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Analysis of factors affecting embryo recovery in superovulated buffaloes

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Abstract

The main objective of this thesis was to investigate the different factors affecting the embryo recovery in superovulated buffaloes. Several hypotheses have been formulated over the years to explain the low embryo recovery recorded in this species after superovulatory treatment, among which the most likely is a failure of ovum capture. Before addressing the major issue, other experiments were carried out in order to select both the donors and the most suitable superstimulation treatment. Indeed, another critical factor limiting the application of this technology in buffalo is the high between-animal variability in the number of embryos produced. Therefore, the aim of Experiment 1 was to evaluate whether the Anti-Mullerian hormone (AMH) can be used as a reliable marker to select good donors in addition to the screening of the antral follicular count (AFC), before enrolling buffaloes in embryo production programs. In order to do so, 54 multiparous Italian Mediterranean Buffalo cows were synchronized by double prostaglandin administration spaced 11 days apart and were slaughtered at a local authorized abattoir 6 days after the second administration. On all animals AFC, recovery rate and oocyte quality were recorded, and both plasma and follicular fluid (FF) were collected for AMH determination. In a representative number (n=22) the mRNA expression of six genes (AMHR2, CYP19A1, FSHR, LHR, TP53INP1, and CASP3) was analysed in granulosa cells (GCs). Finally, buffaloes were classified according to the AFC as good (≥ 12 follicles) and bad (< 12 follicles) donors, in order to evaluate the differences in reproductive parameters. Differences in AMH concentration in FF and gene expression in GCs among classes of follicles were analysed by ANOVA. Pearson correlation analysis was carried out to evaluate the relationship between AMH and the AFC, as well as among other reproductive parameters. The differences in AMH plasma levels and reproductive parameters between good and bad donors were analysed by Student's t test. The results of Experiment 1 showed a positive correlation ($r = 0.31$; $P<0.05$) between intrafollicular AMH concentration and the AFC in buffalo. Interestingly, good

donors had a higher ($P < 0.05$) concentration of AMH in FF and AMHR2 levels in small follicles and higher ($P < 0.05$) LHR levels in large follicles than bad donors. However, no correlation was found with the plasmatic AMH levels, likely due to the poor sensitivity of the available kits. Thus, one of the future objectives could be to assess a more reliable specific assay in order to use this as a marker to screen donors. The aim of Experiment 2 was to evaluate the efficacy of replacing the last four FSH injections with a single administration of 1000 IU of eCG compared to the conventional FSH decreasing doses superovulation protocol. The study was carried out on 20 multiparous Italian Mediterranean buffaloes synchronized with a common double prostaglandin protocol. Then, animals were divided in two groups: one treated with a superovulatory protocol with FSH decreasing doses and one with the eCG replacing the last four FSH administrations. All cows were artificially inseminated at oestrus and ultrasonographic investigations in the two groups were carried out to assess follicular dynamics and to establish the number and size of corpora lutea on the ovaries. Differences between treatments were analysed by Student's t test. It was demonstrated that the replacement of the last four injections of FSH with a single administration of eCG within a superovulation protocol in buffalo is not recommended, due to the presence of a high number of anovulatory follicles at the moment of flushing, and a reduced number of ova compared to the conventional FSH treatment (3.0 ± 0.6 vs 6.6 ± 0.9 and 2.0 ± 1.2 vs 0.8 ± 0.6 respectively). However, a poor recovery rate in relation to the number of corpus luteum (CL) recorded in both protocols remains. In order to select the most reliable control group for the superovulation study, the aim of Experiment 3 was to evaluate the relationship between the ovarian follicular response at the start of an Ovsynch program and the pregnancy outcome. The study involved 116 pluriparous buffaloes that were synchronized by Ovsynch–TAI program, involving the administration of GnRH on Day 0 (GnRH1), PGF2a on Day 7, and GnRH on Day 9 (GnRH2), with timed artificial insemination (TAI) carried out on Day 10. Ovarian ultrasound examinations were undertaken on days 0, 2, 7, 9, 10, and 11 to record total follicles, the dimensions of follicles greater than 0.75 cm, dimensions of the CL,

and the occurrence of ovulation. Blood concentrations of progesterone (P4) were measured on days 0 and 10 and 10 days after TAI. Pregnancy status was determined on days 27 and 45 after TAI to calculate late embryonic mortality. The data were analysed by ANOVA and multiple logistic regression. The results of Experiment 3 showed that only 60% of the buffaloes ovulated after the first GnRH on day 0 of the Ovsynch-TAI program. A higher proportion ($P < 0.01$) of buffaloes that ovulated after GnRH1 had a vascularized corpus luteum (CL) on day 7. Furthermore, a greater proportion ($P<0.05$) of buffaloes that ovulated after GnRH1 was in oestrus and both a higher ovulation rate and pregnancy to AI were observed compared to buffaloes that did not ovulate. The results of the experiment 3 showed how important is the ovulation after the 1st GnRH to the pregnancy outcome, during Ovsynch-TAI program. This is due to both an optimized response of the CL to $PGF_{2\alpha}$ on day 7 and a better response of the preovulatory follicle to second GnRH on day 9, increasing the likelihood of pregnancy to TAI. The results of this experiment allowed us to better plan the experiment 4, by selecting the animals that ovulated after the 1st GnRH as a control group to compare with superovulated animals. Therefore, the aim of Experiment 4 was to compare the morphological parameters of the follicles and the steroid profile (progesterone and oestradiol) both in plasma and follicular fluid in the peri-ovulatory period in superovulated vs synchronized buffaloes. Thirty-five multiparous Italian Mediterranean Buffalo cows were randomLy divided into two groups: a group (n = 25) was synchronized by Ovsynch and another group (n=10) was superovulated with conventional FSH protocol and all of them were sacrificed 18 h after GnRH. On all animals AFC, recovery rate and oocyte quality were recorded, and both plasma and FF were collected for steroid profile determination. In addition, out of 10 animals (5/group), GCs were collected to analyse the mRNA expression of gonadotropin receptors LHR and FSHR while oviducts were collected to evaluate the mRNA expression of steroid receptors ER1 and PGR, VEGF and the VEGF receptor FLK1. Differences in morphological follicular parameters, as well as in the concentration of progesterone and oestradiol both in plasma and in follicular fluid were analysed by ANOVA, while

differences in the recovery rate and in the percentage of oocytes showing cumulus expansion were analysed by Chi Square Test. Experiment 4 demonstrated that in superovulated animals the E2 and P4 concentrations in FF were lower ($P<0.05$) than in synchronized animals. Interestingly, both the recovery rate and the percentage of oocytes exhibiting proper cumulus expansion decreased ($P<0.05$) in the superovulated animals. Finally, in superovulated buffaloes a decreased expression of both ER1 and the VEGF receptor FLK1 in the infundibulum and an increased expression of FSHR in granulosa cells were observed ($P<0.05$). The results of the latter experiment suggest that the exogenous FSH treatment has probably increased the expression of FSHR in granulosa cells that was not followed by a parallel increase of oestradiol synthesis, eliciting an alteration of the granulosa cell function. The consequent altered steroid profile could affect in turn both the cumulus cells expansion during maturation, and the contraction-relaxation of the infundibulum, confirming the hypothesis of a failure in ovum capture in superovulated buffaloes. In conclusion, the results of this thesis taken together highlight the limitations of MOET in buffalo, suggesting that at present the only way to improve the intensity of genetic selection, through the maternal lineage, in this species is the OPU-IVEP technology. Indeed, buffaloes do respond to the gonadotropin treatment with multiple ovulations but the ova capture is severely impaired, resulting in poor embryo recovery. Nevertheless, the outputs of this thesis led to an improved knowledge and understanding of the limiting factors of the superovulation technique in this species.

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 (especially in Egypt) and the rest in Europe (particularly in Italy, but also in Romania, Georgia, and Bulgaria) and Latin America. 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.

In the last years, interest in the world regarding buffalo breeding has increased. This is because in tropical countries environmental and climatic conditions make buffalo an irreplaceable milk producer; indeed, this specie best suit to satisfy animal protein demand, as it is easy fitting, rustic, long-lived and parasite resistant. Moreover, 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 unfavourable 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 relatively low maintenance requirements are attributes, which make them ideal in low-input, low-cost production systems (Zicarelli, 1994a). 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, thanks to long isolation and lack of crossbreeding with other strains, recognized the

"Mediterranean Italian buffalo" breed (Ministerial Decree 201992 of 05/07/2001). 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 Caserta and Salerno provinces, where 66% 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, fine-tuning and perfecting breeding techniques and coming to an efficient intensive or semi-intensive approach. This advance has been made possible selecting only the most productive buffaloes, improving the quantity and the quality of milk, with the aim to increase productive and genetic value and transform a marginal sector into an area with great economic potential. In our country, the profitability of this species is particularly linked to its well-known product: the “Mozzarella di Bufala Campana” cheese, enhanced and protected by a Denomination of Protected Origin (DPO) trademark and, above all, recognized in most of the countries. Therefore, in Italy, the commercialization of milk for mozzarella cheese production represents the main income of buffalo breeding.

It is worth to point out 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 buffaloes with the highly productive Mediterranean buffalo, in order to increase animal proteins and, most of all, meet human needs. 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. In addition, reproductive technologies are fundamental also to meet the constantly increasing requests of the Mediterranean buffalo semen and embryos from many countries.

Reproductive biotechnologies

Reproductive biotechnologies are undoubtedly the most emblematic strategies of applied research in the field of life sciences and animal husbandry. These new technologies have significantly contributed 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. Reproductive biotechnologies allow planning selective directions in a shorter time, allowing the distribution of elite buffalo genes, the reduction in generation interval and providing continued genetic gain, resulting in an increasing production of buffalo meat and milk. 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 (AI)

This technique consists in the collection of semen from animals of high genetic merit and its dilution and utilization in recipient females. Artificial insemination (AI) offers many advantages over natural breeding: it allows the choice of the best bulls to improve the genetic make-up of a buffalo herd and gives the opportunity to quickly obtain multiple generations of the same bull. 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 venereal diseases, as well as other infectious diseases, since the males that are selected for AI undergo severe sanitary controls to verify that they have not been in contact with pathogens, such as vibrio or trichomonas, and that there is no brucellosis, leucosis, tuberculosis or IBR infection going on. These risks are vastly reduced with the use of AI. From a genetic point of view, AI allows a quicker 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. By using AI, taking into consideration the number of straws that can be obtained from a single ejaculate following dilution and freezing, one bull can produce up to 200.000 calves, respect to the 3000 obtained by natural insemination. Another benefit of this reproductive technique is the availability of semen even after death of proven sires and the possibility to transport worldwide frozen semen promoting a genetic improvement worldwide. Another important aspect related to AI is the customization of the best sire to the recipient characteristics. This allows corrections of some morphological and productive defective traits of recipients that will not be carried over to their progeny resulting in calves with different characteristics. 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 often infertile. In addition, they need higher feed requirement, special housing and handling equipment. This was a major stimulus to the initial setting up of AI services. 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.

Despite this, the application of AI has some possible disadvantages. The major limitation is the long time needed in order to prove sires through progeny testing, i.e. testing of their daughters for milk

production. In buffaloes, this process requires at least four to five years, since: over 10 months are required for pregnancy, at least other 20 months are needed to reach puberty and an additional 10 months are required for the subsequent pregnancy of the progeny. Data will be analysed only at the end of the first lactation, and after all this time it could be possible that bull shows negative effects on milk production and hence must be excluded from reproduction. Moreover, currently there are still few proven sires across many farms and this may cause, in the long run, a considerable increase of consanguinity and inbreeding. In order to avoid this problem, a better specific breeding program is needed.

Although buffaloes are probably among the easiest mammals to be trained to serve an artificial vagina (Sansone et al., 2000), natural mating is still widely used in buffalo herds. Indeed, the use of AI in both Swamp and River buffaloes is still marginal for different 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 two decades ago, mainly due to lack of truly superior progeny tested bulls and, most of all, to unacceptable low pregnancy rates, significantly affected by the seasonality of the species (Campanile et al., 2005, 2007a, 2008), when using both natural or synchronized oestrus (Zicarelli et al., 1997a). At our latitude, AI is strongly influenced by seasonality, as shown by the high incidence of embryonic loss (20-40%) during seasons with a higher number of light hours (Campanile et al., 2005, 2007a, 2007b). Furthermore, Rossi et al., (2014) reported a marked seasonal variation in the response of Italian buffaloes to synchronization protocols. Buffalo is a short-day breeder, showing an increase of reproductive activity when daylight length decreases (Zicarelli, 1997; Campanile et al., 2010). The influence of photoperiod results in seasonal cycles in conception, calving and milk production (Campanile et al., 2010). This reproductive pattern depends on the duration and the intensity of the light source. Only around the equatorial belt, where the light/dark ratio is similar throughout the year, the reproductive season is more influenced by

forage availability (Vale et al., 1990). In Italy, in order to meet the market demand, the out-of-breeding season mating strategy is applied, that entails the interruption of natural mating or the use of AI between October and February in adult females and between September and March in heifers. It follows that animals are mainly mated out of the natural breeding season, when reproductive efficiency is low and seasonal anoestrus may occur. This results in high incidence of embryonic mortality (Campanile et al 2005, 2007). Embryonic loss during the unfavourable season is in part due to compromised luteal function, leading to reduced progesterone level (Campanile et al., 2005, 2007), and in part to impaired oocyte competence (Di Francesco et al., 2012). Indeed, an appropriate CL development, vascularization and function during the first weeks after AI affects pregnancy outcome (Russo et al., 2010, Vecchio et al., 2012). In addition, the size that the embryo reaches on day 25 after AI is important for subsequent pregnancy maintenance (Neglia et al 2012). Indeed, a delayed embryonic development, associated to low P4 levels, is reflected in altered proteomic profile of the embryonic chorioamnion and uterine caruncles (Balestreri et al., 2013) and abnormal embryo transcriptome (Strazzullo et al., 2014). In order to counteract early embryonic mortality, hormonal treatments to suppress luteolytic mechanisms have been given either on the day of AI or on day 5 after AI (Neglia et al.,2008; Campanile et al., 2007; Pandey et al., 2015, 2016). However, the major cause of embryonic loss in buffalo is late embryonic mortality (LEM), occurring between 25 and 45 days, where P4 decline occurs later, after day 10 (Russo et al., 2010) affecting embryo attachment, by altering MUC-1 expression in the uterine epithelium. In fact, it was demonstrated that treatment with progesterone, GnRH agonist and hCG on day 25 post-AI was effective to decrease the incidence of LEM (Campanile et al., 2008).

Peculiarities of the oestrous cycle, such as the high variability in the duration of the oestrous cycle and oestrus, the high incidence of double ovulations and the lower expression of oestrous behaviour, also delayed the application of AI in this species. The oestrous cycle in fact varies from 16 to 28 days (Neglia et al., 2007; Baruselli et al., 1997); in buffaloes showing regular oestrous cycles the length of

oestrus is around 10-20 h during the breeding season (Vale et al., 1984), although a large variability (from 2 to 72 h) is observed, particularly out of the breeding season (Baruselli, 2001). Furthermore, the expression of oestrus, as well as the oestrus-associated mounting behaviour are less intense compared with cattle (Ohashi, 1994). It has been reported that only 3.44% of the females present homosexual behaviour (Baruselli, 2001).

Recently though, an improvement of the efficiency in protocols for synchronization of oestrus and ovulation has been reported in buffaloes (Neglia et al., 2008, 2016), 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., 2001a; Campanile et al., 2011; Neglia et al., 2015). Currently, AI is routinely applied in intensive river buffalo systems and the good results obtained with sexed semen (Campanile et al., 2011; 2013) make us foresee an even wider diffusion in the field.

Further aspects still need to be considered and investigated in order to spread diffusion of AI in buffalo management worldwide. Among these, one of the most important is the semen cryopreservation procedures and hence, the quality of frozen-thawed semen that is known to be related to low fertility rates in buffalo (Kumaresan et al. 2005, 2006; Shukla and Misra 2007).

Multiple Ovulation (MO) and Embryo Transfer (ET)

The MOET is the technology currently used to get half of the embryos produced for commercial purposes in the world (IETS, 2015). This technique consists of the administration of exogenous hormones in order to obtain multiple ovulations in animals that normally ovulate a single follicle. MOET schemes are used to increase the intensity of genetic selection, reducing generation intervals, and to select bulls to be used for AI (Gearheart et al., 1989). In this case, previously selected donor dams are inseminated with the semen of bulls showing superior traits. As regarding the offspring, females are used only for production, while males will wait the production results of their sisters

(Smith and Ruane, 1987). The difference with AI is that males are tested and chosen according to the productive performances of their sisters, instead of their offspring and this makes possible to genetically test a bull in 3.5 years instead of 5.5 years. (Smith and Ruane, 1987).

