± 0.3	18.8 ± 2.8^{b}
7.5mM	1600	16.5 ± 3.4^{B}	76.3 ± 2.8^{b}	0.0 ± 0.0	7.2 ± 1.9^{Bc}

$^{A, B}$ Values with different superscripts within columns are different; $P< 0.01$

$^{a, b, c}$ Values with different superscripts within columns are different; $P< 0.05$

Viability and capacitation status (CTC assay)

The results regarding the CTC patterns showed that carnitine supplementation to the freezing extender, prior to cryopreservation, decreased the level of capacitation, in a dose-dependent manner (Table 5). In fact, the percentage of sperm displaying pattern F increased ($P<0.01$), while that of sperm displaying pattern B decreased ($P<0.01$) in both treated groups compared to the control (Table 5). Within treatment groups, the highest concentration was the most effective in reducing cryocapacitation, as indicated by higher ($P<0.01$) percentages of pattern F and lower ($P<0.01$) percentages of pattern B sperm. However, no differences were detected in pattern AR and viability, assessed by CTC/Hoechst technique among groups, as shown in Table 5.

Table 5. Percentages of F-pattern, B-pattern, AR-pattern and viability of buffalo frozen-thawed semen in different Carnitine concentrations

Carnitine concentrations	N.	F- pattern Mean ± SE	B- pattern Mean ± SE	AR- pattern Mean ± SE	Viability Mean ± SE
Control	1600	31.3 ± 2.1^{A}	63.8 ± 1.8^{A}	4.9 ± 0.9	80.0 ± 2.7
2.5mM	1600	49.4 ± 2.2^{B}	46.8 ± 2.2^{B}	3.8 ± 0.6	84.3 ± 2.3
7.5mM	1600	60.3 ± 3.6^{C}	37.2 ± 1.8^{C}	2.6 ± 1.9	84.7 ± 2.2

$^{A, B, C}$ Values with different superscripts within columns are different; $P< 0.01$

Sperm fertilizing ability

Cleavage, total penetration, normospermic and polyspermic penetration rates were similar among control and treated groups as shown in Table 6.

Table 6. Percentages of cleavage, total penetration, normospermic penetration and polispermy penetration in different Carnitine concentrations.

Carnitine concentrations	N.	Cleavage	Total penetration	Normospermic penetration	Polyspermy
		n (%)	n (%)	n (%)	n (%)
Control	429	207(48.4)	239 (55.1)	234 (53.6)	3 (0.9)
2.5mM	430	228(51.3)	253 (56.9)	252 (56.6)	1 (0.3)
7.5mM	403	198(45.2)	233 (53.5)	218 (50.4)	2 (0.6)

Experiment 3- Experimental design

The aim of this experiment was to assess the effect of supplementation of the extender with resveratrol, a natural phytoalexin with antioxidant properties, on freezability of buffalo spermatozoa. Four healthy Italian Mediterranean buffalo (Bubalus bubalis) bulls were selected for the trial and four ejaculates per bull were collected during the favorable season, for the same reasons mentioned above. Each ejaculate was split in 5 aliquots that were diluted at 37°C with an animal protein free extender, BioXcell (IMV-technologies, France), containing 0 (control group), 0.5, 1, 10 and 50 μM Resveratrol, to a final concentration of 30 × 10^6 spermatozoa per mL. The aliquots were frozen according to standard procedures. After thawing sperm viability, motility and capacitation (the latter evaluated both by CTC and the immune-localization of tyrosine phosphorylated protein) were assessed as described before.

On the basis of the results obtained on semen parameters, the concentration of 50 μM was selected to assess the influence of resveratrol on the fertilizing ability of buffalo frozen-thawed sperm, after heterologous IVF. Therefore, frozen-thawed sperm were used to inseminate in vitro matured abattoir-derived bovine oocytes (n =226 and 227, in control and 50 μM Resveratrol group, respectively, over 8 replicates/group). Moreover, to assess the effect on in vivo fertility buffalo cows were synchronized by OVSYNCH protocol and artificially inseminated in two farms (N=30 and N=73 in farm 1 and 2, respectively) , with control and treated semen (50 μM Resveratrol) collected from two different buffalo bulls.

Materials and methods

The materials and methods related to the evaluation of semen parameters such as viability, motility and capacitation and those related to in vitro fertilizing ability are the same described in detail in Experiment 1.

Synchronization protocol and Artificial Insemination

The synchronization protocol used, Ovsynch with timed-AI (OVSINCH-TAI), was similar to that developed for cattle (Pursley et al., 1995) and previously applied in buffaloes (Neglia et al., 2003a). OVSINCH-TAI consists of a GnRH agonist administration (buserelin acetate, 12 μg; Receptal®, Intervet, Milan, Italy) on day 0, a PGF2α analogue (luprostiol, 15 mg; Prosolvin®, Intervet) on day 7 and a GnRH agonist (buserelin acetate, 12 μg; Receptal®, Intervet, Milan, Italy) again on day 9. Artificial inseminations were performed 18-20 h after the second injection of GnRH agonist. Because of the relatively low intensity of estrous behavior in buffaloes, animals were palpated per rectum (immediately before AI) to assess estrous status (follicle >1.0 cm) and a tonic uterus with the presence or absence of mucous vaginal discharge. Twenty-five days after AI, buffaloes underwent transrectal ultrasonography to assess embryonic development. Ultrasonography was conducted with an Aloka SSD-500 unit equipped with a 5.0 MHz linear array probe (Aloka CO., Tokyo, Japan). Pregnancy diagnosis was confirmed on day 45 after AI by ultrasonography. Buffaloes pregnant on day 25, but not on day 45 were considered to have undergone embryo mortality.

Statistical analysis

Tyrosine phosphorylation and CTC patterns data were analyzed after angular transformation of the percentages by a linear mixed model with bull as repeated effect. The Bonferroni method was used to evaluate the differences among groups. In case variances were not homogeneous, within the groups, the non parametyric Kruskal-Wallis was used.

The percentages of cleavage, total, normospermic and polyspermic penetration were analyzed by Chi Square test. The differences of pregnancies rates after AI in control and treated groups were analyzed by Chi square test.