In cattle MOET programs have improved over the years; however, wide ranges in superovulatory response and embryo yield in several different species have been reported (Armstrong, 1993). In fact, Gordon (1975) stated in 1975 "although superovulation as a method of obtaining a supply of eggs has taken many forms over the years; it still leaves much to be desired. In fact, it must still be regarded as a major problem blocking progress in exploiting egg transfer in increasing numbers of progeny from genetically superior cattle". Although considerable recent progress has been made in the study of ovarian physiology, manipulation of ovarian function and gonadotropin biochemistry, factors inherent to the donor animal which affect superovulatory response are only partially understood. Thus, a high degree of unpredictability in superovulatory response still exists more than 35 years later, creating problems, which affect the efficiency and profitability of commercial embryo transfer. However, currently, MOET is still the technique that produces more embryos in the world, for a total of 600,000 embryos per year, of which about 460,000 are transferred (IETS, 2015). Traditionally, superovulation was performed between the 9^{th} and the 11^{th} day of the bovine oestrous cycle, in coincidence with the emergence of the second follicular wave. Subsequently, on day 6-7 after AI embryos are recovered by flushing the uterine horns of the donors with isotonic buffered solutions. The recovery of embryos is performed in different ways: in the early development stages of the technique, embryos were recovered either surgically, or by laparoscopy, or by transvaginal insertion of a catheter into the uterus. Further development resulted in a technique involving 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 oestrus synchronization of the recipients, the embryos are transferred in surrogate females for the gestation.

Several extrinsic and intrinsic limiting factors influence the response to MO in buffalo that will be discussed deeply in the next chapter. Indeed, although the birth of the first embryo transfer buffalo calf occurred more than three decades ago (Drost et al. 1983), the application of MOET in this species is still limited, due to poor efficiency compared to cattle (Drost 2007; Madan et al 1996; Carvalho et al. 2002). In fact, using this technique in bovine, the mean embryo recovery per animal is 5, whereas in buffalo there is an average recovery of 1-2.5/donor, (Zicarelli 1997; Baruselli et al., 1999; Carvalho et al., 2002; Misra and Pant, 2003; Misra and Tyagi, 2007; Neglia et al., 2010; Qin et al., 2012). Due to the low and inconsistent efficiency of MOET treatments in this species (Misra et al, 1997; Zicarelli et al, 1997), 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.

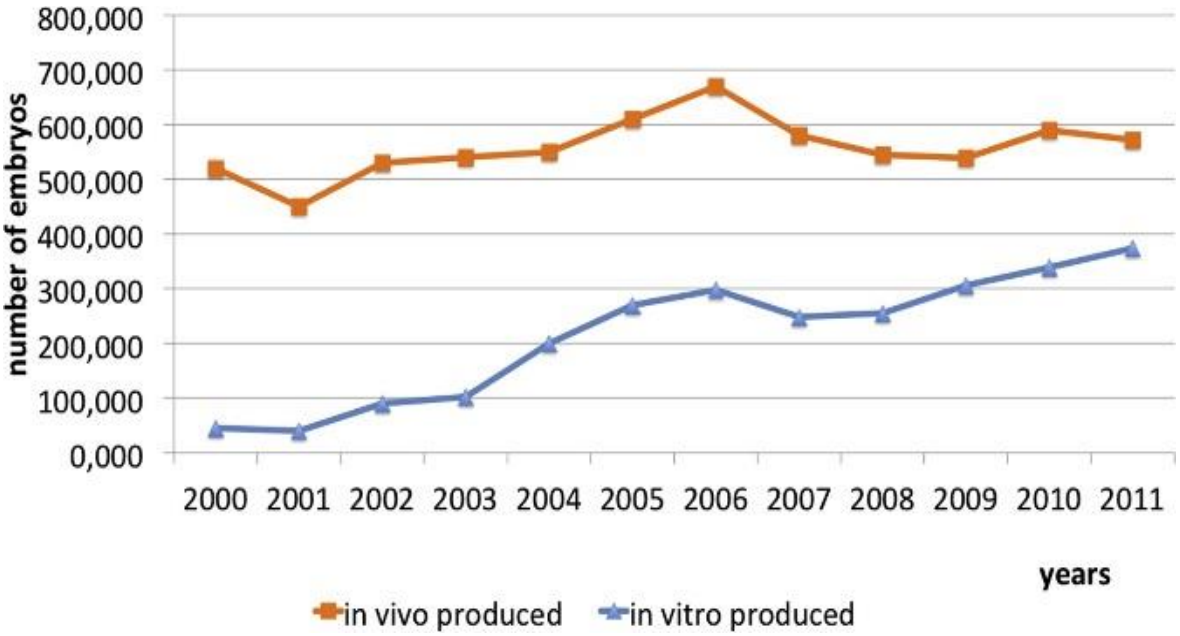
Ovum-pick up and in vitro embryo production

The advent of Ovum Pick-Up (OPU), i.e. the in vivo oocyte collection from live donors, has effectively increased the worldwide interest in the *in vitro* embryo production (IVEP) procedure, as a valid alternative to the *in vivo* embryo production. It consists in an ultrasound guided transvaginal procedure to recover oocytes from a live donor. It is an alternative technique to MOET because it can be performed on a wider typology of donors, such as acyclic, prepubertal animals (Armstrong et al., 1992), pregnant cows up to the 3^{rd}-4^{th} month of gestation, animals with non-perfect conditions of the reproductive tract (Galli et al., 2001) or at the end of their productive career and animals that are not sensitive to the hormonal stimulation treatments. Moreover, since it does not interfere with animal reproductive and productive cycles and does not require any hormonal pre-treatment, unlike MOET method, it has no negative impact on the treated animal and may have a therapeutic effect in animals with ovarian cysts or similar diseases. The optimal interval between aspiration sessions is 3-4 days

(Boni, 1997; Galli et al., 2001). In fact, the aspiration of all follicles with diameter >2 mm, resets the oestrous cycle and avoids the dominance of one follicle over the subordinates.

The combined OPU and IVEP technology is currently the most efficient tool for increasing the number of transferable embryos obtainable per donor. According to the International Embryo Transfer Society statistics, the number of embryos produced in vitro and transferred into recipients has increased more than 10 times (Stroud, 2011) in the last dozen years, approaching the number of embryos produced in vivo by superovulation (Fig. 1).

Figure 1. Comparison of the number of in vivo and in vitro embryos transferred annually for the last decade.



This indicates that OPU and IVP is considered a reliable and cost-effective technique and has acquired a role in cattle breeding.

In buffalo, this technology has even greater potentials because, as mentioned before, superovulation gives poor results compared with cattle (Gasparrini 2002) and it has never had a real impact on

buffalo breeding programs. This is both because of the limited number of embryos that can be recovered and low proportion of animals responding to the hormonal stimulation (Zicarelli, 1997b; Techakumph et al., 2001), as well as 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 anoestrus. Because of these limits, the OPU technology has always been of great interest also in buffalo breeding. Boni et al. (1994) reported the first OPUs in buffalo and the procedure is performed exactly in the same way as in cattle. Because of the great value of the female offspring in buffalo herds, the combination of OPU with cryopreservation (Galli et al., 2012) and use of sexed semen for IVF (Liang et al., 2008) offer the opportunity to accelerate the genetic gain in the buffalo industry. The competitiveness of OPU-IVEP becomes overwhelming when donors are selected based on their folliculogenetic potentials. Earlier OPU trials performed in the Mediterranean Italian Buffalo reported an extreme individual variability in the number of follicles (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 two 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. Moreover, 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, 2008).

However, although the buffalo IVEP system has greatly improved through the years, leading to high blastocyst yields (Gasparrini et al., 2006a; Neglia et al., 2003) 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. As mentioned before 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. Is it known that *in vitro* produced embryos are still less viable and cryotolerant than their *in vivo* counterparts (Leibo and Loskutoff, 1993; Hasler, 2000). In addition, *in vitro* produced embryos display several alterations in morphology, metabolism and gene expression that may result in poorer cryotolerance, higher incidence of embryonic loss and hence lower pregnancy to term after ET, as well as foetal abnormalities, post-implantation defects and long term effects, all symptoms of the well described large offspring syndrome (Bertolini et al. 2002; Crosier et al. 2002; Rizos et al., 2008). It is also worth pointing out that, although IVEP allows a greater embryo yield over a medium-long term, resulting in a higher number of offspring, the relative efficiency is lower and costs of production much higher than MOET.

In addition, unlike cattle, for economic reasons, buffalo cows are usually slaughtered when they are old or when their fertility and productivity are compromised (12 years in Italy and even longer in China and South East Asia). 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.

Indeed, MOET could be a cheaper alternative or a complementary approach for embryo production, suitable for practitioners, as it does not require expensive laboratory facilities. It follows that an improvement of MOET efficiency in buffalo, through a better comprehension of the factors involved in the failure of embryo recovery may provide a valid parallel tool to satisfy the increasing embryo demand from many countries of the world. As this is the major topic of this thesis, a more detailed update of MOET technology in cattle and buffalo is provided below.

Basic concepts on superovulation

In order to achieve a better comprehension of the superovulation schedules some information on follicular dynamics should be provided. In most domesticated animal species (e.g. cow, sheep, goat, horse) follicular development during the oestrous cycle takes place in a wave-like pattern (Evans et al., 1994). Normally, bovine cycles present two or three growth waves but cycles with one or four waves have also been observed (De Rensis and Peters, 1999). As well as the other species, buffalo follicular cycles are organized in a wave-like pattern, in both natural and synchronized oestrus (Baruselli et al., 1997; Neglia et al., 2007, Campanile et al., 2010). More than 60% of buffaloes show two waves for cycle and the remaining shows three waves for cycle. The length of the cycle is normally 23-24 days, although shorter (16 days) and longer (26-30 days) cycles have been observed (Neglia et al., 2007). In buffaloes having a two-wave growth pattern, the emergency of the first growth wave occurs on day 1 and the second on days 12-14. In those with a three-wave growth pattern the second and the third wave come out on days 11 and 20, respectively (Neglia et al., 2007). During each wave, the phenomena of recruitment, selection and dominance occur (Ginther et a., 2001). At the moment of the recruitment, the increasing circulating levels of FSH allow the growth of a cohort of 6-8 follicles (Baruselli et al., 1997). Later, the phenomenon of selection and dominance takes place: in buffalo, a monovular species, normally only a single follicle will develop into a dominant follicle, causing the regression of the other. The dominance occurs when the dominant follicle, after 3 days of growth, reaches the diameter of approximately 7.4 mm (Gimenes et al., 2011). During the deviation phase, the follicle acquires the LH receptors. LH plays a key role since, before this time, all growing follicles could become dominant, whereas after deviation only the largest follicle reaches the dominance phase, letting the others regress. Moreover, some studies carried out in bovine heifers demonstrated that LH receptors emerge in granulosa cells of the future dominant

follicle about 8 hours before the beginning of deviation (Ginther et al., 2001). Moreover, the intrafollicular oestradiol: progesterone ratio declines, laying the basis for the ovulation (Mihm et al., 2006).

Hormonal control for optimizing superovulation

As introduced above, the aim of this technique is to overcome the physiological limitations of one ovulation per buffalo oestrous cycle. For this reason, the follicular dynamics is altered and modulated by the exogenous administration of hormones some days in advance of the anticipated normal oestrus in order to obtain multiple ovulations in the donor. This is inevitably achieved by avoiding the dominance phase, thus allowing the growth of a large number of dominant follicles to acquire ovulatory capability. In the first trials carried out on superovulation in both cattle and buffalo the treatment conventionally started during mid-cycle. Bó et al. (1995) reported that a greater superovulatory response is obtained when the superstimulatory treatments begun 8 to 12 days after oestrus. Using ultrasonography, it was demonstrated that these days would correspond approximately to the emergence of the second follicular wave, when on the ovary there is a cohort of growing follicles. Due to this reason, the protocols used nowadays involve the initiation of superstimulatory treatments after the exogenous control of follicular wave emergence. Different methods have been used to elude the effect of the dominant follicle and reduce the atresia (see below), such as hormones administration (oestradiol, progesterone, GnRH, eCG, etc.), or mechanical techniques, as the ablation of the dominant follicle by ovum pick-up (Zicarelli et al., 1995).

Only with a synchronized cycle, the superovulatory treatment could be initiated. Normally, MOET is carried out with an intravaginal device releasing progesterone, on one hand to avoid an endogenous LH peak and ensure synchronous growth of all follicles and, on the other hand, to ensure high progesterone levels that are beneficial for subsequent embryo development (Bó and Mapletoft, 2014).

Several hormones have been utilised to manipulate the follicular development in both bovine (Mapletoft and Bó, 2012; Bó and Mapletoft, 2014) and buffalo (Zicarelli, 1997; Misra and Tyagi 2007). In particular, three different types of gonadotropins are used:

1) Pituitary extracts of porcine or other domestic animal origin;
2) equine chorionic gonadotropin (eCG);
3) human menopausal gonadotropin (hMG).

These hormones show different characteristics that will be analysed more in details below.

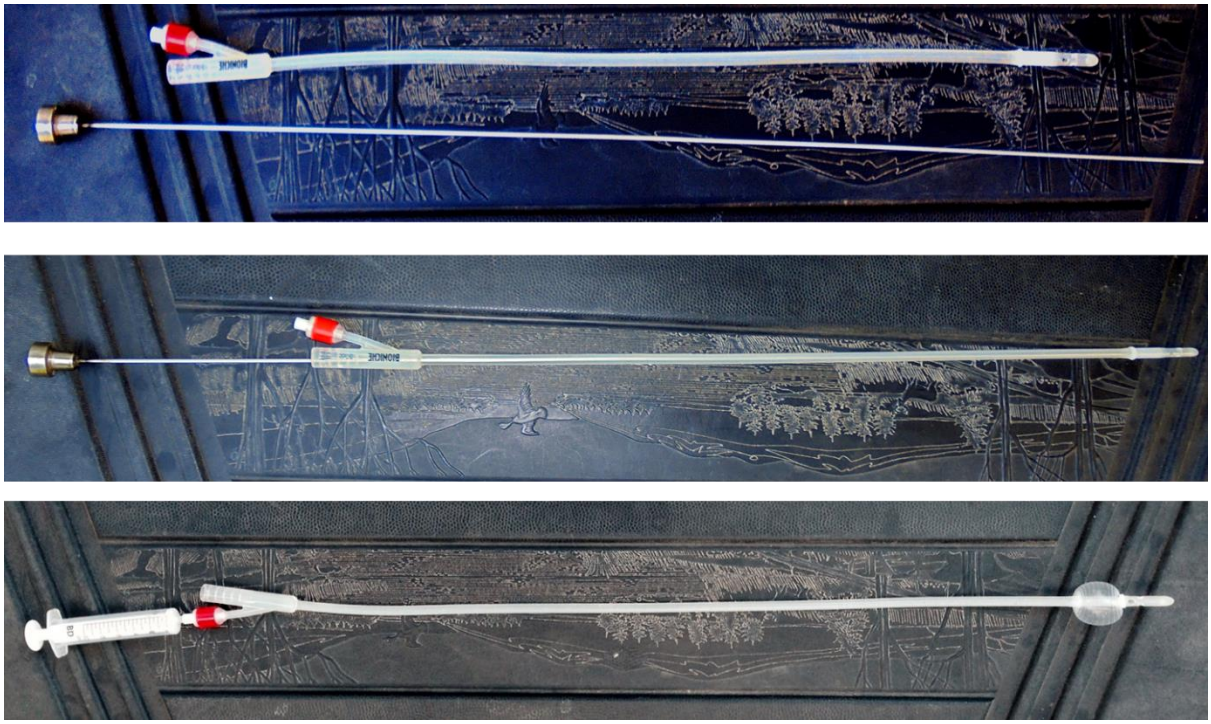
Conventionally an administration of prostaglandin $F_{2\alpha}$ (PGF2α) is made one day before the end of the superovulatory treatment, to ensure corpus luteum (CL) regression and the donors are naturally or artificially inseminated about 60 and 74 hours after this administration. In order to apply fixed-time AI a GnRH injection can be given to synchronize the ovulation, on the day before AI.

Uterine flushing

Six days after insemination, the non-surgical embryo collection is carried out by performing a uterine flushing with the aid of a Fooley catheter. This is a flexible tube that is inserted in the uterus by using a mandrel until about 2/3 of each uterine horn. The Fooley catheter is characterized by two separated channels (2-way catheter), running down its length. One channel is open at both ends and allows the inflow and outflow of flushing medium. The other lumen has a valve on the outside end and connects to a balloon at the tip; the balloon is inflated with air when it lies inside the uterine horn in order to stop it from slipping out. The Fooley catheter (Fig. 2) is inserted through the vagina, cervix and into one of the uterine horns where the balloon is inflated. Due to the presence of an intercornual ligament buffaloes have the uterine horns less extensible and elastic than those of cattle (Vittoria, 1997). This anatomic feature makes the insertion of the catheter not as easy as in cattle and expose the basis of

the horn to the risk of laceration if the balloon pressure is not suitably regulated (Zicarelli, 1994). Usually 8 to 22 cc of air are used in buffalo species (Zicarelli, 1997).

Figure 2. Fooley catheter



The inflated balloon will seal off the anterior portion of the uterine horn, avoiding fluid from getting out of the uterus when the flushing medium is inserted into the uterine horn to recover the embryos. After a certain amount (around 500-750 mL) of medium is "flushed" into the uterus, the fluid is then recovered from the uterus and this process is repeated on the other horn. Each uterine horn is flushed with commercially available, complete, and ready-to-use flushing media containing antibiotics and bovine serum albumin as a source of protein. Some of them also contain surfactants to minimize the formation of foam and bubbles when the embryo is searched in the Petri dish. In the past, the flushing medium consisted primarily of Dulbecco's phosphate buffered saline (PBS) with antibiotics and 1%

foetal/calf bovine serum (FBS/FCS) or alternatively 0.1% bovine serum albumin (BSA). This solution is actually largely utilized in the world for its low costs. It is worth to point out that the flushing solutions should be maintained at temperature of 30-35°C, to avoid thermal shock to the embryos at the recovery.

Buffalo embryos have a faster rate of development compared to bovine (Chantaraprateep et al., 1989; Drost and Elsden, 1985; Anwar and Ullah, 1998; Misra et al., 1998) and these results have been confirmed from further researches performed *in vitro,* demonstrating that buffalo embryos are 12 to 24 hours more advanced than the bovine counterparts (Galli et al., 2001). Moreover, oocytes and embryos in buffaloes reside in the oviduct for 74-100 hours post-fertilization (Karaivanov et al., 1987) and thus reach the uterus 4.5-5 days after fertilization. Likewise, in Nili-Ravi buffaloes (Anwar and Ullah, 1998), the embryos are in the oviduct after 85 hours post insemination and at 108 hours most of them (78%) descend into the uterus. It seems that buffalo embryos are at morula stage when they reach the uterus (Anwar and Ullah, 1998), similarly to that described in bovine at 120 hours (Betteridge and Flechon, 1988). This would confirm that the descent of the embryos into the uterus might occur a few hours earlier in buffalo than in cattle. Compact morulae are observed from 125 to 152 h post-oestrus and blastocysts from 141 hours. For this reason, it is reasonable to anticipate the flushing in buffaloes on day 6 rather than on day 7 as usually performed in cattle.

Embryo collection

When the flushing is completed, the cuff is deflated and the contents of the catheter are carefully allowed to flow into a sterile holder located at the end of the outflow tubing. Usually, an embryo filter is located at the end of the Fooley catheter so that the flushed medium can be decanted and subsequently filtered. The filtered medium is dispensed into a search dish with grid and visualized using a stereoscope (dissecting microscope) at 10× magnification. All embryos are transferred to a

clean Petri dish containing holding media with higher concentrations of FBS/FCS (10%–20%) or BSA (0.4%). Embryos are then visualized at a higher magnification (40–60×) and classified according to their morphology, stage of development (unfertilized oocytes, early morula, tight morula, blastocyst, expanded blastocyst, hatched blastocyst), and quality (excellent, good, fair, poor and degenerate) (Stringfellow and Seidel, 2007). The quality score is based on morphologic assessment of the physical integrity of embryos and morphologic characteristics according to the stage of embryonic development, compaction status, colour of cytoplasm, areas of cellular degeneration, number of extruded blastomeres, size of perivitelline space, and the size and sphericity of the embryo. Only embryos classified as fair, good, or excellent should be transferred. Gandolfi (1997) showed that *in vivo* produced embryos are characterized by higher resistance to cryopreservation compared to the *in vitro* counterparts, probably because they develop into a natural environment. Embryos are kept in holding media after being "washed" at least three times into different, clean wells containing holding media (Stringfellow and Seidel, 2007). This procedure is recommended by the International Embryo Technology Society because a good washing could remove cellular debris and potential pathogens adhered to the zona pellucida. Embryos are kept in holding media at room temperature until they are transferred to recipients or prepared for freezing (Stringfellow and Seidel, 2007). This allows overcoming the problem of not being able to synchronize an adequate number of subjects as recipients (Neglia, 2006). In fact, it has to be underlined that embryo recipients need to be in the same stage of the oestrus cycle of the donor: the variability in terms of response to MOET does not allow to foresee the number of animals that have to be synchronized.

Factors affecting MOET efficiency in buffalo

Superovulatory response in buffalo could be affected by intrinsic and extrinsic factors. Intrinsic factors, known to influence the response to MOET, are the age of the donors, the stage of the

oestrous cycle, the steroid profile, the presence of the dominant follicle at the start of treatment and genetic factors. Among the extrinsic factors, the MOET schedules, the source of hormones, as wells as season, nutrition, days in milk etc. affect the efficiency.

Intrinsic Factors

Age of the donor

Since the bovine follicular population is inversely related to age (Erickson, 1966), several authors have suggested that the age of the donor would influence the superovulatory response and the quality of embryos (Gordon, 1975; Du-Mesnil Du-Buisson et al., 1977). In buffalo, heifers show better response to MOET in terms of ovulations and recovery rate than pluriparous cows (Zicarelli, 1997), but a high incidence of unfertilized oocytes (UFO) is recovered. This could be due to the difficulty in establishing their sexual maturity, for the large variability of the population (Zicarelli, 1997). The high recovery of UFO after MOET treatment may be hence explained by a failure related to the time of fertilization, due to incompetent oocytes or not capacitated sperm, or moreover to an inadequate uterine environment. In addition, the reproductive apparatus in buffalo has lower dimensions than the bovine counterpart (Vittoria, 1997): if the animals do not show an adequate uterus development, it could be difficult performing the flushing. In any case, no differences have been shown in animals of different age or parity in terms of embryo recovery (Misra and Tyagi 2007).

Stage of the oestrous cycle

As the number of follicles responsive to exogenous gonadotropins varies during different stages of oestrous cycle, the stage of cycle is one of the most important sources of variability in determining the superovulatory response. Although superovulatory treatment has been initiated during various stages of oestrous cycle, the greatest superovulatory response is reported when superstimulatory treatments are initiated 8 to 12 days after oestrus, in correspondence with the emergence of the second follicular wave (Alexiev et al., 1988; Misra et al., 1994). Similar results in terms of embryo recovery rate are reported when MOET treatment is initiated between days 12 and 15 after oestrus (Misra et al., 1990). Although the day of emergence of the second wave differ between buffaloes with two or three waves/cycle, this condition does not seem to be mandatory to improve MOET response in buffalo, since superovulated animals with 2 or 3 follicular waves (Heleil and El Deeb, 2010) show similar number of follicles (7.50 vs. 7.33), ovulations (5.81 vs. 6.08), corpora lutea (3.94 vs. 4.42), recovered embryo + ova (2.69 vs. 2.69) and transferable embryos (1.06 vs. 1.17). Moreover, because of the large variability in terms of oestrus duration in buffaloes, the prediction of ovulation can hardly be achieved (Baruselli et al., 1997; Campanile et al., 2010). Nasser et al. (1993) have shown that the superovulatory response is definitely higher when gonadotropin treatments are initiated precisely at the time of follicular wave emergence rather than 1 or 2 days before or later, making it necessary to synchronise the timing of follicular wave emergence. More recently, Jiang et al. (2006) observed that, in buffaloes, the initiation of the treatment on days 7 to 8 seems to give higher superovulatory response compared to days 9 to 10 or 13 to 14.

Progesterone levels during MOET treatment

If the stage of the oestrus cycle when starting the MOET protocol is not yet well defined, the influence of progesterone on superovulatory response is ascertained. Although a former study failed

to establish a correlation between progesterone concentration on the day of FSH treatment and superovulatory response (Ullah et al., 1992), a positive correlation between superovulatory response and circulating progesterone levels at the time of gonadotropin treatment has been demonstrated in both cattle (Goto et al., 1988) and buffalo (Misra et al., 2000; Madan, 1996). In agreement with this, it has been proven *in vitro* by Park et al (1996) that progesterone is able to act on the β-subunits of the pituitary gland, by inducing LH secretion and storage in rat. Furthermore, *in vivo* progesterone decreases the frequency of GnRH pulses secreted into the hypothalamic–hypophyseal portal circulation (Karsch et al., 1987). Since GnRH is also an important regulator of the number of GnRH receptors, it is hypothesized that progesterone could decrease pituitary responsiveness to GnRH by reducing the frequency of GnRH pulses which in turn would lead to reduced synthesis of GnRH receptors (Nett et al., 2002). Therefore, progesterone high levels during the first days of superovulation treatment may increase LH storage in pituitary gland and increase GnRH induced LH release (Nett et al., 2002). Buffaloes with more than 2 ng/mL plasma progesterone levels show better superovulatory response compared to those with less than 2 ng/mL, although the presence of a functional corpus luteum has been recorded (Madan et al., 1988). The administration of exogenous progesterone by intravaginal device can also be used to ensure high progesterone levels on the day of starting MOET treatment (Neglia et al., 2010), resulting in a higher number of corpora lutea and recovered embryos on day 6 post-oestrus (flushing).

Presence or absence of dominant follicle

Assessment of follicular status prior to the initiation of a superovulatory treatment may be important for improving the superovulatory response. During a normal follicular wave, the decreasing concentrations of FSH, caused by the secretions of the cohort but especially by the dominant follicle (oestradiol and inhibin), lead to the regression of the subordinate follicles. Therefore, gonadotropin

treatments should initiate at the time of follicular wave emergence, optimizing the number of follicles that can be recruited. The presence of a dominant follicle on the day of starting MOET schedule significantly influences embryo recovery rate, reducing the number of follicles that can be recruited and increasing the incidence of those that are encountering atresia. Italian Mediterranean buffaloes that underwent MOET in the absence of a dominant follicle show higher recovery rate (3.0 vs. 1.87, in absence or presence of dominant follicle, respectively) and number of transferable embryos (2.92 vs. 1.74, in absence or presence of dominant follicle, respectively), than those superovulated in its presence (Zicarelli, 1997). The influence of dominant follicle is still not clear in Murrah buffaloes. A study carried out by Taneja et al (1995) showed no differences in terms of serum progesterone concentration, recovery rate or transferable embryos, but a higher number of corpora lutea on the day of flushing in the absence of a dominant follicle. In another study, despite a similar number of corpora lutea and embryos, a higher number of large follicles was recorded regardless of the presence or absence of a dominant follicle during the MOET treatment (Manik et al., 1998). It is possible to hypothesize that these discrepant results may result from a difference in the functional status of the dominant follicle at the beginning of the superovulatory treatment. Ginther et al (2001) highlight that during a normal follicular wave the dominant follicle has a growth phase, followed by a static and regression phases. Clearly, the beginning of MOET treatment during one of these phases could result in different response, due to the different activity of the dominant follicle on the subordinates. Moreover, in cattle the number of follicles of 3 to 8 mm in diameter is highly correlated with the absence of a dominant follicle, detected by a single ultrasound examination (Bungartz and Niemann, 1993). The number of small and medium follicles prior to initiation of superovulation in buffaloes is not always different between subjects that show and those that do not show a dominant follicle (Manik et al., 1998). Therefore, it can be concluded that the use of morphological criteria, based on the size of the largest follicle alone, are not sufficient to predict the response of buffalo to MOET. Numerous methods have been used to remove the influence of the dominant follicle. Previously it has

been specified that MOET is normally carried out in presence of progesterone releasing intravaginal device, with or without oestradiol. If it is possible to use oestradiol (forbidden in many countries), it should be administered before initiating treatment by progesterone releasing intravaginal device, causing the regression of the dominant follicle (Baruselli et al., 1999). In countries where oestradiol cannot be used, practitioners have turned to alternative treatments for the synchronization of follicle wave emergence, such as mechanical follicle ablation or the administration of GnRH to induce ovulation. High progesterone levels suppress follicular growth, maintaining a high number of small follicles on the ovary when MOET treatment is initiated.

Genetic factors

There are two main types of domestic buffalo: the River buffalo of the Indian subcontinent with 50 pairs of chromosomes and the Swamp buffalo of the South Asian region with 48 pairs of chromosomes. Crossbreeding by mating River and Swamp buffalo has been done in many countries. This mating produces a hybrid F1 with a chromosome complement of 2n = 49. Slight differences have been observed after SO in these three genotypes. Usually, crossbred and Swamp buffaloes show a lower ovary diameter compared to the River, although this is not a good indicator to predict the number of embryos collected. In fact, both the number of corpora lutea and embryos are not different among the three genotypes (on average 6 corpora lutea and 2-2.7 embryos/donor) (Situmorang et al., 2003). Genetic factors have to be considered in terms of individual differences among subjects. It is reported that cows and buffaloes which respond poorly to first superovulation treatment have tendency to respond poorly to subsequent superovulation, while those which respond well to first superovulation continue to do so during subsequent treatments (Moor et al.,1985, Zicarelli, 1997). Probably the reason for such variations could be due to genetic constitution of a donor that may have more or less sensitivity to exogenous gonadotropins. This conclusion is substantiated from the reports

that Booroola Merino and Cambridge sheep are known for high prolificity and Booroola gene in these sheep has been shown to be associated with higher superovulatory response (Bindon et al., 1986). Similarly, beef breeds are reported to be more sensitive to eCG stimulation than dairy breeds (Mariana et al., 1970; Sreenan and Beehan, 1976). Some studies have shown that IGF-1, the main receptor of IGF, is involved in some physiological processes, including ovarian follicular development (Mazerbourg et al., 2003; Beg and Ginther, 2006), ovulation (Echternkamp et al., 2004), pre-implantation embryo development (Velazquez et al., 2009), conception and growth. These reports concluded that oocyte quality and embryo viability are associated with blood concentrations of IGF-1 in domestic ruminants undergone superovulation treatments.

Extrinsic Factors

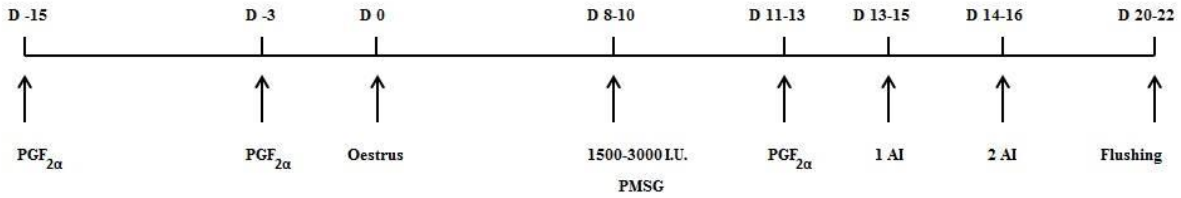
MOET schedules and different types of gonadotropins

Three different types of gonadotropins from various sources have been used to induce superovulation:

Equine chorionic gonadotropin (eCG), or pregnant mare serum gonadotropin (PMSG), is a complex glycoprotein with both FSH and LH activity (Murphy and Martinuk, 1991) that has been largely used to induce SO because of its low cost and easy availability. In cattle, eCG induces follicular growth primarily due to its FSH like activity by preventing or reversing the process of atresia in ≥ 1.7 mm diameter follicles and thereby increasing the number of follicles capable of responding to the gonadotropin (Moor et al., 1985). Its main characteristic is the long half-life that is about 40 hours in cattle, persisting in the circulation until 10 days after the end of the superovulation treatment. On one hand, a single eCG administration is enough to ensure an optimal superovulatory

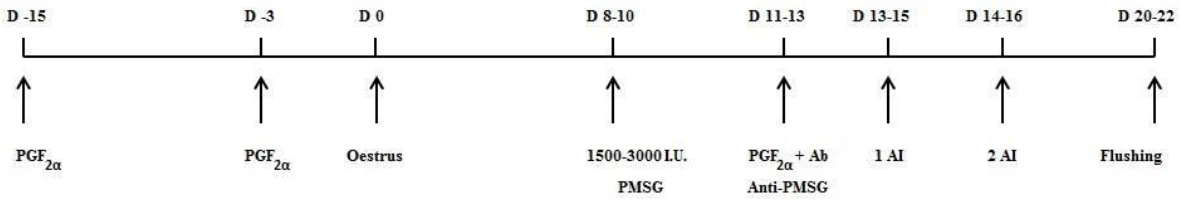
response, facilitating animals' management and handling, on the other hand the prolonged high eCG circulating levels can be associated with ovarian hyperstimulation, further growth of extra follicles after ovulation, abnormal endocrine profiles and high incidence of anovulatory follicles (Karaivanov et al., 1987). Indeed, after eCG treatment some follicles tend to luteinize and others develop in cysts, rather than ovulate (Monniaux et al., 1983) altering the oestradiol: progesterone ratio. Moreover, consequent to luteinisation of some of the rescued follicles, elevated concentration of P4 at the time of LH surge may account for asynchrony of the LH surge leading to impaired transport of gamete in the fallopian tube and poor fertilization rate (Mapletoft and Murphy, 1993). In contrast, a study demonstrated that high progesterone levels (more than 2.3 ng/mL) on the day of eCG administration seem to be associated with higher embryo recovery (Heleil and El Deeb, 2010). In addition, eCG treatment in buffaloes results in elevated plasma inhibin from fully developed follicles which continue for a long time and culminate in the inhibition of FSH, leading to poor ovulation in the remaining follicles (Singh et al., 2000). One limitation with eCG is that its FSH/LH activity varies not only among pregnant mares, but also among bleedings in the same mare collected at different times during gestation (Gonzales-Mencio et al., 1978). Since hormone concentration could be different at different times of gestation and, hence, among different batches, there is variability in terms of superovulatory response and embryo recovery. In buffalo, the treatment schedule consists in the intramuscular injection of dosages variable between 2,500 to 4,000 IU, however, 3000 IU of eCG by intramuscular route is commonly practiced (Karaivanov et al., 1990). A schematic representation of eCG treatment is shown in Fig. 3.