Fertility parameters

No differences among groups were found in sperm motility (51.3±4.6; 55.6± 5.5; 52.5±4.4; 51.2±5.2; 50.6±4.2 control, 0.5, 1, 10 and 50 μM resveratrol, respectively). Similarly viability, evaluated by Trypan blue/Giemsa technique, was not affected by the treatments (80.0±2.4; 74.8± 3.1; 77.3±2.9; 77.8±2.8; 73.2±3.6 control, 0.5, 1, 10 and 50 μM resveratrol, respectively).

Capacitation status (localization of tyrosine phosphorylated proteins)

No differences were observed among groups in non-fluorescent sperm (on average 4.8±0.5 %) and in pattern E sperm (on average 4.9±0.6 %), as shown in Figure 5. Overall, the more relevant changes in tyrosine phosphorylated proteins patterns regarded pattern A and EA (Figure 6). In particular, resveratrol increased pattern A, while decreasing pattern EA in a dose-dependent manner (Figure 6). In fact, the two higher concentrations of resveratrol tested (10 and 50 μM) increased ($P<0.01$) the percentage of sperm displaying pattern A compared to the control. When extender was supplemented with 10 μM resveratrol the percentage of sperm showing pattern EA was lower than in the control ($P=0.055$). However, treatment with 50 μM resveratrol was much more effective in decreasing ($P<0.01$) the proportion of sperm exhibiting pattern EA. No significant differences were observed in both pattern A and EA between the control and the lower concentrations of resveratrol (0.5 and 1 μM).

Figure 5. Effect of resveratrol on non-fluorescent and E pattern

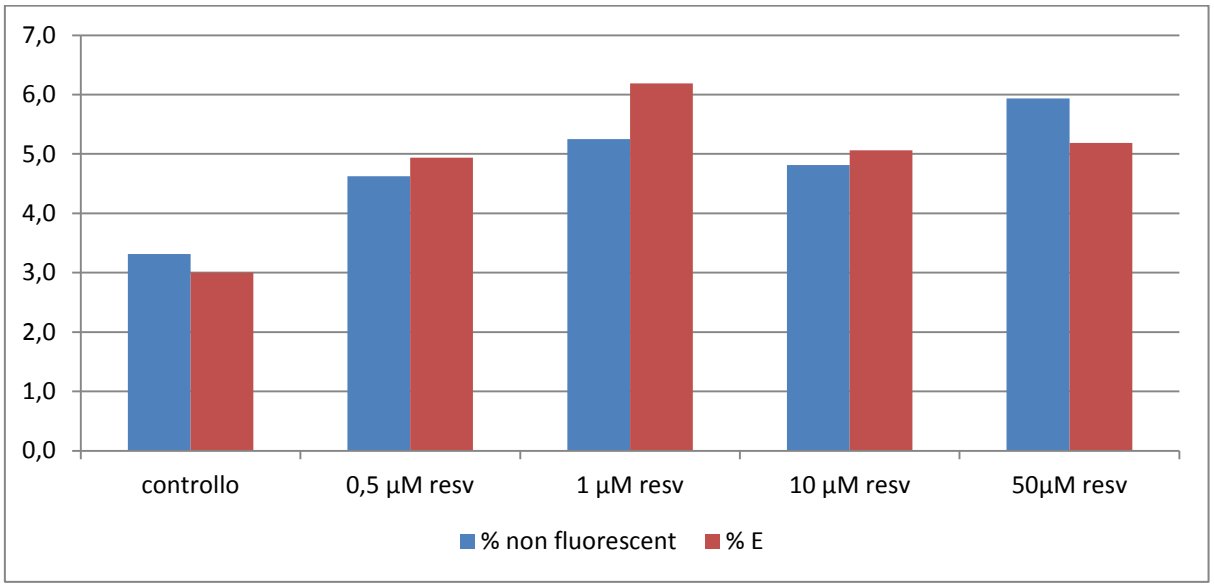
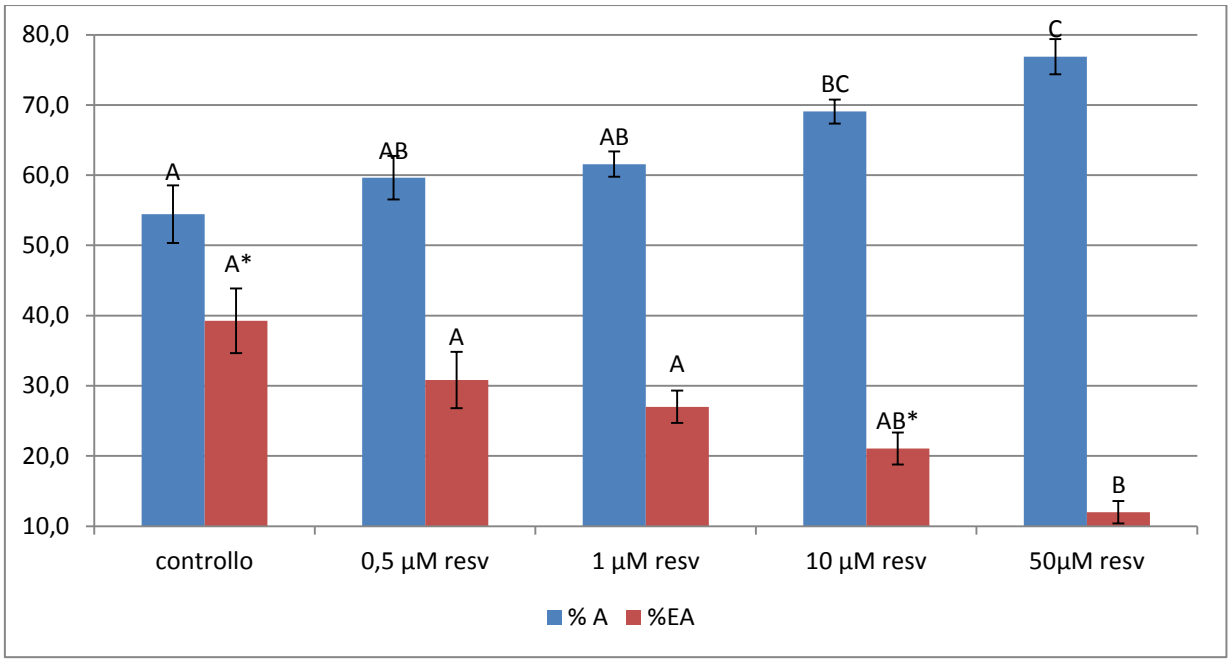