Figure 3. The eCG superovulatory protocol.



Mean embryo recovery rate is between 0.7 (Karaivanov et al., 1987) and 2 (Schallenberger et al., 1990; Heleil and El Deeb, 2010) embryos. The ovarian response to eCG treatment has also been tested on pre-pubertal buffalo heifers in association with GnRH (Singh and Madan, 1999), reporting a mean number of 4.5 to 5 ovulations/heifer, suggesting that the embryo recovery would be similar from that reported in adult buffaloes. Furthermore, GnRH administration has no influence on the total ovulation rate, although it allows a reduction in the interval between the first and the last ovulation (about 20 hours compared to 40 in GnRH treated and control buffaloes, respectively). Attempts have been made to overcome the side effects of the long half-life of eCG by the intravenous injection of antibodies (polyclonal or monoclonal) to eCG at the time of the first insemination, 12 to 18 hours after the onset of oestrus (Fig.4).

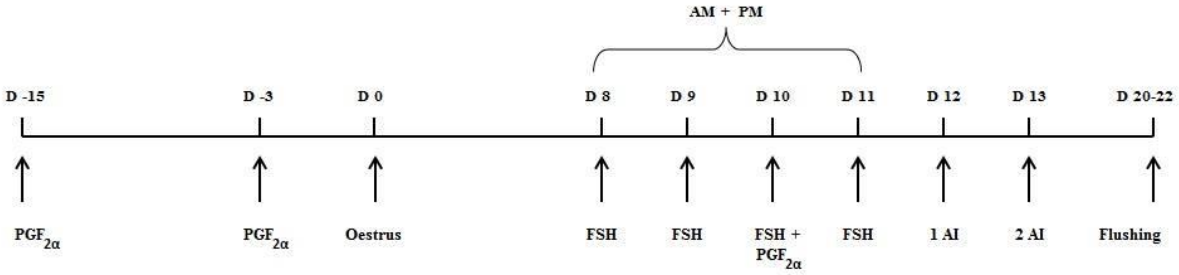
Figure 4. Use of antibodies during the eCG superovulatory protocol.



However, results are conflicting because of considerable variation in the interval between the injection of PGF2α and LH peak and also because of variation in the interval from the time of first standing heat to the LH peak (Dieleman et al., 1993). Treatment with Neutra-eCG results in a significant decrease in the peripheral inhibin concentration at 84 to 120 hours after prostaglandin and in the number of large anovulatory follicles at 168 hours, although the number of large follicles is lower than that recorded in buffaloes treated by eCG alone (Palta et al., 1997) and no differences in terms of corpora lutea number is recorded. Furthermore, embryo recovery rate by using antibodies versus eCG is about 1.51/donor, with 1 transferable embryo/donor, thus similar to that obtained with eCG alone (Palta and Madan, 1995).

Pituitary extracts of FSH and LH have given a better superovulatory response compared to other gonadotropins and therefore they have been more widely used for buffalo MOET. The two most commonly used commercial FSH preparations are made from porcine pituitary extracts and have some LH activity which varies between manufacturing batches (Chupin et al., 1984). Although high amounts of LH in FSH preparations interfere with optimal superovulatory response, it is believed that a low level of LH contamination does not interfere and may even be needed for superovulation (Herrier et al., 1991). In any case, the maximum acceptable level of LH contamination in pituitary extract for SO would range between 15 and 20%. This variability may be reduced by using bovine FSH produced by recombinant DNA technology (Looney et al., 1990). However, no trials have been performed till now in buffalo species. The mechanism of this inhibitory effect of LH contamination is not clearly known, however, it is postulated that elevated LH may disrupt the balance of androgen and oestrogen production necessary for the prevention of atresia in follicles (Farookhi, 1981) or LH may induce down-regulation of its receptors on theca and/or granulosa cells (Murphy et al., 1984). Because of its short half-life, pituitary extracts are administered twice a day, usually for schedules of 3 to 5 consecutive days (Fig. 5).

Figure 5. Superovulatory protocol based on pituitary extracts.



Usually the administration of a decreasing dosage is preferred, to increase follicular recruitment and development. Regimens with constant, decreasing or increasing dosages have been used (from 800 to 1200 IU), although no differences have been recovered in terms of embryo recovery (Zicarelli, 1997). In buffalo, dosages lower than 700 IU of FSH are not able to induce a good response. Moreover, it has to be considered a further increase or decrease of about 20%, if buffaloes weighing more than 800 kg or heifers are treated, respectively. Several attempts have been done in cattle in order to reduce the number of FSH administrations, by injecting the hormone together with some slow releasing substances (e.g. PVP or hyaluronic acid) subcutaneously or intramuscularly (Hill et al., 1985; Biancucci et al., 2016). However, a single injection seems to result in lower response to MOET compared to multiple dose injection regimens (Misra and Tyagi, 2007). However, embryo recovery rate is similar to that reported for treatment by eCG, varying from 0.9 to 3.5 embryos or ova/donor (Zicarelli, 1997; Baruselli et al., 1999; Carvalho et al., 2002; Neglia et al., 2010). Mediterranean buffaloes treated for MOET by a commercial pituitary extract preparation (Zicarelli, 1997) responded with 1.9 embryo/donor (and only 1.51 transferable embryos).

Human menopausal gonadotropin has also been used to induce MO, with results similar to those obtained by pituitary extracts (Filicori et al., 2003). However, commercial hMG preparations are highly expensive and are scarcely utilized in practice.

Influence of the season

The season may influence the superovulatory response of buffaloes for both seasonality and weather conditions. It is known that buffalo is a short day breeder, that tends to increase its reproductive activity when day light hours decrease (Campanile et al., 2009) because of its tropical origins. Indeed, they originate from North equatorial areas, where the availability of forage coincides with the period in which dark hours increase. Therefore, it has been supposed that only the animals calving in the most suitable period for survival of the offspring were selected (Zicarelli, 1974). This characteristic was retained even when they were transferred to places where forage is always (Italy) or less (South Equator areas like Sao Paulo, Br) available. In fact, in Italy, buffalo shows its maximum reproductive efficiency in the autumn-winter period, in which daily light hours are lower (Campanile et al. 2010). Therefore, it is reasonable that the application of reproductive biotechnologies would also be more successful in this period. In contrast, embryo recovery rate after MOET in periods of decreasing daylight length was lower compared to other periods of the year (Zicarelli et al., 1993), likely because in this period also hypofertile buffaloes are cycling and hence are enrolled for SO, leading to low efficiency (Zicarelli, 1997). Since only cycling subjects are selected for superovulation, the application of treatments out of the breeding season allows to select high fertile subjects, that are able to show high reproductive activity also when day light hours increase. The influence of extreme weather conditions in tropical countries on the superovulatory response in buffalo is controversial. In fact, although an effect of high temperature and high humidity have been reported (Misra, 1993; Mutha et al., 1994), it is known that because of its tropical origin,

buffalo does not undergo heat stress like cattle. It is likely that the daily light length rather than weather conditions influences the superovulatory response.

Days open

Another factor that could affect the response to MOET treatment is the number of days open (Zicarelli et al., 1993). Zicarelli (1997) reported that the higher superovulatory response, together with both the higher number of transferable embryos per donor and higher transferable embryos/corpora lutea ratio were obtained from animals between 61 and 220 days post-partum. Buffaloes that undergo MOET schedules within 60 days post-partum produce mainly unfertilized oocytes, probably for inadequate uterine environment for sperm survival or inadequate oocyte competence. While the lack of response in buffaloes at more than 220 days post-partum could be due to uterine inflammations or deep anoestrus, phenomena that these subjects encounter when they are not pregnant for long time (Zicarelli, 1997).

Influence of nutrition

Nowadays, the influence of nutrition on reproductive efficiency is well established (Campanile and Neglia, 2004), although some mechanisms remain to be understood. For optimum superovulatory response a donor should be on a well-defined plane of nutrition. Diets characterized by low starch content (<15.5%) and high cellulose content (>23%) allow to obtain the maximum superovulatory response in buffaloes treated by FSH (Di Palo et al., 1994). This is particularly evident in buffaloes within 120 days post-partum, thus during the catabolic phase of lactation, when the animals encounter the negative energy balance. This interesting observation is supported by the evidence that diets characterized by low energy density result in an increase of small follicles and high quality

oocytes (Campanile et al., 1999) improving blastocyst yield *in vitro* (Di Palo et al., 1994). Consequently, the utilization of high quality forages (with high nutritive value and relatively low crude fibre level), and low concentrates content in the diet is recommended (Zicarelli, 1997): these types of diets meet nutritional requirements, without impairing reproductive efficiency. Furthermore, meeting nutritional requirements is fundamental in order to ensure a proper body condition score (BCS), that was demonstrated to affect the reproductive efficiency (Baruselli et al., 2001). The BCS system is a scoring method of evaluating the energy reserves of dairy animals which provides a better understanding of biological relationships between body fat, milk production and reproduction (Anitha et al., 2010). In buffalo, body condition score (BCS) is known to affect fertility; in fact, pregnancy rate improved from 39.7, 53.9 and 56.7% with the improvement of BCS from 3.0 to 3.5 and 4.0, respectively (Baruselli et al., 1999). A lower BCS is responsible for lower LH pulsatility, impairing the efficiency of reproductive biotechnologies, such as AI and MOET.

r-BST priming

To increase the response of the follicular population to MOET treatment, recombinant bovine somatotropin (r-BST) priming has been used in both cattle (Gong et al., 1993) and buffalo (Songsasen et al., 1999). Recombinant bovine somatotropin acts increasing the concentration of Insulin-like Growth Factor I (IGF-I) together with FSH, increasing both the number of small follicles (Gong et al., 1997) and the number of LH receptors on the granulosa cells and improving follicular growth and oocyte. Moreover, IGF-I promotes cumulus cells expansion (Izydar et al., 1998) and this can contribute to oocyte adhesion to the fimbriae after ovulation (Carvalho et al., 2007). In buffalo, results on r-BST influence during superovulation are controversial. Treatment with r-BST in Swamp buffalo increased the number of corpora lutea and recovered ova and significantly the number of transferable embryos (Songsasen et al., 1999), leading to a mean recovery rate of about 3 transferable

embryos/donor. Similarly, a study of Zicarelli (1994) showed that superovulated Mediterranean buffaloes treated with r-BST showed a lower incidence of ovarian cysts and higher recovery rate, in terms of both recovered ova and transferable embryos. However, the dosage utilized seems to affect the superovulatory response. Good results have been obtained by using 250 mg, administered on the day of progesterone releasing device insertion (Day 0) and starting the superovulation schedules on both day 4 (Carvalho et al., 2007) or day 9-11 (Songsasen et al., 1999). In contrast, contradictory results are reported with the use of 500 mg, since in some studies a great improvement in terms of ovulation rate and embryo recovery was reported (Baruselli et al., 2003), whereas no effect was observed in others (Carvalho et al., 2007).

Ovum Pick-up (OPU) priming

OPU technology allows the visualization and the aspiration of all follicles with diameter >2 mm, which resets the oestrous cycle and avoids the dominance of one follicle over the subordinates. In most of the species the optimal interval between aspiration sessions is 3–4 days (Galli et al., 2001), but in buffaloes higher intervals may be necessary (Neglia et al., 2011). For this reason, with this technology, it is hypothetically possible to avoid the atresia of subordinate follicles and increase the follicular waves from 2-3 to 6 for each cycle. This leads to the collection of a higher number of cumulus oocyte complexes usable for in vitro embryo production (IVEP), as well as to an improved oocyte quality (Galli et al., 2001). Moreover, OPU priming can be utilized to reset follicular population before initiating superovulatory treatment, avoiding the negative influence of the dominant follicle on the subordinates and improving oocyte quality. Zicarelli et al (1995) have shown that OPU priming in buffalo increases the number of animals able to respond with at least one embryo (more than 68% vs. 53%, in buffaloes undergone OPU priming and controls, respectively), and this is particularly evident during periods of decreasing daylight length (84 vs. 15%, in buffaloes

undergone OPU priming and controls, respectively). Furthermore, in the same period, OPU primed buffaloes showed a higher ova/corpora lutea ratio (47 vs. 12%, in buffaloes undergone OPU priming and controls, respectively) and transferable embryos (3.2 vs. 0.5, in buffaloes undergone OPU priming and controls, respectively). However, no differences were observed between buffaloes undergone OPU priming and the control counterparts during periods of increasing daylight length.

Influence of interval between PGF and oestrus

Another factor to take into account for a good superovulatory response is the interval between prostaglandin administration and oestrus. Usually more than 90% of animals exhibits oestrus within 48 hours after $PGF_{2\alpha}$ administration (Misra and Pant, 2003). Interestingly, these animals give higher recovery rate and number of viable embryos compared to those that exhibit oestrus later. Probably late oestrus signs could be associated with a delayed (or failure) ovulation and lack of oocyte fertilization.

Repeated superovulation

To obtain full benefits of non-surgical embryo collection, an elite donor should respond optimally to repeated gonadotropin treatment. Usually, it would be reasonable to carry out the hormonal treatment on the same subject at least 80 or 100 days apart, in cattle and buffalo respectively. First of all, it has to be considered the type of gonadotropin that is utilized. A study carried out by Cruz et al (1991) demonstrated that Swamp buffaloes, treated by eCG for four successive superovulatory treatment, undergo a high incidence of anovulatory large follicles (cysts) and reduced embryo recovery rate probably because of the long half-life of eCG and, consequently, the continuous ovarian stimulation. Therefore, the utilization of pituitary extracts is preferred. When buffaloes were treated repeatedly for

SO by FSH for six times 77 days apart, a marked decline (about 50%) in ovulation rate, corpora lutea and embryo recovery was reported (Mutha et al., 1994). However, it is worth pointing out that the repetitive superovulatory treatments up to the 6^{th} time did not significantly affect the fertility of the donors, since more than 80% pregnancy rate and about 1.8 services/conception after the last treatment were recorded.

Utilization of exogenous LH

A study carried out by D'Occhio et al (1999) demonstrated an improvement in MOET treatment in cattle, obtained by using a GnRH agonist – LH protocol. This is based on the block of the preovulatory endogenous LH surge by treatment with a GnRH agonist bio implant, hence making ovulation possible only by the exogenous administration of LH after follicular superstimulation. The advantage of this protocol is that the ovulations occur over a relatively short and predictable period of time after LH administration (D'Occhio et al., 1997). It results in higher ovulation rate (about 70%) and higher embryo recovery rate (3.7 embryos on average) compared to subjects treated by a conventional MOET protocol or in those in which LH is administered 36 hours after the superovulatory protocol (Carvalho et al., 2002). A further improvement could be obtained by the incorporation of the oestradiol treatment at the start of the superstimulatory treatment: in this case it is likely that higher follicular responses and ovulation rate is recorded.

Administration of prostaglandin on the day of artificial insemination

Early return-to-oestrus of superovulated donors after embryo collection is necessary to improve efficiency of embryo production. If an administration of $PGF_{2\alpha}$ or its analogues causes CL regression after days 5-6 till days 15-17 of the oestrous cycle, by increasing the intraluteal production of

vasoactive molecules, such as endothelin-1 (ET-1) and Angiotensin-II (Ang-II) (Ohtani et al., 1998; Hayashi et al., 2001), its role during the oestrous phase is still unclear. Ainsworth et al (1979) reported that inhibitors of prostaglandin synthesis inhibit ovulation and that the administration of $PGF_{2\alpha}$ on the day of AI increases CL dimensions and progesterone production at the end of a standard (Ovsynch-TAI) synchronization treatment in buffalo (Neglia et al., 2008), probably via ET-1 and Ang-II gene expression inhibition (Girsh et al., 1996; Meidan et al., 1999; Miyamoto and Shirasuna, 2009). Moreover, the stimulation of α-receptors in the ampulla and fimbriae by prostaglandins increases the contractility of smooth muscle, thus affecting gametes transportation (Riehl and Harper, 1981). Probably, the influence of prostaglandin during the ovulation could be due to its action during follicular rupture. Indeed, the ovulatory process involves a complex series of biochemical and biophysical events that ultimately lead to the rupture of the preovulatory follicle and the release of the maternal germ cell into the oviduct. The LH peak results in the activation of a novel form of cyclooxygenase (COX-2) enzyme, in the granulosa cells of the preovulatory follicle (Hedin et al., 1987), increasing the intrafollicular concentrations of prostaglandin $F_{2\alpha}$ and E2 and these are responsible for the increased contraction of the myoid (smooth muscle) components of the ovary, leading to ovulation (Senger, 2005). To verify the possibility of increasing oocyte capture by the fimbria, the administration of prostaglandin $F_{2\alpha}$ during the peri-ovulatory period has been also utilized during superovulatory treatment (Soares et al., 2013). A higher number of ova (3.5 vs. 2.3, in buffaloes treated with prostaglandin and not treated, respectively) and transferable embryos (2.7 vs. 1.8, in buffaloes treated with prostaglandin and not treated, respectively) was recovered in buffaloes treated with 4 doses of prostaglandins 12 hours apart, starting from the end of the superovulatory treatment. This is probably due to both: 1) improved contractions of the follicular wall, which then ruptures, expelling the ovum and accompanying follicular fluid containing high levels of $PGF_{2\alpha}$ into the tubal ampulla (LeMaire and Marsh, 1975) and 2) increased contractile activity of the tubes. In fact, it is believed that elevated $PGF_{2\alpha}$ levels in the follicular fluid are necessary to enhance ovum

capture by tubal fimbriae. In addition, the increased contractility of the oviductal smooth muscles, results in increased speed of embryonic transport and development (Weber et al., 1991; Kissler et al., 2004).

Immunisation against inhibin

Despite an equal number of ovarian follicles recruited in a cohort wave in buffalo and cattle (Baruselli et al., 1999; Campanile et al., 2010; Mapletoft and Bó, 2012), the number of recovered embryos in the former is very low. The developing dominant follicle produces inhibin, a substance that inhibits both pituitary FSH secretion and follicular development of subordinates. The immunoneutralisation against inhibin in cattle stimulates follicle development (Medan et al., 2004), embryo yield (Mantovani et al., 1997) and embryo quality (Li et al., 2009), reducing the phenomenon of follicular atresia. The immunisation against inhibin improves superovulatory response also in water buffaloes (Li et al., 2011), in terms of improving superovulation rates and number and percentage of transferable embryos, as well as elevating plasma E2 concentrations during oestrus and P_4 concentrations during diestrus. In particular, the utilisation of primary immunisation with 2 mg inhibin fusion protein is able to increase the ovulation rate (6.5 vs 4.8, in treated and not treated animals, respectively), the number of recovered ova (4.5 vs. 2.8, in treated and not treated animals, respectively) and the number of transferable embryos (3.3 vs. 1.6, in treated and not treated animals, respectively). On the other hand, as described above, the utilization of antibodies anti-eCG reduces peripheral inhibin concentration and the number of anovulatory follicles (Palta et al., 1997), slightly improving the superovulatory response.

Causes of poor embryo recovery rate in superovulated buffaloes

As reported in the introduction of this chapter, several attempts have been performed to optimize the MOET technique in buffalo species. However, although embryo recovery improved in the last thirty years, from less than 1 viable embryo to 2.5-3/donor, the MOET typically results in a relatively low recovery of both embryos and unfertilized ova in buffalo (Zicarelli 1997; Misra and Tyagi, 2007; Alexiev et al., 1988 Misra et al., 1994; Madan et al., 1996; Neglia et al., 2010; Taneja et al., 1995; Palta et al., 1997; Baruselli et al., 1999; Carvalho et al., 2002; Misra and Pant, 2003; Beg et al., 1997; Qin et al., 2012) compared with cattle (Mapletoft and Bó, 2012; Bó and Mapletoft, 2014) throughout the world. In addition, while in cattle 88% of subjects produced at least 1 embryo after superstimulation, the proportion of responsive buffaloes varies between 30 and 50% (Zicarelli, 1997).

Several hypotheses have been formulated to explain the low embryo recovery recorded in buffalo species after SO. It is known that oogenesis in cattle and buffalo gonads starts during the foetal life, resulting in the formation of a pool of primordial follicles, which represents the source of gametes throughout the life of the female. The number of primordial follicles represents the first great difference between cattle and buffalo. In fact, while in cattle the number of primordial follicles from the birth to about 4^{th} year of life is 133,000 (Erickson, 1966; Driancourt et al., 1993), only 26,600 (Ty et al., 1994) or 40,000 (Samad and Nasseri, 1979; Danell, 1987) primordial follicles have been recorded, in Swamp and River buffaloes, respectively. Therefore, if buffaloes have about 20-30% of the total pool of primordial follicles compared with cattle, it would be reasonable that the response to SO treatment in terms of embryo recovery in the former is about 1/5 compared to the latter.

However, since the response of buffalo in terms of follicles > 1cm at the end of the MOET treatment is quite satisfactory, this hypothesis leaves some doubts. Some studies (Baruselli et al., 1999; Carvalho et al., 2002; Neglia et al., 2010) demonstrated that superovulation schedules usually result in the growth of a high number of ovulatory follicles, together with a relatively high ovulation rate

(50-70%), although embryo/ova recovery rate is very low (13-35%). Therefore, it has been supposed that a limited capacity for follicular growth and ovulation may not be the causes of the relatively low embryo recovery recorded in superovulated buffalo. It is more likely that the recruitment of the oocytes by the fimbriae and/or the transport of ova and embryos within the oviduct are compromised (Carvalho et al., 2002). Plasma 17-β-oestradiol (oestradiol) levels recorded in buffaloes are lower than those reported in cattle during normal oestrus cycles (Batra and Pandey, 1983). On the contrary, oestradiol plasma concentrations are particularly high during SO (Beg and Ginther, 2006), resulting in an altered oestradiol: progesterone ratio, that may influence oocyte recruitment by the fimbriae (Misra et al., 1998). This would be further confirmed by the negative correlation observed between the presence of a high number of large (>8 mm) follicles on the day of embryo recovery and the number of recovered embryos (Misra et al., 1998; Baruselli et al., 1999). Furthermore, it is worth noting that the quality of the oocyte and especially the cumulus cell layers surrounding it are factors that may affect oocyte capture by the fimbriae (Priedkalns, 1987). Grade I and II oocytes (those surrounded by several granulosa cell layers) represent only about 50-60% (Neglia et al., 2011) of the total, whereas about 80% is recorded in cattle.

In conclusion, the application of MOET in buffalo still needs to be improved, to increase both the number of subjects that are able to produce at least one embryo and, possibly, the number of recovered embryos.

Experimental Part

Aim of the work

The main objective of this thesis was to investigate the different factors affecting the embryo recovery in superovulated buffaloes. Several hypotheses have been formulated over the years to explain the low embryo recovery recorded in this species after superovulatory treatment, among which the most likely is a failure of ovum capture.

Before addressing the major issue, other experiments were carried out in order to select both the donors and the most suitable superstimulation treatment. Indeed, another critical factor limiting the application of this technology in buffalo is the high between-animal variability in the number of embryos produced (Govignon et al., 2000; Gasparrini et al., 2014). It was recently reported that follicular recruitment is predetermined within each individual, suggesting the possibility to select good donors (Neglia et al., 2011; Gasparrini et al., 2014). Therefore, the aim of Experiment 1 was to evaluate whether the Anti-Mullerian hormone (AMH) can be used as a reliable marker to select good embryo donors in addition to the screening of the antral follicular count, before enrolling buffaloes in embryo production programs. In order to do so, the association between AMH concentration in both plasma and follicular fluid and the antral follicular count was evaluated and the reproductive parameters, including AMH concentration and gene expression in granulosa cells, were compared between good and bad donors ($\geq$ 12 and $<$ 12 follicles, respectively). Another objective was to investigate the population of AMH-producing antral follicles in buffalo ovaries by determining AMH concentration in follicular fluid recovered from different size follicles and evaluating the mRNA expression profiles of development-related genes in the corresponding granulosa cells.

With regard to the MOET treatment, as the follicular development switches from FSH-dependence to LH dependence after deviation, in order to better mimic the follicular dynamics in superovulated animals, FSH should be given first to ensure follicular growth, followed by LH administration at the

time of presumptive follicular deviation. This approach, that can be achieved by using eCG during the final stages of follicular growth, has never been investigated in buffalo. Therefore, the aim of Experiment 2 was to evaluate the efficacy of replacing the last four FSH injections with a single administration of 1000 IU of eCG compared to the conventional FSH decreasing superovulation protocol in Italian Mediterranean Buffalo. The efficacy of the two treatments was assessed by evaluating the follicular dynamics, as well as the number of corpora lutea and embryo yields.

As previously stated, the main aim of this thesis was to analyse at different levels the various factors involved in the failure of ovum capture, as an improved knowledge may allow the development of strategies to improve the embryo recovery rate in superovulated buffaloes. Thus, in order to select the most reliable control group for the superovulation study, the aim of Experiment 3 was to evaluate the relationship between the ovarian follicular response at the start of an Ovsynch program and the pregnancy outcome. The results of this experiment allowed us to better plan the Experiment 4, by selecting the animals that ovulated after the 1st GnRH as a control group to compare with superovulated animals. Finally, the aim of Experiment 4 was to compare the morphological parameters of the follicles and the steroid profile (progesterone and oestradiol) both in plasma and follicular fluid in the peri-ovulatory period in superovulated vs synchronized buffaloes. Furthermore, to investigate whether the failure is linked to lack of capture, the oocyte morphology and expansion, as well as the mRNA expression of gonadotropins receptors (*FSHr* and *LHr*) in granulosa cells were evaluated. In addition, the mRNA expression of factors playing a role on contraction-relaxation like *ER1, PGR, VEGF* and its receptors *FLK1* and *FLT1* was analysed in the oviduct.

EXPERIMENT 1

Anti-Mullerian Hormone (AMH) concentration in follicular fluid and mRNA expression of AMH receptor type II and LH receptor in granulosa cells as predictive markers of good buffalo donors

As previously mentioned, one of the most critical factors limiting the application of embryo technologies in buffalo is the high between-animal variability in the number of embryos produced (Govignon et al., 2000; Gasparrini et al., 2014). Moreover, the intrinsic species-specific lower number of primordial (Danell, 1987) and antral follicles (Van Ty et al., 1989; Baruselli et al., 1997) compared to cattle accentuates the problem of the high variability in follicular recruitment in this species. It was recently demonstrated that follicular recruitment is predetermined within each individual, allowing desirable and undesirable donors (Neglia et al., 2011; Gasparrini et al., 2014) to be distinguished, as in cattle (Tamassia et al., 2003). Therefore, in addition to the screening of the antral follicular count (AFC), identification of a reliable marker to select good embryo donors is fundamental before enrolling buffaloes in embryo production programs, to offset laboratory costs.

Very recently, attention has focused on the anti-Mullerian hormone (AMH), a molecular marker of the ovarian follicular pool and follicular responsiveness to superovulatory treatments in women, cattle, goats and other species (Fanchin et al., 2005; Monniaux et al., 2010, 2011). The anti-Mullerian hormone is a 140-kDa glycoprotein that belongs to the transforming growth factor (TGF)-β family and is expressed only in the gonads (Cate et al., 1986). In cattle, as in other mammalian species, AMH is specifically expressed by ovarian granulosa cells (Vigier et al., 1984; Takahashi et al., 1986); its expression is highest in granulosa cells of preantral and small antral follicles, and decreases during terminal follicular growth (Baarends et al., 1995), with low AMH concentrations found in the

follicular fluid of large antral and preovulatory bovine follicles (Monniaux et al., 2008). It is also known that AMH expression in granulosa cells and AMH concentration in follicular fluid are both low in atretic follicles (Rico et al. 2009). Anti-Mullerian hormone is known to play a critical role in folliculogenesis by modulating the FSH function and thereby limiting follicular recruitment (Weenen et al., 2004; Bentzen et al., 2013). In addition, AMH is a key mediator in regulating steroidogenesis, as it inhibits oestradiol secretion by reducing the expression of the aromatase enzyme CYP19 (Eilsø Nielsen et al., 2010) and progesterone production in granulosa cells *in vitro* (Yding Andersen et al., 2008). Furthermore, AMH is negatively associated to the expression of LH receptors in small human antral follicles (Yding Andersen et al., 2008). The pattern of expression in the granulosa cells of growing follicles and the role in regulating folliculogenesis and steroidogenesis make AMH an ideal marker for the size of the ovarian follicle pool. Moreover, AMH has been shown to be a reliable endocrine marker of oocyte and embryo quality in humans (Lehmann et al., 2014; Lie Fong et al., 2008).

The only work carried out so far in buffalo reported a correlation between plasma AMH concentration and antral follicular count in Murrah heifers, suggesting that AMH is a potential marker of follicular reservoir in this species (Baldrighi et al., 2014). However, many aspects still need to be investigated to clarify the role of AMH in influencing follicular growth and oocyte quality in buffalo. Therefore, the aim of our study was to characterize the population of AMH-producing antral follicles in buffalo ovaries by determining AMH, concentration in follicular fluid recovered from different size follicles and evaluating the mRNA expression profiles of four follicular development-related genes (*AMHR2*, *CYP19A1*, *FSHR* and *LHR*) in the corresponding granulosa cells (GCs). Another objective was to evaluate whether AMH concentration in both plasma and follicular fluid (FF) and gene expression of granulosa cells are associated to the AFC. Furthermore, we compared reproductive parameters, including AMH concentration and gene expression, in good vs bad donors (≥ 12 and < 12 follicles, respectively). Finally, apoptosis-related genes, such as tumour

protein p53-inducible nuclear protein 1 (*TP53INP1*), and caspase 3 (*CASP3*) were assessed in granulosa cells from good and bad donors.

Materials and Methods

The Ethical Animal Care and Use Committee of the University of Naples Federico II (Naples, Italy) approved the experimental design and animal treatments.

Experimental design

The study was carried out in Southern Italy (latitude 40.5° - 41.5° N and longitude 13.5 - 15.5) during autumn, i.e. the breeding season, on 54 multiparous Italian Mediterranean Buffalo cows, with a mean weight and age of 565.3 ± 14.5 kg and 5.3 ± 0.3 years, respectively, over four replicates. To induce luteolysis and synchronize the occurrence of oestrus, buffaloes received a double administration of a PGF2α analogue (Cloprostenol, 0.250 mg/mL Schering-Plough Animal Health, Milan, Italy), spaced 11 days apart and were slaughtered at a local authorized abattoir six days after the second administration, i.e. after the emergence of the first follicular wave but before development of a dominant follicle (Monniaux et al., 2008; Rico et al., 2011). The Ethical Animal Care and Use Committee of the University of Naples Federico II (Naples, Italy) approved the experimental design and animal treatments.

On all animals AFC, recovery rate and oocyte quality were recorded, and both plasma and FF were collected for AMH determination. In a representative number (n=22) the mRNA expression of six genes (*AMHR2*, *CYP19A1*, *FSHR*, *LHR*, *TP53INP1*, and *CASP3*) was analysed in GCs. Finally, buffaloes were classified according to the AFC as good (≥ 12 follicles) and bad (< 12 follicles)

donors, in order to evaluate the differences in reproductive parameters. The criterion used to classify the donors was chosen on the basis of our previous experience on abattoir-derived ovaries during the favourable season.