Figure 6. Effect of resveratrol on A and EA pattern



A, B, C Bars with different letters are significantly different; P<0.01

* P=0.055

Capacitation status (CTC assay)

The effect of resveratrol supplementation on the localization of tyrosine phosphorylated proteins suggested to focus the attention only on the two higher concentrations. Therefore, the CTC assay was performed only on the control semen and on semen treated with 10 and 50 μM resveratrol. No differences in CTC patterns and viability among groups were recorded, as shown in Table 7.

Table 7. Percentages of F-pattern, B-pattern, AR-pattern and viability of buffalo frozen-thawed semen in control and resveratrol-treated groups.

Resveratrol concentrations	N.	F- pattern Mean ± SE	B- pattern Mean ± SE	AR- pattern Mean ± SE	Viability Mean ± SE
Control	1600	33.9 ± 2.5	62.3 ± 2.1	3.9 ± 0.9	77.4 ± 2.2
10 μM	1600	36.1 ± 2.1	60.2 ± 1.9	3.7 ± 0.9	75.7 ± 2.4
50 μM	1600	34.6 ±2.2	62.1 ± 2.1	3.6 ± 0.7	72.9 ± 3.1

Effect of resveratrol on sperm fertilizing ability

Although cleavage, total penetration, and polyspermy rates after heterologous IVF were similar among groups, the normospermic penetration improved ($P<0.05$) when semen was treated with 50 μM resveratrol, as shown in Table 8.

Table 8. Percentages of cleavage, total penetration, normospermic penetration and polispermy after heterologous IVF with control and 50 μM resveratrol-treated semen.

Groups	N.	Cleavage	Total penetration	Normospermic penetration	Polispermy
		n (%)	n (%)	n (%)	n (%)
Control	226	104 (46.0)	125 (55.3)	116 (51.3)[a]	9 (4.0)
Resveratrol	227	118 (52.0)	141 (62.1)	138 (60.8)[b]	3 (1.3)

[a, b,] Values with different superscripts within columns are different; $P< 0.05$

Pregnancy rates after AI

No differences in pregnancy rates both at 25 and 45 days were found between bulls within the control (45.9 and 43.2%, respectively for bull 1; 28.6 and 28.6, respectively for bull 2) and the resveratrol-treated semen (51.4 and 48.6, respectively for bull 1; 40.0 and 40.0, respectively for bull 2). Although a tendency to greater pregnancies was recorded with resveratrol-treated semen in

both farms, differences in terms of pregnancy rates at 25 and 45 days were not statistically different (Table 9). However, a farm effect was observed, with higher pregnancy rates at both 25 and 45 days in farm 1 compared to farm 2. The incidence of embryonic mortality was very low in both groups and farms.

Table 9. Pregnancies rates at 25 and 45 days in control and resveratrol-treated semen.

Farms	Groups	N.	Pregnancies at 25 days	Pregnancies at 45 days	Embryo mortality
		n (%)	n (%)	n (%)	n (%)
1	Control	15	10 (66.7)[a]	10 (66.7)[a]	0 (0.0)
	Treated	15	11 (73.3)[a]	11 (73.3)[a]	0 (0.0)
2	Control	36	11 (30.6)[b]	10 (27.8)[b]	1 (2.8)
	Treated	37	14 (37.8)[b]	13 (35.1)[b]	1 (2.7)
Totale	Control	51	21 (41.2)	20 (39.2)	1 (2.0)
	Resveratrol	52	25 (48.1)	24 (46.2)	1 (1.9)

[a, b] Values with different superscripts within columns are different; $P < 0.05$

Experiment 4 - Experimental design

Since cholesterol efflux from the sperm membranes plays an important role in sperm capacitation, it was hypothesized that increasing sperm cholesterol content, using cholesterol loaded cyclodextrins (CLC) technology prior to cryopreservation, may reduce premature sperm capacitation, thereby increasing the lifespan of cryopreserved sperm cells. Therefore, the aim of this study was to investigate the effect of CLC on freezability of buffalo spermatozoa.

Four healthy Italian Mediterranean buffalo (Bubalus bubalis) bulls were selected for the trial and four ejaculates were collected from each bull during the favorable season (autumn), like in experiment 2 and 3. Each ejaculate was split in 3 aliquots that were diluted at 37°C with an egg yolk tris extender, BULLXcell (IMV-technologies, France), containing 0 (control group), 1.5 and 3 mg/mL CLC, to a final concentration of 30×10^6 spermatozoa per mL. The aliquots were frozen according to standard procedures.

Sperm viability, motility and capacitation (the latter evaluated both by CTC and the immune-localization of tyrosine phosphorylated protein) were assessed on both fresh and frozen sperm just after thawing. Moreover, to assess the effect on in vivo fertility buffalo cows were synchronized by OVSYNCH protocol and artificially inseminated with control and CLC-treated semen(n=33, n=34, n=30 in control, 1.5mg/mL and 3mg/mL groups, respectively).

Materials and methods

Unless otherwise stated, reagents were purchased from Sigma-Aldrich® (Milano, Italy).

The materials and methods related to the evaluation of semen parameters such as viability, motility and capacitation were previously widely described in Experiment 1. The synchronization protocol and artificial insemination material and methods were previously reported in Experiment 3.

CLC preparation

Methyl-β-cyclodextrin was loaded with cholesterol as described earlier (Purdy and Graham, 2004a). In a glass tube 200 mg of cholesterol was dissolved in 1 mL of chloroform while in a second glass tube, 1 g of methyl-β-cyclodextrin was dissolved in 2 mL of methanol. A 0.45 mL aliquot of the cholesterol solution was added to the cyclodextrin solution, and the mixture was stirred until the combined solution appeared clear. This was followed by pouring of mixture into a glass petri dish and removing of solvents using a stream of nitrogen gas. The resulting crystals were allowed to dry for an additional 24 h and then were removed from the dish and stored in a glass container at 22°C. A working solution of CLC was prepared by adding 50 mg of CLC to 1 mL of Sperm-Talp at 37°C and mixing the solution briefly using a vortex mixer.

Statistical analysis

Tyrosine phosphorylation and CTC patterns data were analyzed after angular transformation of the percentages by a linear mixed model with the bull as repeated effect. The Bonferroni method was used to evaluate the differences among groups. In case variances were not homogeneous the non-parametric Kruskal-Wallis was used. The differences of pregnancies rates after AI in control and treated groups were analyzed by Chi square test.

Results

Fertility parameters

The motility of fresh semen was very high (on average 85.6±2.4%) and decreased ($P<0.01$) in frozen-thawed semen control (66.5± 5.6; $P<0.01$) and 1.5 and 3 mg/mL CLC treated groups (68.8±4.8 and 68.8±4.8, respectively; $P<0.05$). Moreover viability, evaluated by Trypan blue/Giemsa technique, did not differ among groups (91.5±1.7, 83.6±1.7, 85.2±2.6 and 85.9±2.6 in fresh semen and frozen-thawed control, 1.5 and 3 mg/mL CLC groups, respectively).

Capacitation status (localization of tyrosine phosphorylated proteins)

The results regarding the effect of cholesterol supplementation on the localization of tyrosine phosphorylated proteins are shown in Table 10. Treatment of sperm with 3 mg/mL CLC increased ($P<0.01$) the percentage of non-fluorescent sperm compared to fresh semen. Within frozen-thawed groups,

the control showed a lower percentage of non-fluorescent sperm compared to 1.5 mg/mL ($P<0.05$) and 3 mg/mL ($P<0.01$) CLC groups. An increased ($P<0.05$) percentage of sperm displaying pattern A was detected in semen treated with 3mg/mL compared to fresh semen ($P<0.05$). No differences were observed among groups in pattern E. Moreover, the percentage of sperm with pattern EA was reduced ($P<0.01$) in both CLC-treated groups compared to the control, showing similar values to those recorded in fresh semen (Table 10).

Table 10. Tyrosine phosphorylated proteins patterns of fresh semen and frozen-thawed control and CLC-treated semen.

Groups	N.	NF-pattern Mean ± SE	A-pattern Mean ± SE	E-pattern Mean ± SE	EA-pattern Mean ± SE
Fresh	800	11.0 ± 6.9^{A}	78.0 ± 8.2^{a}	0.0± 0.0	11.0 ± 3.5^{B}
Control	800	1.6 ± 0.7^{Aa}	60.9 ± 6.9^{ab}	0.1 ± 0.1	37.3 ± 6.9^{A}
1.5mg/mL	800	24.6 ± 4.3^{b}	64.6 ± 1.8^{ab}	0.0 ± 0.0	10.8 ± 3.3^{B}
3 mg/mL	800	41.8 ± 3.6^{B}	63.9 ± 3.3^{b}	0.5 ± 0.5	5.6 ± 1.6^{B}

$^{A, B}$ Values with different superscripts within columns are different; $P< 0.01$

$^{a, b}$ Values with different superscripts within columns are different; $P< 0.05$

Capacitation status (CTC assay)

As regards CTC patterns, the results are shown in Table 11.

As expected, in fresh semen a higher ($P<0.01$) percentage of sperm showing F pattern and a lower percentage of sperm displaying B pattern ($P<0.05$) was found compared to all frozen-thawed groups.

Within frozen-thawed groups, treatment with CLC increased ($P<0.01$) the proportion of pattern F and decreased ($P<0.01$) that of pattern B compared to the control, and the more effective concentration was 1.5 mg/mL. Furthermore, no differences in sperm viability were observed among cryopreserved groups.

Table 11. Percentages of F-pattern, B-pattern, AR-pattern and viability of fresh semen and frozen-thawed control and CLC-treated semen

Groups	N.	F- pattern Mean ± SE	B- pattern Mean ± SE	AR- pattern Mean ± SE	Viability Mean ± SE
Fresh	800	78.1 ± 3.3^{A}	21.9 ± 33^{Aa}	0.0 ± 0.0	95.1 ± 1.7^{a}
Control	800	28.6 ± 3.3^{B}	69.6 ± 3.4^{B}	1.8 ± 0.7	86.5 ± 1.9^{b}
1.5mg/mL	800	61.5 ± 1.7^{Ca}	37.8 ± 1.5^{ACb}	0.8 ± 0.4	87.6 ± 1.5^{b}
3 mg/mL	800	47.6 ± 4.3^{Cb}	51.3 ± 4.7^{C}	1.1 ± 0.6	88.4 ± 2.3^{ab}

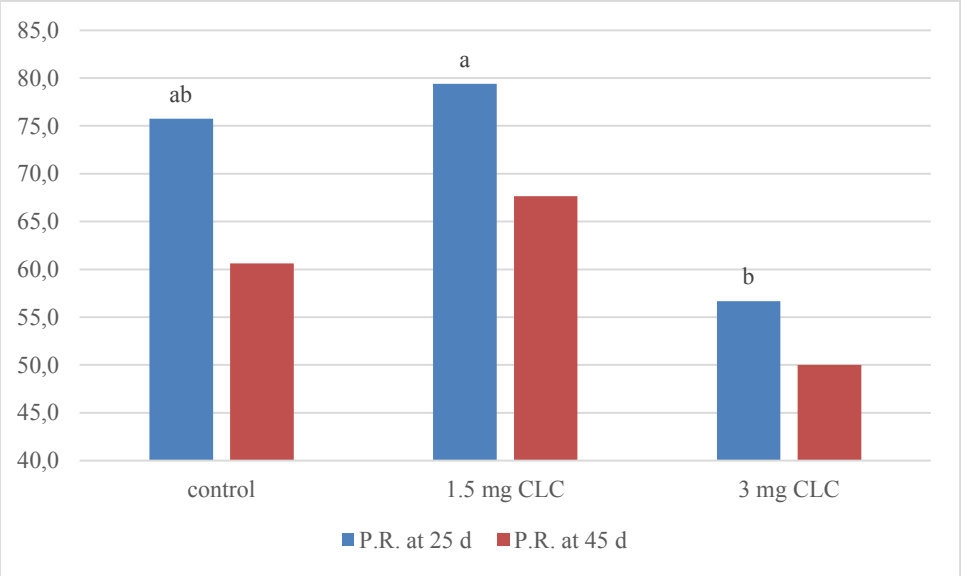
$^{A, B, C}$ Values with different superscripts within columns are different; $P< 0.01$