Collection of follicular fluid and granulosa cells

At slaughter, ovaries were recovered individually and transported to the laboratory in physiological saline supplemented with 150 mg/L kanamycin at 30-35°C. All the ovaries were weighed and measured: ovarian size was assessed by measurement of two perpendicular diameters of each ovary with a millimetre scale. For each antral follicle, antrum size was estimated by measurement of two perpendicular diameters with a millimetre scale. Thus, all follicles with an antrum larger than 3 mm were counted (AFC) and allocated to three size classes: small (3-5 mm), medium (5-8 mm), and large follicles (> 8 mm). The FF of each follicle was aspirated by a syringe with a 21-gauge needle, poured into a petri dish for a quick search of the oocyte, recovered in a vial and centrifuged at 600 X g for 10 min to separate the follicular fluid and the granulosa cells. After centrifugation, the FF recovered was directly snap frozen and stored at - 80°C until AMH determination, while the GCs-containing pellet was previously washed with PBS, centrifuged again at 600 X g for 10 min, then snap frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Small and medium follicles were respectively pooled (from 2 to 7 and 1 to 2 follicles, respectively) in each buffalo in order to get sufficient FF for AMH determination and GCs to isolate RNA. Follicular fluids from large follicles (> 8 mm) were recovered individually.

Evaluation of oocyte quality

The FF recovered from different classes of follicles was inspected for the presence or absence of the cumulus-oocyte complex (COC) by microscope. The COCs recovered were scored for quality on the basis of morphological features, as previously described (Di Francesco et al., 2011). For each run, the recovery rate, i.e. the percentage of total number of COCs in relation to total number of aspirated follicles, and the percentage of superior quality oocytes (Grade A+B) were recorded. Briefly, according to our classification (Di Francesco et al., 2011), Grade A are oocytes with homogeneous cytoplasm and the entire surface surrounded by multiple layers of cumulus cells and Grade B those with homogeneous cytoplasm and with at least 70% of the surface surrounded by multiple layers of cumulus cells.

Detection of AMH in follicular fluid and plasma

Anti-Mullerian Hormone in follicular fluid was measured by a specific AMH Gen II ELISA kit according to the manufacturer's instructions (Beckman Coulter, A73818, USA), as previously described for bovine species (Monniaux et al., 2008; Rico et al., 2009). Briefly, AMH was measured on 20 μL follicular fluid diluted at 1:100 for small follicles, 1:10 for medium follicles, and not diluted for large follicles. Each sample was run in duplicate in this study. The intra-assay and inter-assay coefficients of variation were <5% and <10%, respectively. For validation of the assay in buffalo, serial dilutions of different buffalo follicular fluids were analysed using the kit. The follicular fluid dilution curves were linear and parallel to the standard curve: the correlation coefficient r was 0.99 and the model was given by the equation $y = 1.004x - 0.173$.

Blood samples were collected into tubes containing EDTA at the slaughterhouse. The samples were immediately placed in ice, and later centrifuged at 600g for 15 min for separation of plasma. Plasma

samples were frozen at -20°C until later analysis. Anti-Mullerian Hormone in plasma was first measured with the same kit used for the FF (AMH Gen II ELISA kit) according to the manufacturer's instructions (Beckman Coulter, A73818, USA), as previously described. Unfortunately, the sensitivity of this kit was too low to detect any AMH concentration in plasma, so we decided to use a new kit, already used in Murrah buffaloes by Baldrighi et al (2014). Therefore, plasma AMH was evaluated using an enzyme-linked immunosorbent assay ELISA kit according to the manufacturer's instructions (Ansh Labs, Milan, Italy) as previously described for buffalos (Baldrighi et al., 2014). The sensitivity of the AMH assay was 0.011 ng/mL and intra-assay CV was <5%.

RNA Isolation and detection

Total RNA was purified for each GC sample by using RNeasy Mini Kit (Qiagen, 74104, Germany). The protocol was performed according to the manufacturer's instructions. The final elution step was repeated and the purified total RNA was subsequently stored at -80°C. Quantity of the total RNA samples was evaluated by Thermo Scientific™ NanoDrop™ 2000 spectrophotometers. The level of degradation was analysed using agarose electrophoresis. Samples with Clear 28S and 18S ribosomal RNA bands and band intensities of 28S:18S close to two were chosen in this study.

cDNA synthesis

One µg of detected RNA was used to synthesize first-strand cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen, 205311, Germany) according to the manufacturer's protocol. To eliminate genomic DNA contamination in RNA samples, the gDNA elimination reaction was performed using 2 µL of gDNA wipe-out buffer, 1 µg template RNA and RNase-free water to adjust the final volume to 14 µL at 42°C for 2 min. Reverse transcription was carried out in a total of 20 µL reaction

containing 1 µL Quantiscript Reverse Transcriptase, 4 µL Quantiscript RT buffer with Mg^{2+} and dNTPs, 1 µL RT Primer Mix and 14 µL Template RNA (Entire genomic DNA elimination reaction) at 42°C for 15 min and 95°C for 3 min. All steps were performed on ice. The cDNA was stored at -20°C until real-time PCR analysis.

Real-Time PCR analysis

Real-time quantitative PCR reactions were run using QuantiFast® SYBR® Green PCR Kit (Qiagen, 204054, Germany) on BIORAD CFX Manager Machine. The *AMHR2, FSHR, LHR, CYP19A1* and *TP53INP1, CASP3,* as well as *ACTB* primers (shown in Table 1) were designed by Primer Premier 5 software and synthesized by TSINGKE Biological Technology Company (Wuhan, China). Samples were prepared according to the manufacturer's instructions. A total reaction volume of 20 µL contained 10 µL Master Mix, 2 µL primers and 8 µL diluted cDNA (1:30). The samples were then centrifuged at 1000 X g at 4°C for 1 min. All cDNA samples were amplified under the following conditions: 95°C for 5 min, 40 cycles at 95°C for 10 s and 60°C for 30 s. The data were subsequently quantified according to the comparative cycle threshold (CT) method and expression levels were presented as $2^{-\Delta CT}$ normalized to the *ACTB* housekeeping gene.

Table 1. Primers used in this study for Real time PCR.

Gene		Sequence (5'to3')	Accession No.	Product size (bp)
AMHR2	F	attgtccgctttatcaccgc	XM_006047467.1	213
	R	tggtttgtactggccatcct		
CYP19A1	F	gttgcaattcatcggcatgc	NM_001290963.1	204
	R	gtcaacacgtccacatagcc		
FSHR	F	aaacgtgttctccaacctgc	NM_001290962.1	157
	R	cttgtgaacagctggcaagt		
LHR	F	gctctacctgctgctcattg	XM_006078413.1	216
	R	ttcagtcgcagcttttggtc		
TP53INP1	F	ataacccaggcagtcccaga	XM_006076259.1	181
	R	tggcgacgaaggctatttct		
CASP-3	F	cagcgtcgtagctgaacgta	XM_006075118.1	248
	R	ccgcatccacatctgtacca		
ACTB	F	atgatgatattgccgcgctc	NM_001290932.1	216
	R	cgtgctcaatggggtacttg		

Statistical Analysis

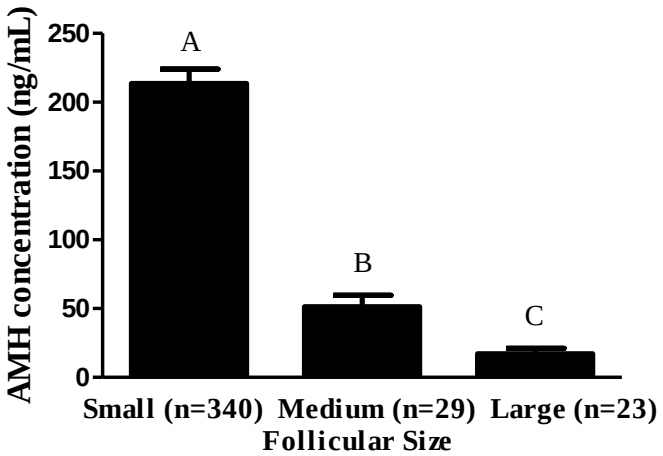
The differences in AMH concentration in FF and gene expression in GCs among classes of follicles were analysed by ANOVA, and Least Significant Difference (LSD) was used as a post-hoc test. Pearson correlation analysis was carried out to evaluate the relationship between AMH and the AFC, as well as among other reproductive parameters on samples from 54 animals. To evaluate the correlation between AMH concentration in FF and gene expression in GCs, samples from 22 animals were used. The differences in AMH plasma levels and reproductive parameters, including, between good (≥12 follicles) and bad (<12 follicles) donors were analysed by Student's t test. Differences in gene expression levels between good and bad donors were also analysed by Student's t test. Differences in the percentages of grade A+B COCs between good and bad donors were analysed by Chi Square test. In all cases a P-value <0.05 was accepted as statistical significance.

Results

AMH concentration in FF and gene expression in GCs in relation to follicular size

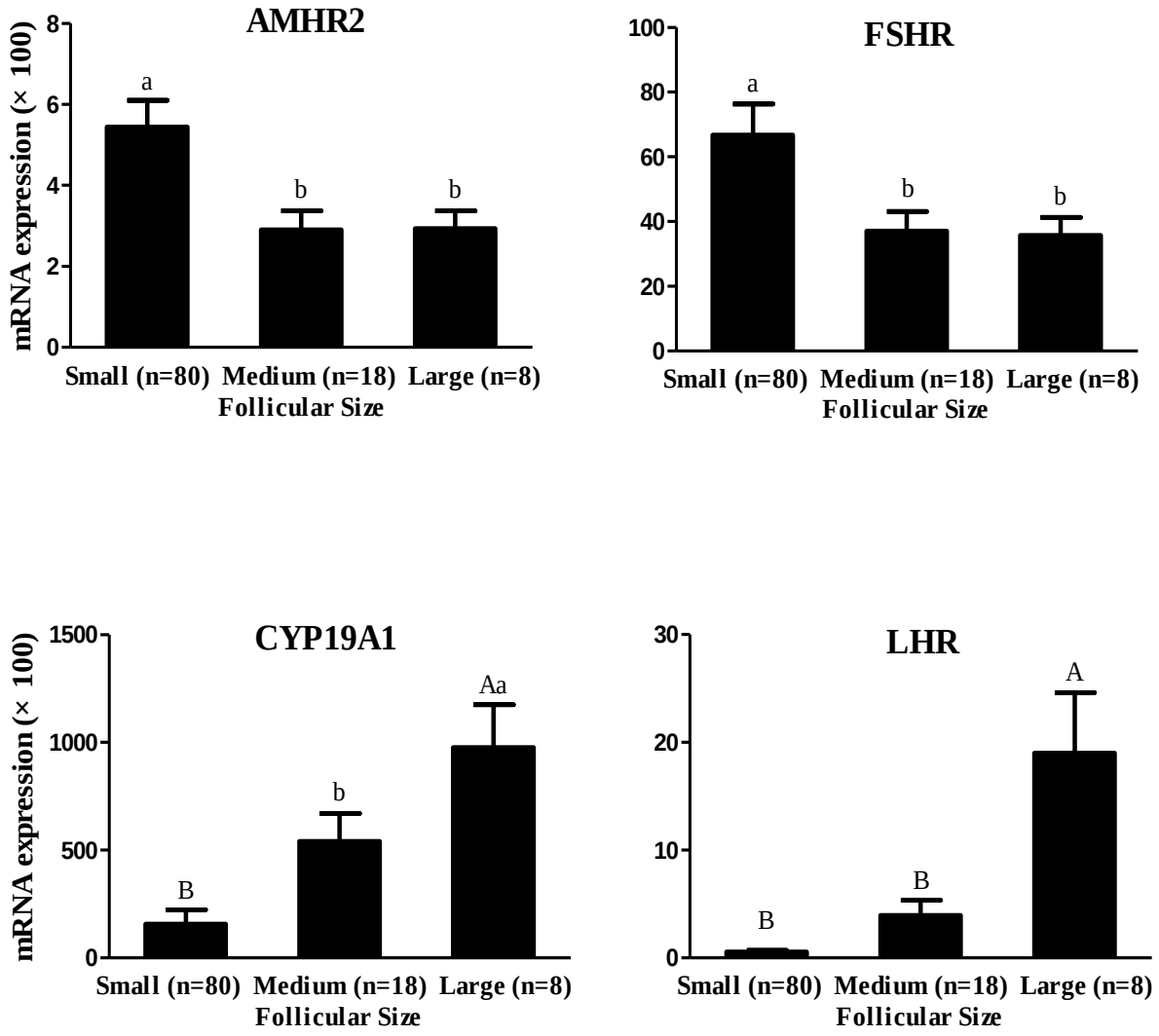
Anti-Mullerian hormone concentration in FF decreased (P<0.01) at increasing follicular diameter, as shown in Figure 6. Indeed, AMH concentration in small follicles was 4 and 12 times higher than that recorded respectively in medium and large follicles. As shown in Figure 7, mRNA expression of *AMHR2* and *FSHR* was higher (P<0.05) in small follicles than in medium and large follicles. In contrast, mRNA expression of *LHR* and *CYP19A1* was higher (P<0.05) in large follicles than in small and medium follicles.

Figure 6. Intrafollicular AMH concentration (ng/mL) in relation to the follicular size.



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

Figure 7. Relative mRNA expression of *AMHR2*, *FSHR*, *CYP19A1* and *LHR* genes in GCs from total 106 buffalo antral follicles in relation to follicular diameter.



a,

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

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

Correlation analysis

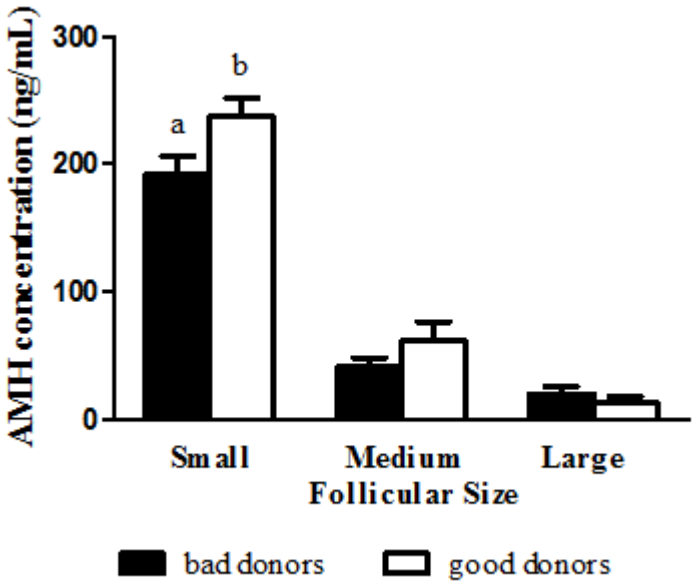
Unexpectedly, no correlations were found between plasmatic AMH levels and all the other parameters. In contrast, the AMH concentration in FF of small follicles was positively correlated with the AFC ($r = 0.31$; $P<0.05$) and with the number of small follicles ($r = 0.29$; $P<0.05$). In addition, the AFC and the average number of small follicles were positively correlated with the number of COCs ($r = 0.76$; $P<0.001$ and $r = 0.78$; $P<0.001$, respectively) and Grade A+B COCs ($r = 0.52$; $P<0.001$ and $r=0.46$; $P<0.001$, respectively). However, no correlation was found between AMH concentration and both the number and percentage of Grade A+B COCs.

The intrafollicular AMH concentration was positively correlated with *AMHR2* ($r= 0.537$; $P<0.01$) and *FSHR* ($r= 0.385$; $P<0.05$) and negatively correlated with *CYP19A1* ($r=-0.486$; $P<0.01$) and *LHR* ($r=-0.499$; $P<0.01$). The *AMHR2* was positively correlated with *FSHR* ($r=0.560$; $P<0.01$), while *CYP19A1* was correlated with *LHR* ($r= 0.535$; $P<0.01$).

Differences between good and bad donors

No differences were found between good (≥12 follicles; n = 26) and bad (<12 follicles; n = 28) donors in the mean weight (5.4 ± 0.2 vs. 5.0 ± 0.3), length (3.3 ±0.1 vs. 3.2 ± 0.1), and width (2.1 ± 0.1 vs. 2.0 ± 0.1) of the ovaries. Unexpectedly, good and bad donors showed similar AMH plasma level (0.155± 0.01 and 0.168 ± 0.01, respectively) but it is worth pointing out that reading was only possible at the lower points of the curve and, according to our experience, the assay cannot be considered reliable. However, good donors had a higher ($P < 0.05$) concentration of AMH in small follicles than bad donors (Fig. 8).

Figure 8. Intrafollicular AMH concentration (ng/mL) in different size follicles in good (≥ 12 follicles; n =26) and bad (<12 follicles; n =28) buffalo donors.



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

As expected, good donors also had higher numbers of small follicles and COCs (P < 0.01), as well as of grade A and B COCs (P < 0.05), as shown in Table 2. However, no differences were found in the percentage of superior quality oocytes (grade A and B).