$^{a, b}$ Values with different superscripts within columns are different; $P< 0.05$

Pregnancy rates after AI

As differences between bulls were not recorded data were pooled for the analysis. Pregnancy rates at 25 and 45 days after AI with conventional (control) and CLC-treated semen were similar, as shown in Figure 7. However, within CLC-treated semen, pregnancy rate at 25 d decreased ($P<0.05$) with the highest concentration (Figure 7). The incidence of embryonic mortality was not different among groups (15.2, 11.8 and 6.7, in the control, 1.5 mg/mL CLC and 3 mg/mL CLC, respectively).

Figure 7. Pregnancy rates at 25 and 45 days after AI with control and CLC-treated semen



[a, b] Bars with different letters are significantly different; $P< 0.05$

Discussion

Currently, the competitiveness of buffalo breeding highly depends on genetic improvement that can be achieved through the application of reproductive biotechnologies. The availability of genetic material from bulls of proven quality is a necessary development to continue the process of selective breeding already begun by farmers. The use of artificial insemination is critical to enhance the paternal contribution to genetic improvement, while OPU and IVEP are the best tool to speed up the genetic progress through the maternal lineage. However, AI in buffalo breeding is limited to just the 10% of the buffaloes enrolled to the Herd Book and this restricted number of buffalo bulls available for semen is affected by a high re-breeding rate, since bulls come from farms with a high percentage of inbreeding. Furthermore, a major limitation of IVEP technology is the high variability of the fertilizing ability of buffalo bulls in vitro that, together with the lack of data referred to the males currently utilized as semen producers in Italy, imposes to perform an accurate and time-consuming screening in the laboratory before a certain bull can be used for IVF programs. It is specified that in our experience only 10 % of the bulls tested is suitable for IVF (Gasparrini, 2002). Ongoing analysis seems to confirm that a high degree of variation exists also in the fertility in vivo after AI, as largely reported in cattle (Killian et al., 1993; Cancel et al., 1997).

Moreover, as reported before the reproductive efficiency of buffalo species is strongly related to seasonality (Baruselli et al., 2001a; Campanile et al., 2005; 2007a; Di Francesco et al., 2012; Rossi et al., 2014) and to the quality of frozen-

thawed semen (Muer et al., 1988; Andrabi et al., 2001; Ahmad et al., 2003; Senatore et al., 2004; Kumaresan et al., 2005; 2006; Shukla and Misra, 2007).

As the reproductive seasonality of the species, in males, is characterized by the reduction of the libido (Sansone et al., 2000), it often happens that, in increasing day length months bulls stop mounting and, hence, do not produce ejaculates. This has hampered the evaluation of semen in relation to the season. Therefore, the aim of the first experiment was to evaluate the seasonal influence on the quality of fresh and frozen buffalo semen at our latitudes.

The first result of this study was the evident decline in the quality of semen after cryopreservation. In fact, regardless of season, freezing of semen decreased sperm motility, viability and induced an increase of capacitation-like changes, as indicated by the differences in both tyrosine phosphorylation and CTC patterns.

It is known that freezing–thawing of buffalo spermatozoa causes considerable damage to motility apparatus, plasma membrane, acrosomal cap (Rasul et al., 2000) and leakage of intracellular enzymes (Dhami and Kodagali, 1990). Indeed, buffalo spermatozoa are more susceptible to hazards during freezing–thawing than cattle spermatozoa (Raizada et al., 1990; Kumar et al., 1992). Several mechanisms have been attributed to the reduced fertility of cryopreserved semen; however, cryopreservation-induced capacitation-like changes in frozen spermatozoa gained momentum recently (Watson, 2000; Cormier and Bailey, 2003; Thomas et al., 2006). Various methods have been used to differentiate capacitated and non-capacitated spermatozoa. For a long time, capacitation of both fresh and frozen/thawed sperm has been evaluated indirectly by estimating the capability of spermatozoa to undergo acrosome reaction following incubation

with LPC, a fusogenic lipid known to induce AR in capacitated sperm without affecting motility (ref). Later, the CTC fluorescence assay was developed. The fluorescent antibiotic CTC was used to assess the destabilization of sperm membrane (Fraser et al., 1995) based on its ability to cross over the cell membrane, enter intracellular compartments and bind to free calcium ions. This method has been used to assess sperm capacitation in most domestic species (Vadnais et al., 2005; Miah et al., 2011), including buffalo (Kadirvel et al., 2011; Kaul et al., 2001). Moreover CTC technique allows distinguishing three stages of sperm activation: non-capacitated (F pattern), capacitated acrosome intact (B pattern), and capacitated acrosome reacted (AR pattern). As it is well established that tyrosine phosphorylation of sperm proteins is a key event of sperm capacitation, the localization of phosphotyrosine containing protein detected by an indirect immunoflourescence assay has been recently successfully used (Kadirvel et al., 2011; Tardif et al., 2001). Several studies have correlated the degree of tyrosine phosphorylation with the capacitative state of spermatozoa. This technique differentiate four levels of sperm capacitation: none (no fluorescent), low (A Pattern), medium (E Pattern) and high (EA Pattern).

As regards seasonal variation, no differences in sperm motility and viability were observed in fresh semen, unlike to what reported by Heuer et al., (1986). Moreover, sperm concentration did not vary statistically between autumn and spring, similarly to what observed under tropical conditions in Thailand (Koonjaenak at al., 2007a). Furthermore, the ejaculate volume reported in this study was not influenced by seasonality according to previous studies in swamp buffaloes (Jainudeen et al., 1982), as well as in Murrah (Bhosrekar et al., 1992)

and Nili-Ravi buffalo breeds (Khan et al., 1997). Conversely, a strong effect on post-thaw motility was recorded, with a significant increase in the favorable season (autumn at our latitudes), as previously described by several authors (Sagdeo et al., 1991; Tuli and Mehar, 1983; Di Francesco, 2010).

Moreover, in fresh semen, the higher percentage of sperm displaying pattern A found in our study suggested a lower level of capacitation during the favorable season. Nevertheless, in each season a higher proportion of sperm displayed low capacitation level, confirming that associated protein tyrosine phosphorylation event starts very early compared to cattle (Roy and Atreja, 2009). On the contrary, negligible differences were observed in frozen semen. With regard to CTC patterns, no seasonal variation was recorded in fresh semen, but the percentage of capacitated spermatozoa, as well as the incidence of acrosome reacted sperm, significantly decreased in autumn compared to spring in frozen-thawed semen. However, despite the season effect, the frozen-thawed semen still contained significantly higher number of sperm displaying pattern associated to capacitation status compared to fresh semen, as reported before.

Our results confirmed that freezing–thawing induced capacitation-like changes in buffalo spermatozoa, as previously reported in bull (Cormier and Bailey, 2003), boar (Maxwell and Johnson, 1997), stallion (Thomas et al., 2006) and ram (Perez et al., 1996). However, to our knowledge, in buffalo there were no reports about the influence of season on the cryocapacitation damages. Therefore, this study revealed that season increased the cryoresistance of the sperm membrane as showed by the reduction of the percentage of spermatozoa displaying capacitation-like patterns.

The importance of these results is confirmed by the findings on fertilizing ability following heterologous IVF, using buffalo sperm to inseminate bovine in vitro matured oocytes, as previously reported (Mariotti et al., 2009). The reason behind this choice is mainly the relative scarcity of buffalo gamete availability in our settings/location, due to the lower number of heads slaughtered, together with the low number of oocytes recovered per ovary (Totey et al., 1992; Jainudeen et al., 1993), the latter arising from physiological features intrinsic to the species (Gasparrini, 2011). Furthermore, the IVEP system is well established in cattle and it was previously demonstrated that the cleavage and penetration rates after heterologous IVF are highly correlated to those observed after homologous IVF (unpublished data). It is well known that sperm acquire fertilizing ability during capacitation and hence, the assessment of oocyte penetration, as well as cleavage rates, is of the utmost importance. Indeed, when IVF was carried out by using semen collected in autumn, the overall penetration rate, as well as the normal fertilization and cleavage rates, were significantly improved compared to those recorded using semen collected in spring. In addition, no effect on polyspermy was observed. Therefore, there is an overwhelming evidence that the highest IVEP efficiency corresponds to the shortest day length, while the worst efficiency is recorded when the number of daily light hours is the highest, perfectly reflecting the seasonal pattern expressed in vivo by the species at our latitudes (Zicarelli et al., 2007a; 2007b). Our results agree with those reported in the sheep, another short-day breeder that shares many reproductive traits with buffalo (Zicarelli, 1994a; 1997a; 2002), in which a

significant decrease in efficiency has been described in the unfavorable seasonal period after IVF (Stenbak et al., 2001).

The results obtained in the first experiment suggested to limit the collection of the ejaculates for the next experiments during the favorable season, to reduce the variability related to the seasonal effect and use a superior quality semen in order to assess the effectiveness of the substances tested. Another objective of the thesis, in fact, was to develop innovative strategies to optimize freezing semen extender following two different approaches: the enrichment with either antioxidants or sterols.

The antioxidant supplementation in freezing extender has been used to defend against free radicals that potentially reduce sperm fertilizing ability. Therefore, the second part of the thesis was performed to investigate whether carnitine (Experiment 2) or resveratrol (Experiment 3) would provide protection against temperature shock and cryocapacitation damages to buffalo sperm.

In Experiment 2, the results demonstrated that the supplementation of carnitine to the freezing extender in buffalo sperm did not improve fertility parameters, such as motility and viability, as previously reported in Angora goat, where carnitine addition did not influence subjective and CASA sperm motilities (Bukac et al., 2010a). However, several authors showed that carnitine supplementation led to higher motility and viability in bovine (Bucak et al., 2010), human (Matalliotakis et al., 2000; Vicari and Calogero, 2001; Vitali et al., 1995), rat (Palmero et al., 1990) chicken (Tabatabaei and Aghaei, 2012) rabbit (El- Nattat et al., 2011) and boar spermatozoa (Newsletter, 2000). Likewise, in Experiment 3, resveratrol supplementation did not improve sperm motility and viability. These findings are

different from those reported in the bovine (Tvrdá et al., 2012), where sperm viability increased, while motility decreased at concentrations higher than the doses tested in our study. Both viability and motility of rat and human sperm showed a linear decrease at increasing concentrations of resveratrol, including 50 μM, which was the most effective concentration in our work (Collodel et al., 2011). With this regard, it is worth pointing out that resveratrol displays an important dichotomy: low doses improve cell survival, yet high doses increase cell death, as proven in in vitro culture studies (Brown et al., 2009). It is known that species-specific differences exist in dose response to substances and hence, it is likely that, as the most effective concentration was the highest tested, we could not detect any deleterious effect.