Interestingly, good donors had higher (P<0.05) *AMHR2* levels in small follicles and higher (P<0.05) *LHR* levels in large follicles than bad donors (Figure 9). However, there were no significant differences of *FSHR* and *CYP19A1* gene expression between good and bad donors. Likewise, no differences were observed in the relative expression of *TP53INP1*, and *CASP3* in GCs between good and bad donors (Figure 10).

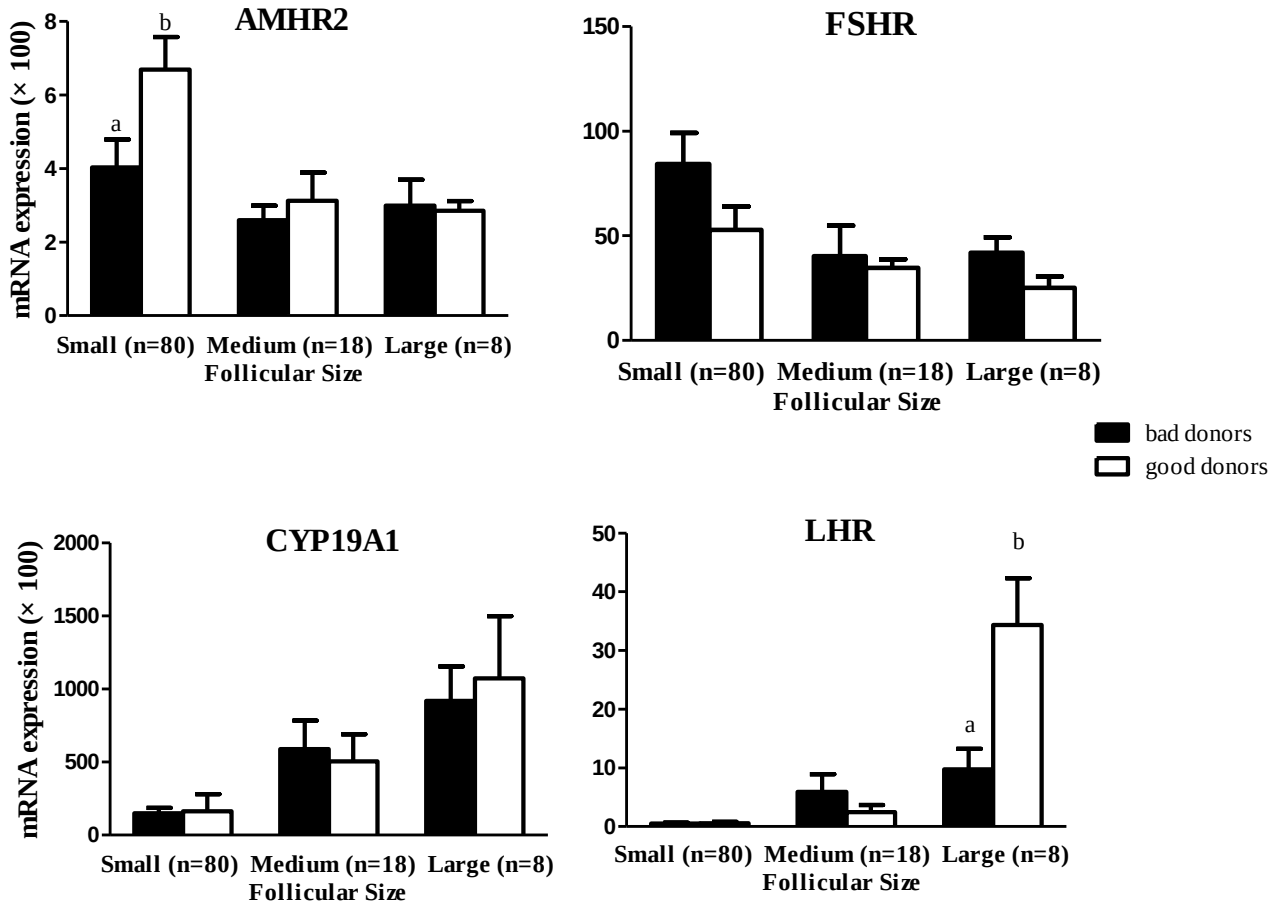
Table 2. Differences between good (≥ 12 follicles; n=26) and bad (< 12 follicles; n=28) donors in the number of total (TFL), small (SFL), medium (MFL) and large (LFL) follicles, COCs, good quality COCs (G. A+B), recovery rate and the percentage of good quality COCs. Data are presented as mean ± SE.

Donors	SFL n.	MFL n.	LFL n.	TFL n.	COC n.	Recovery rate %	G. A+B n.	G. A+B %
Bad	7.9 ± 0.3^{A}	0.8 ± 0.2	0.8 ± 0.1	8.9 ± 0.4^{A}	5.5 ± 0.6^{A}	62.5 ± 7.2	1.9 ± 0.3^{a}	36.3 ± 5.7
Good	14.8 ± 0.9^{B}	1.4 ± 0.4	0.6 ± 0.1	16.2 ± 0.8^{B}	11.4 ± 1.2^{B}	68.1 ± 4.9	3.4 ± 0.6^{b}	31.9 ± 5.0
Total	11.2 ± 0.7	1.1 ± 0.2	0.7 ± 0.1	12.4 ± 0.7	8.3 ± 0.8	65.2 ± 4.4	2.6 ± 0.4	34.1 ± 3.8

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

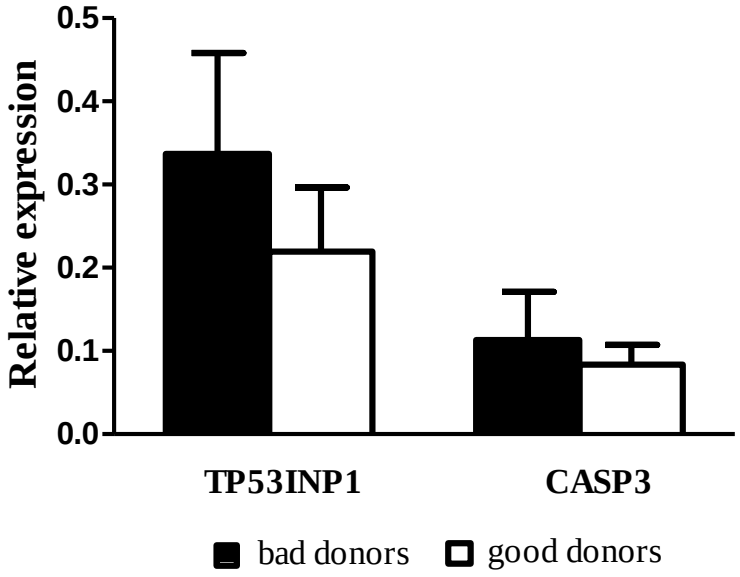
$^{a, b}$ Values with different superscripts are significantly different; P<0.05

Figure 9. Relative mRNA expression of *AMHR2*, *CYP19A1*, *FSHR* and *LHR* in good (≥ 12 follicles; n=12) vs bad (< 12 follicles; n =10) donors.



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

Figure 10. Relative mRNA expression of *TP53INP1* and C*ASP3* in GCs of small follicles from good (≥ 12 follicles; n=12) and bad (< 12 follicles; n =10) donors.



Discussion

To our knowledge, this is the first study to integrate information of intrafollicular concentration of AMH in different size follicles with the expression of four genes, known to be involved in folliculogenesis and steroidogenesis, namely *AMHR2*, *FSHR*, *LHR* and *CYP19A1* by corresponding GCs in buffalo. Knowledge of factors involved in regulating follicular growth may be useful to gain insights into the causes of failure in response to hormonal stimulation in this species. Furthermore, as the ovarian follicular reservoir in buffalo is low, strongly limiting the application of reproductive technologies, and high individual variability in follicular recruitment is recorded (Gasparrini et al., 2014),

identification of a reliable marker predictive of the population of the small antral gonadotrophin-responsive follicles is fundamental. Therefore, another goal of the work was to assess whether plasmatic and intrafollicular AMH concentrations were correlated to the AFC and to evaluate differences in reproductive parameters between good and bad donors.

The results of this study demonstrated that intrafollicular AMH concentration decreases at increasing follicular size, with the small follicles (3-5 mm) contributing most of the AMH, similar to other species (Eilsø Nielsen et al., 2010; Monniaux et al., 2011; Rico et al., 2011). The total AMH content per follicle significantly decreased in medium follicles (5-8 mm) and was further reduced in large follicles (> 8 mm). The small follicles, in addition to the highest intrafollicular AMH concentration, also showed the highest expression of *AMHR2*, which encodes the specific AMH type II receptor, suggesting an autocrine mechanism in regulating follicular development. Interestingly, the highest expression in GCs of *FSHR* was also observed in small follicles, while it decreased 2-fold in both medium and large follicles. A decrease in *FSHR* expression in GCs was also previously reported in large compared to small follicles in other species (Monniaux et al., 2011; Catteau-Jonard et al., 2008). The positive correlation between the intrafollicular AMH concentration and the expression of *AMHR2* and *FSHR* in GCs confirms these findings and suggests that these may be functional markers of immature GCs. As expected, both the expression of *LHR* and *CYP19A1*, markers of maturation and differentiation of GCs (Rico et al., 2009), increased in follicles > 8 mm, indicating that they had reached the maturity stage of preovulatory follicles. It is indeed known that the expression of *CYP19A1* gene, encoding the aromatase, increases in GCs since the stage of follicular selection, leading to enhanced oestradiol secretion. Moreover, the GCs become more responsive to LH, through an increased expression of *LHR* gene, encoding the LH receptor, in the preovulatory follicle (Clément and Monniaux, 2013).

The negative association between AMH concentration and the expression of *CYP19A1* in the corresponding GCs, observed in this work confirms previous reports (Rico et al., 2009; Jeppesen et al., 2013) and suggests a role of AMH within small follicles to prevent premature oestrogen production. In the present study, a negative correlation was also found between AMH concentration and the expression of *LHR* by GCs. This is in line with an earlier in vitro study that demonstrated an inhibitory effect of AMH on the induction of *CYP19A1* and LH receptors by FSH in rat and porcine GCs (Di Clemente et al., 1994).

Another interesting finding arising from the study was the positive correlation found between the intrafollicular AMH concentration and both the AFC and the number of small follicles, suggesting that AMH can be used as a marker of the population of gonadotrophin-responsive follicles in buffalo. The importance of this finding is highlighted by the high correlation between the AFC and both the number of COCs and Grade A+B COCs. However, in contrast to previous works in other species (Rico et al., 2009; Monniaux et al., 2011), including Murrah buffalo (Baldrighi et al., 2014), the AMH levels in plasma were not correlated to the AFC. It is worth to point out that two different kits were used in the present study, including the kit previously employed to measure the plasmatic AMH level in Murrah buffaloes (Baldrighi et al 2014). The first kit (AMH Gen II ELISA kit, Beckman Coulter, A73818, USA), reliable to detect AMH in FF, did not allow to measure AMH in plasma. For this reason, we repeated the analysis with the kit (Ansh Labs, Milan, Italy) used by Baldrighi et al (2014). This allowed to measure AMH but, as reading was only possible at the lower points of the curve, according to our experience the assay was not reliable. It is worth mentioning that the other authors also encountered the same problem in measuring plasmatic AMH levels in buffalo. It is known that AMH concentration is much lower in plasma than in FF in other species (Monniaux et al., 2012) but these results suggest that in buffalo the AMH

concentration in plasma is even lower and that, in order to use this as a marker to screen donors, a more reliable specific assay should be developed.

The importance of selecting buffalo cows to enrol in embryo production programs suggested comparing the most relevant reproductive parameters between good (≥ 12 follicles) and bad (< 12 follicles) donors. Despite the larger AFC, the ovaries of good donors had similar weight and size to those of bad donors. Nevertheless, they showed larger numbers of small follicles, COCs and Grade A+B COCs compared to bad donors, confirming previous findings (Gasparrini et al., 2014). However, the increased number of Grade A+B COCs depends on the greater AFC, as no effect was observed on the percentage of superior quality oocytes, evaluated by morphological criteria. As previously discussed, the failure in highlighting differences in plasmatic AMH levels between good and bad donors may be due to the sensitivity of the kit. Interestingly, good donors had both higher levels of AMH in the FF and higher expression of *AMHR2* in corresponding GCs in small follicles, as well as higher expression of *LHR* in large follicles. The latter finding indicates that good donors, in addition to the expected higher AFC, also differ from bad donors in improved maturation of large follicles and hence in oocyte competence, suggesting a better response to embryo production technologies. It has been reported elsewhere that AMH expression is higher in healthy follicles than atretic follicles, speculating that AMH inhibits apoptosis in granulosa cells (Rico et al., 2009; Lehmann et al., 2014). The higher AMH concentrations in FF and the expression of *AMHR2* in corresponding GCs found in this study in good donors suggest that these may have a lower atresia rate. To evaluate the phenomenon, we have chosen an upstream regulator of apoptosis, i.e. *TP53INP1*, a good molecular candidate of atresia, according to Hatzirodos et al (2014) and *CASP3*, known to be a downstream executioner caspase responsible for degradation of cellular proteins (Creagh et al., 2001; Riedl and Shi, 2004). As no significant change in the expression of both apoptosis-related genes was

detected in small follicles between good and bad donors, this hypothesis appears to be ruled out.

In conclusion, it was demonstrated that the intrafollicular AMH concentration shows an inverse pattern to follicular size in buffalo. Moreover, small follicles have a higher expression of *AMHR2* and *FSHR* and a lower expression of *LHR* and *CYP19A1* in corresponding GCs compared to medium and large follicles. Good donors showed higher levels of AMH and greater mRNA expression of *AMHR2* in small follicles and higher expression of *LHR* in large follicles. The positive correlation found between intrafollicular AMH concentration and the AFC suggests that AMH is indeed a marker of follicular reserve also in buffalo. However, no correlation was observed with the plasmatic AMH levels, likely because of the poor sensitivity of the available kits. The use of intrafollicular AMH concentration would imply the collection of FF by transvaginal aspiration prior enrolment of donors, which is certainly less practical than the AFC itself. Therefore, at present the only reliable practical way to select donors to for embryo production programs is to evaluate the AFC.

EXPERIMENT 2

Utilization of eCG during the final stages of follicular growth in a superovulation protocol

It is well known that FSH plays a pivotal role during the early stages of follicular development, ensuring follicular recruitment and growth (Adams et al 1992, 1993) until the dominant follicle reaches a certain size, i.e. 8.5 mm diameter in Bos Taurus (Ginther et al 1996) 6.2 mm in Bos indicus (Sartorelli et al 2005, Gimenes et al 2008) and 7.2 mm in buffalo (Gimenes et al 2011). At this stage, after acquisition of LH receptors, the dominant follicles switch to LH-dependence (Mihm and Evans 2008). It follows that, in order to better mimic the follicular dynamics in superovulated animals, FSH should be given first to ensure follicular growth, followed by LH administration at the time of presumptive follicular deviation. Equine chorionic gonadotrophin, characterized by both FHS and LH activity, can be used for this purpose. Recent studies (Barros et al., 2008; Sartori et al., 2009; Mattos et al., 2011) have evaluated the possibility of replacing the last injections of FSH with eCG during the superovulatory treatment in Bos Indicus with opposing results. In fact, some authors (Barros et al., 2008) have observed a better response to superovulation in animals treated with eCG, while others (Sartori et al., 2009) have not described any favourable effect. In both cases, the last two injections of FSH were replaced with 2 injections of 150 IU eCG. In Italian Mediterranean buffalo eCG has been used in progestin-based synchronization protocols at a dose of 1000 IU (Neglia et al., 2003). It was recently observed that a single administration of 500 IU of eCG on Day 7 of an Ovsynch synchronization protocol does not increase follicular growth until the day of fixed time insemination (Neglia et al., unpublished results). To our knowledge, however, the inclusion of eCG near the end of the superovulation

protocol has still not been evaluated in buffalo. Therefore, the aim of the present experiment was to assess the efficacy of replacing the last four FSH injections with a single administration of 1000 IU of eCG compared to the conventional FSH decreasing superovulation protocol in Italian Mediterranean Buffalo. In particular, the comparison of the two superovulation protocols was based on the study of follicular dynamics and the evaluation of CL and embryo yields.

Materials and Methods

The Ethical Animal Care and Use Committee of the University of Naples Federico II (Naples, Italy) approved the experimental design and animal treatments.

Animals and housing

The study was carried out on multiparous Italian Mediterranean buffaloes (n = 20) located at two commercial buffalo dairy farms in the South of Italy (between 40.5° N and 41.5° N parallel). The study coincided with the transition to seasonal anoestrus (from January to May) when many buffaloes become non-cyclic as a result of increasing day-length. Buffaloes were milked twice daily and were at 95±38 days post-partum at the beginning of the study. Animals were maintained in open yards on a cement pad that allowed 15 m^2 per animal and fed with a total mixed ration that consisted of 50-55% forage and 50-45% concentrate, containing 0,90 UFL/Kg of dry matter (DM) and 15% crude protein/DM.