Furthermore, it is intriguing to note that in our experiments, data showed a carnitine dose dependent effect on sperm capacitation status, assessed by both CTC and tyrosine phosphorylation proteins assays. Also resveratrol acted in a dose dependent manner but the reduction of the cryocapacitation damages was observed only in the tyrosine phosphorylation protein patterns. In particular, a significant decrease of the capacitation level was detected when the highest concentrations, 7.5mM carnitine and 50μM resveratrol, were tested. We can speculate that the reduction of the cryopreservation-induced modifications to sperm membranes was due to the antioxidant properties of both compounds, which may protect sperm membranes from toxic oxygen metabolites generated during cryopreservation. In fact, it has been reported a relationship between in vitro cryocapacitation and superoxide anion production (de Lamirande and Gagnon, 1992b; de Lamirande et al., 1998) which suggested that free radicals

could play a role in the cryopreservation-induced damages. Some authors have proposed that cryocapacitation is at least partially induced by ROS originated during semen processing (Bailey et al., 2000; Allamaneni et al., 2005) and that frozen sperm showed higher sensitivity to lipid peroxidation (Trinchero et al., 1990; Salamon and Maxwell, 1995).

It is well-known the role of carnitine to reduce the availability of lipids for peroxidation by transporting fatty acids into the mitochondria for β-oxidation to generate Adenosine Triphosphate (ATP). This transport of fatty acids into the mitochondria for catabolism reduces the amount of lipids available for peroxidation (Kalaiselvi and Panneerselvam, 1998). Lipids are a basic component of semen, contributing to the membrane structure of spermatozoa, the metabolism of the sperm cells, and their ability to fertilize the female gamete (Kelso et al., 1997; Sarica et al., 2007). Although the exact mechanism of action is not completely elucidated, it is known that resveratrol reduces sperm DNA damage during the process of cryopreservation (Branco et al., 2010), prevents lipid damages induced by cryopreservation (Garcez et al., 2010) and protects sperm against oxidative stress (Collodel et al., 2011), despite its disrupting effect on calcium homeostasis (Hidalgo et al., 2013). Moreover, it is known that polyphenols compounds, such as resveratrol, are easily incorporated into the membrane's lipid bilayer, inhibiting the formation of lipid radicals and keeping the membrane integrity and the ionic equilibrium of the cell (Halliwell et al., 1999). Therefore, we can assume that the reduction of the capacitation damages detected in experiment 2 and 3 could be related to the ability of both carnitine and resveratrol to reduce lipid peroxidation in sperm. Buffalo spermatozoa are

very susceptible to peroxidation damage because of the high concentration of long chain polyunsaturated fatty acids within the phospholipids (Jain and Anand, 1976). There is considerable evidence to indicate that the lipid composition of the sperm membrane is a major determinant of the cold sensitivity, motility, and overall viability of spermatozoa (Kelso et al., 1997). Moreover, lipid peroxidation plays a key role in the aging of spermatozoa by shortening lifetime both in vivo and in vitro (Aitken et al., 1989; Cecil and Bakst, 1993).

Furthermore, sperm in vitro fertilizing capability of carnitine and resveratrol treated semen was investigated after heterologous IVF. Data showed that carnitine supplementation did not improve in vitro fertility of buffalo sperm, in agreement with a previous in vivo study in cattle (Bucak et al., 2010).

On the contrary, resveratrol extender supplementation improved the in vitro normospermic penetration; therefore AI was performed to assess the effectiveness of this compound on in vivo fertility. Although no significant differences were observed in pregnancy rates both at 25 and 45 days compared to the control, a tendency to greater pregnancies was recorded with resveratrol-treated semen. The number of inseminated animals in this study was probably too low to draw definitive conclusions and therefore an increase of the case records is worth. Moreover, the incidence of embryonic mortality was very low in both groups, highlighting the improvement of the artificial insemination efficiency in buffalo.

As previously stated, the other approach we followed to improve buffalo semen freezability was the extender supplementation with sterols, i.e. CLC complex. In Experiment 4, CLC did not improve fertility parameters, as revealed by similar

percentages of motility and viability among groups, contrary to what reported in a previous study in buffalo. Rajoriya et al., (2014) detected an increase in progressive motility and viability of buffalo spermatozoa following cryopreservation after incorporation of cholesterol. The difference may be attributed to the various factors that affect motility and viability like bull's age, season, frequency of collection and sexual excitement before semen collection (Kumar, 2012).

The most interesting result arising from this study was the strong reduction of sperm cryocapacitation observed when extender was supplemented with CLC. The treatment was highly effective, with frozen-thawed sperm showing a capacitation status closer to that of fresh semen. In particular, the maximum beneficial effect on semen cryopreservation was recorded with the dose of 3 mg/mL CLC when the capacitation status was assessed by the protein tyrosine phosphorylation assay. However, according to the CTC assay, extender supplementation with 1.5 mg/mL CLC was reported to be more effective. The discrepancy between these results could be partially explained because of the differences between the two techniques that detect the capacitation status at two different steps of the process: the ion calcium influx that occurs earlier (CTC assay) and protein tyrosine phosphorylation that is the culminating event. It is worth to remember that the capacitation process begins with an efflux of cholesterol from the plasma membrane of sperm with consequent decrease in the ratio of cholesterol/phospholipids, followed by an increase of the processes of methylation and an increase of the synthesis of phosphatidylcholine from phosphatidyl-ethanolamine (Aitken and Nixon, 2013). This involves: a

membrane hyperpolarization and an increase in its fluidity, with an increase of the ion flux of calcium and bicarbonate activating adenylate cyclase for the formation of cAMP from ATP. The cAMP activates a protein kinase A, which induces the phosphorylation of tyrosine proteins that are involved in both the subsequent acrosome reaction and hyperactivation (Aitken and Nixon, 2013). At this point, the sperm is attracted and crosses oocyte cumulus cells. The acrosome reaction is triggered by the interaction of the sperm capacitated with the zona pellucida. The interaction of gametes is a species-specific event that starts with the recognition and, therefore, the binding of complementary molecules present on the plasma membrane of sperm (receptors) and on the surface of the zona pellucida (ligands). This triggers a process of signal transduction mediated by Ca^{++} which results in the acrosome reaction, i.e. in the release of acrosomal enzymes, such as esterases, acrosine and neuraminidase, necessary because the sperm penetrates the zona pellucida and, therefore, the perivitelline space to reach the membrane oocyte plasma.

The reduction of the level of capacitation, detected in Experiment 4, suggested that treating sperm with cholesterol before cryopreservation reduces the sensitivity of sperm membranes to cooling damage. This is particularly evident in buffalo sperm, due to the lower cholesterol: phospholipids ratio (Rajoriya et al., 2014). It is known that cryopreservation induces cholesterol loss in boar (Cerolini et al., 2001), stallion (Moore et al., 2005) and buffalo (Rajoriya et al., 2014) sperm, and this loss of cholesterol could be at least partially responsible for the capacitated state observed in cryopreserved sperm. Several authors reported that increasing the cholesterol content in sperm prior to freezing

maintained higher cholesterol levels after cryopreservation in stallion (Moore et al., 2005), boar (Tomàs et al., 2014), ram (Mocé et al., 2010) and buffalo (Rajoriya et al., 2014) sperm. Therefore, it is likely that treatment of sperm with CLC modifies the capacitation pattern as shown in Experiment 4. As expected, our results showed that CLC treatment retards capacitation of frozen-thawed buffalo sperm, unlike to what reported in bovine sperm. In fact, Purdy and Graham (2004b) reported that both cryopreserved control and CLC-treated bull sperm underwent capacitation and acrosome reaction in the same time frame, even if the amount of cholesterol in the CLC-treated sperm was threefold higher than in the control sperm (Purdy and Graham 2004a).

Because of the fact that CLC treatment affects sperm capacitation, it is likely that it might also affect the fertilizing capacity of the sperm. In most in vitro studies sperm treated with CLC exhibited higher oocyte penetration rates and/or higher numbers of sperm bound to the zona pellucida or egg perivitelline membranes in stallion and boar (Moore et al., 2005; Tomàs et al., 2009e). However, other studies reported similar in vitro fertilization rates in cattle (Purdy and Graham, 2004b) and zona pellucida binding rates in stallions (Spizziri et al., 2010) for CLC-treated and control sperm.

Our results of the in vivo fertility trial showed that although sperm quality, in general, is higher for CLC-treated sperm after cryopreservation, this is not translated into an increased fertilizing ability in vivo. In fact, when 1.5 mg/mL CLC-treated sperm were used for artificial insemination using standard breeding procedures, pregnancy rates were similar to control sperm. However, when 3 mg/mL CLC-treated sperm were used, pregnancy rates were reduced although

not significantly. Although the number of cows inseminated in each treatment group was too small to detect a difference between the control and CLC-treated sperm, the trend is at least in the desired direction, suggesting that CLC treatment before freezing may improve fertility rates. However, a large fertility trial is required to verify our hypothesis. Our results are in agreement with most studies in which CLC-treated and control sperm exhibit similar fertility rates (Müller et al., 2008; Purdy and Graham, 2004b; Purdy et al., 2010; Spizziri et al., 2010). Nevertheless, lower fertility rates for CLC-treated sperm compared to control sperm have been reported in stallion (Zahn et al., 2002).

The reason that increased sperm quality, as shown by the lower percentage of cryocapacitation damages reported in our study, does not translate into a higher fertilizing ability is not known. Some authors suggested that cholesterol treatment might modify certain sperm plasma membrane domains, but not those important for sperm fertilization (Müller et al., 2008), and others that the CLC-treated sperm have altered capacitation requirements before they can acrosome react in vivo (Spizziri et al., 2010). It should be noted that the AI protocols used in all studies to date applied protocols developed for control sperm and hence the insemination time may not have been optimal for CLC-treated sperm, which may require more time to accomplish the capacitation process in the female reproductive tract. It is likely therefore that Nevertheless, we would like to emphasize that even if preliminary results show similar fertility rates, the CLC technology could be useful for bulls, with sperm that do not normally freeze well and undergo high cryocapacitation damages, to stabilize membranes prior to freezing. As these damages are very intense in buffalo, this in turn may allow to

increase the number of bulls to enroll as semen donors. This might be especially important in several types of breeding programs, when males having valuable genetic merits might be eliminated because of their sperm not fulfilling threshold sperm quality parameters after cryopreservation, as well as for creating genetic resource banks, when it is mandatory to keep as much genetic variability as possible.

Conclusions

In conclusion, it was shown that cryopreservation affects sperm quality, capacitation status and fertility in buffalo hence, influencing the efficiency of advanced reproductive technologies, such as IVEP and AI. The results of experiment 1 demonstrated that, despite the evident decline in sperm quality due to the cryopreservation procedures, season affects fertility parameters, capacitation status and in vitro fertility, with an improved sperm quality during autumn compared to spring. This suggests to limit the semen collection during the favorable season to improve benefit: cost ratio and at the same time to focus the attention on strategies aimed to improve the quality of semen during the unfavorable season.

The results of experiment 2 and 3 demonstrated that the supplementation of carnitine or resveratrol to the freezing extender in buffalo sperm significantly reduces the cryocapacitation damages induced by freezing. Within the antioxidants tested, resveratrol also increased the normospermic penetration rate after heterologous IVF, indicating an improvement of the fertilizing capability. This suggests that incorporation of resveratrol in the extender for buffalo semen may be useful to improve IVEP efficiency in buffalo, particularly for hypofertile bulls. In addition, a tendency to increased pregnancy rates was also found after AI.

Finally, the results of experiment 4 showed that treating sperm with cholesterol-loaded cyclodextrins increased sperm membrane integrity as indicated by the reduction of sperm displaying high levels of capacitation. The treatment was very

effective, with the capacitation status of frozen-thawed sperm approaching that of fresh semen. However, treating sperm with CLC has not resulted in higher in vivo fertility rates. This may be due to CLC treated sperm requiring more time to capacitate within the female reproductive tract and therefore the optimization of the insemination time may be pursued in order to increase the efficiency. However, a tendency to improve pregnancy rates was observed when 1.5mg/mL CLC - was used to treat sperm.

Larger AI trials with both resveratrol and CLC-treated sperm are ongoing to demonstrate their effectiveness on in vivo fertility. Furthermore, our results suggest in perspective studies to apply the new formulations of the extenders to cryopreserve semen collected during the unfavorable season, in order to improve sperm quality. The improvement of sperm quality using these innovative corrective strategies will result in higher AI and IVEP efficiencies facilitating the diffusion of these technologies in the field.

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