Synchronization and MOET scheme

Animals were synchronized with a double injection of 0.524 mg of a PGF2α analogue (Cloprostenol, Estrumate® Schering-Plough Animal Health, Milan, Italy) spaced 12 days apart, to induce luteolysis and synchronize the occurrence of oestrus. Because of the low intensity of oestrous behaviour of buffalo (Ohashi 1994), 60 h after the last injection of PGF2α, animals were subjected to trans-rectal examination of the genital tract by ultrasound (Aloka SSD-500, Tokyo-Japan) to assess oestrus status (follicle >1.2 cm and a tonic uterus with the presence or absence of mucous vaginal discharge). Only buffaloes that responded to synchronization underwent the superovulation treatment, while the remaining animals were excluded from the trial. Therefore, on the day of ultrasonographic study (day of oestrus or day 0), an intravaginal progesterone implant (CIDR®, Zoetis, Rome, Italy) was inserted only in the animals selected for the trial on the basis of the response to synchronization. Superovulation treatment started 8 days after oestrus detection and insertion of the CIDR®. In particular, animals were divided into two groups (A and B) homogeneous for days open, number of lactations and average production recorded in the last 7 days:

a) The Group A (n = 6) received a double daily dose of 400 mg of FSH (Folltropin ®, Bio98, Milan, Italy) in a dose dependent manner for four consecutive days. Briefly, the scheme was 80 mg on day 8, 60 mg on day 9, 40 mg on day 10, and 20 mg on day 11, given twice daily. At day 10 (72 and 84 hours after the start of treatment with gonadotropins) all treated buffaloes were subjected to a double injection of 0.524 mg PGF2α (Cloprostenol, Estrumate®, Schering-Plough Animal Health, Milan, Italy) to induce luteolysis.

b) The buffaloes of Group B (n = 7) received a superovulation treatment lasting four days. In particular, the treatment was the same as in Group A for the first two days, while the last four injections of FSH were substituted with a single injection of 1000 IU of pregnant mare serum

gonadotropin (eCG; Ciclogonina, Zoetis, Rome, Italy). At day 10 (72 and 84 hours after initiation of treatment with gonadotropins) all treated buffaloes were subjected to a double injection of 0.524 mg PGF2α (Cloprostenol, Estrumate®, Schering-Plough Animal Health, Milan, Italy) to induce the luteolysis.

All cows were artificially inseminated at oestrus with single straw semen from bulls of proven fertility.

Ultrasound examination

Ultrasonographic investigations in the two groups were carried out at the time of CIDR insertion to verify the response to synchronization treatment. Furthermore, the follicular dynamics in the two groups was assessed daily, from the beginning to the end of treatment. Further ultrasound examinations were carried out 48 hours after oestrus, to check the ovulation rate. Ovulations occurring 48 hours after oestrus were not considered. In every ultrasound examination the total number and the size of follicles were recorded. In particular, follicles were classified into three categories depending on the diameter:

- Small: with a diameter between 0.20 and 0.50 cm;
- Medium: with a diameter between 0.51 and 1.00 cm;
- Large: with a diameter greater than 1.00 cm.

Assessment of CL and embryo yields

Six days after oestrus ultrasound examination was carried out to establish the number and size of corpora lutea on the ovaries. Then ova and embryos were collected by a nonsurgical

procedure (Newcomb et al., 1978), evaluated and classified as quality I embryos (freezable), quality II embryos (transferable) and degenerated embryos based on the IETS guidelines (Stringfellow and Seidel, 2007).

Statistical Analysis

All statistical analyses were performed using SPSS Software (SPSS 2005). In particular, data regarding the incidence of the different follicular categories were analysed by chi-square test. The average number of total follicles and that of all follicular categories were compared between groups using the Student's t test. Differences in the average follicular diameter for each category of follicles during the superovulatory treatment until oestrus were also compared using Student's t test. Differences were considered statistically significant when they reached at least the 5% confidence level. Differences of less than 10 % were considered to be indicative of a trend.

Results

The response to synchronization was 65 % (13/20), in agreement with previous studies (Neglia et al., 2010). Both the number of total follicles and the number of follicles of different categories, recorded at the beginning of the superovulatory treatment (day 8), and after the first four injections of FSH (day 10) were similar in Group A and Group B (Table 3). At the time of oestrus (Day 13), animals treated with eCG (Group B), despite a similar number of total follicles, had a higher incidence ($P = 0.06$) of large follicles compared to Group A (Table 3).

Table 3 - Average number of small (SFL), medium (MFL), large (LFL) and total (TOT FL) follicles recorded in in buffaloes superovulated with FSH (Group A) and a FSH + eCG (Group B), at the beginning of superovulatory treatment (day 8), at the beginning of eCG treatment in group B (day 10) and at oestrus (day 13).

Group	**SFL**			**MFL**			**LFL**			**TOT FL**		
	Day			**Day**			**Day**			**Day**		
	8	10	13	8	10	13	8	10	13	8	10	13
A (n=6)	7.8±1.5 (47)	4.7±1.1 (28)	3.2±0.7 (19)	1.5±0.3 (9)	8.5±0.7 (51)	5.5±1.4 (33)	1.0±0.0 (6)	1.2±0.2 (7)	3.3±0. 7[a] (20)	10.3±1.5 (62)	14.3±1.2 (86)	12.0±1.5 (72)
B (n=7)	7.7±1.8 (54)	4.7±1 (33)	2.6±1.2 (18)	1.6±0.6 (11)	8.3±1.2 (58)	5.0±1.1 (35)	1.0±0.0 (7)	1.0±0.0 (7)	5.6±1.6[b] (39)	10.3±2.1 (72)	14.0±1.6 (98)	13.1±2.0 (92)

[a,b] Values with different superscripts are significantly different; P=0.06.

Although the number of dehiscent follicles within 40 hours after oestrous was not different between the two treatments (2.83±0.60 vs. 4.43±0.78, Group A and Group B, respectively), the animals of Group B showed a higher number of ovulations within the first 28 hours (0.50±0.22 vs. 3.14±0.74; $P<0.01$ in Groups A and B, respectively). The mean follicular diameter within follicular categories (Table 4) was similar in the two groups at the beginning of the superovulatory treatment (day 8) and after the first four injections of FSH (day 10). The mean diameter of the small and medium follicles was also not affected by treatment at the time of oestrus (Table 4). On the contrary, the average diameter of large follicles, recorded at the day of oestrus was greater ($P <0.05$) in Group B compared to Group A (1.34±0.07 vs. 1.54±0.06, Group A and Group B, respectively).

Table 4 - Average diameter (cm) of small (SFL), medium (MLF) and large (LFL) follicles, recorded in buffaloes superovulated with FSH (Group A) and a FSH + eCG (Group B) at the beginning of superovulatory treatment (day 8), at the beginning of eCG treatment in group B (day 10) and at oestrus (day 13).

Group	**SFL**			**MLF**			**LFL**		
	Day			**Day**			**Day**		
	8	10	13	8	10	13	8	10	13
A (n=6)	0.3±0.0 (47)	0.4±0.0 (28)	0.3±0.0 (19)	0.6±0.0 (9)	0.7±0.0 (51)	0.7±0.0 (33)	1.4±0.1 (6)	1.5±0.1 (7)	1.3±0.1[a] (20)
B (n=7)	0.3±0.0 (54)	0.4±0.0 (33)	0.3±0.0 (18)	0.6±0.0 (11)	0.7±0.0 (58)	0.7±0.0 (35)	1.4±0.1 (7)	1.5±0.1 (7)	1.5±0.1[b] (39)

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

Furthermore, six days after oestrus, at the time of flushing, ultrasound examination of the ovaries highlighted a higher number (P = 0.07) of large follicles (anovulatory follicles) in Group B compared to Group A (Table 3). Moreover, the average diameter of medium and large follicles was higher (P<0.01) in buffaloes of the Group B compared to Group A (Table 5).

Table 5. Average number and diameter of small (SFL), medium (MFL), large (LFL) and total (TFL) follicles, recorded in buffaloes superovulated with FSH (Group A) and a FSH + eCG (Group B) at the time of flushing (6 days after oestrus).

Group	**n. SFL**	**Diameter (cm) SFL**	**n. MFL**	**Diameter (cm) MFL**	**n. LFL**	**Diameter (cm) LFL**	**n. TOT FL**	**Diameter (cm) TFL**
A (n=6)	2.3 ± 0.9	0.3 ± 0.0	1.3 ± 0.6	0.7 ± 0.0^{A}	1.7 ± 0.5^{a}	1.4 ± 0.1^{A}	5.3 ± 0.7	0.8 ± 0.1^{A}
B (n=7)	1.4 ± 0.5	0.3 ± 0.0	2.3 ± 0.6	0.8 ± 0.0^{B}	4.3 ± 1.1^{b}	1.9 ± 0.1^{B}	8.0 ± 1.3	1.3 ± 0.1^{B}
Total	3.7 ± 0.7	0.3 ± 0.0	3.6 ±0.6	0.8 ± 0.0	6.0 ± 0.9	1.7 ± 0.1	13.3 ± 1.0	1.0 ± 0.1

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

$^{a, b}$ Values within columns with different superscripts are different; P = 0.07

No differences were recorded in the number and area of CL, in the number of embryos recovered and in the recovery rate between groups; however, a higher (P=0.06) number of ova was collected in group A (Table 6).

Table 6. Number and area of corpora lutea (CL), embryos and ova recovered and recovery rate (RR) on day 6 after oestrus in buffaloes superovulated with FSH (Group A) and a FSH + eCG (Group B).

	n. CL	**CL area (cm^2)**	**n. Embryos**	**n. Ova**	**RR (%)**
Group	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**
A (n =6)	6.3 ± 2.4	1.3 ± 0.2	1.3 ± 1.3	2.0 ± 1.2[a]	31.4 ± 24.6
B (n =7)	4.4 ± 0.7	1.5 ± 0.3	0.6 ± 0.4	0.8 ± 0.6[b]	12.6 ± 8.5
Total	5.35 ± 1.5	1.4 ± 0.3	0.9 ± 0.5	1.3 ± 0.6	19.6 ± 10.1

[a,b] Values within columns with different superscripts are different; P =0.06

Discussion

The results of this experiment demonstrated that the strategy to replace the last FSH doses with a single administration of eCG (group B) was effective in increasing the response to superovulation, as indicated by the increased number and greater diameter of large follicles at oestrus and by the higher incidence of ovulations occurring within 28 h post-oestrus. Nevertheless, the number of ovulations within 40 h post-oestrus, as well as the number and area of CL found on day 6 post-oestrus were similar in the two groups. In addition, a higher number of large follicles was observed on day 6 post oestrus in group B. Finally, although the number of viable embryos recovered was

similar, the average number of ova was higher (P= 0.06) in group A, i.e. conventional FSH treatment.

The main objective of the superovulatory treatment is to obtain a high number of embryos from high genetic merit subjects (Mapletoft et al., 2002; Mapletoft & Bo, 2012). Although this technique is routinely used in cattle, with an average recovery of 5 good quality embryos (Galli and Lazzari, 2005), in buffaloes, despite the numerous studies carried out over the past 20 years by researchers around the world, the average recovery is around two embryos per animal (Misra, 1993; Zicarelli et al., 1997b; Carvalho et al., 2002; Neglia, 2006; Misra & Tyagi, 2007; Neglia et al., 2010). One of the limitations of MOET in buffalo species is the selection of cyclical subjects that are more likely to be responsive (producing at least one embryo) to the superovulatory treatment (Zicarelli, 1997b; Neglia et al., 2010). In our study, we synchronized the buffaloes with a double injection of an analogue of PGF2α. This allowed us to select cyclical subjects and then discard those in anoestrus, which could misrepresent the results of follicular dynamics. In this study, buffaloes that received eCG in the last two days of the superovulatory treatment presented a greater number of large follicles at the time of oestrus, and thus a better response to superovulation, compared to those who received four FSH injections. The hormonal treatments mainly used in superovulation programs are based on gonadotrophins (FSH and LH) and on equine chorionic gonadotropin. The need to administer FSH and LH at different times during superovulatory treatment has been a much debated topic. Basic studies on follicular development showed that the follicular growth, when follicles have a diameter of at least 0.4 cm, is closely FSH-dependent (Lucy, 2007), since FSH allows follicular recruitment (Adams et al., 1992; Adams et al., 1993). This is true until the dominant follicle reaches 0.85 cm in diameter in Bos Taurus (Ginther et al. 1996) and 0.62 cm

in Bos Indicus (Sartorelli et al. 2005; Gimenes et al. 2008). Recent studies carried out in Murrah buffalo heifers (Gimenes et al., 2011) have shown that the deviation of the dominant follicle takes place when the latter reaches the diameter of 0.72 cm. In contrast, a positive response to GnRH in terms of ovulation rates was observed in follicles of about 0.5 cm in diameter in the Italian Mediterranean buffalo (Campanile et al., 2008). In any case, after the selection and deviation phase, the dominant follicles acquire LH receptors on the granulosa and theca cells and become LH-dependent (Lucy, 2007; Mihm & Evans, 2008). Studies carried out in the 90s (Xu et al., 1995) using in situ hybridization technique demonstrated that, at least in bovine, FSH receptors continue to be expressed on the granulosa cells throughout follicular development, while disappear in swine upon the transition of the follicle from FSH to LH dependent-phase (Liu et al., 2004). It is clear, therefore, that the follicles of superovulated animals could benefit from the LH administration, especially at the end of the superovulation protocol. Equine chorionic gonadotrophin is a complex glycoprotein, characterized by a long half-life (over 40 hours), which has both LH and FSH-like action (Steward et al. 1976; Licht et al. 1979; Murphy & Martinuk 1991) and could be a constant stimulus to the LH receptors of growing follicles towards the end of a conventional FSH-based superovulation treatment.

After the first two days of FSH treatment, over 60% of medium follicles with a diameter greater than 0.65 cm were recorded both in Groups A and B. It is likely, therefore, that these follicles passed the stage of deviation and acquired the LH receptors. The substitution of the last four doses of FSH, which represent the 30% of the total of the superovulatory treatment, with a single dose of eCG has allowed the growth of a greater number of follicles, as also demonstrated by the fact that the diameter of large follicles in Group B was greater compared to Group A. These results

would be, at least in part, in agreement with a recent study in the Bos Indicus in which the last two FSH administration were replaced by two injections of 150 IU of eCG and the animals treated with eCG showed a greater number of large follicles than those that received FSH injections (Mattos et al., 2011). However, the last two doses of FSH were replaced by only two injections of 300 IU eCG in total, while in our study we used a single dose of 1000 IU eCG. Moreover, during the oestrous cycle Bos Indicus has a number of follicles considerably greater than Bos Taurus (Sartori and Barros, 2011) and buffalo (Baruselli et al., 1997; Neglia et al., 2007) and hence the administration of only 300 IU could be sufficient to ensure adequate follicular growth in this species in the last days of the superovulatory treatment. In this experiment, due to the low population of primordial follicles in buffalo (Danell, 1987) it was thought to support the follicular growth with a greater amount of eCG. It is worth pointing out that the greatest diameter, reached by large follicles in Group B, also resulted in a higher rate of ovulation within 28 hours. The phenomenon of ovulation involves a complex series of biochemical and biophysical events that leads to dehiscence of the preovulatory follicle (Lucy, 2007; Sirois et al., 2004). It is known that the induction of ovulation via exogenous administration of GnRH or hCG of follicles with a diameter less than 1.15 cm could determine the formation of smaller corpora lutea that produce lower amounts of progesterone than dominant follicles showing a greater size (Vasconcelos et al., 2001). Furthermore, the diameter of the dominant follicle is closely related to the oestradiol blood concentration (Perry et al., 2006), which is able to act by positive feed-back at the hypothalamic level on the LH release. The action of LH causes the activation of the cyclooxygenase-2 (COX-2) in the ovary (Sirois et al., 2004) and, consequently, the synthesis of prostaglandins that promote ovulation. It is conceivable, therefore, that the presence of a larger size dominant follicles in the

group of buffaloes treated with FSH / eCG has favoured the release of increased amounts of oestradiol and hence, LH, leading to anticipation of the activation of COX-2 and then ovulation. In Group A, on the contrary, the highest rate of ovulation occurred within 40 h. In any case, the administration of eCG was not able to avoid the problem of anovulatory follicles. The long half-life of eCG is an advantage in some cases, since it allows to implement the treatment of superovulation by performing a single administration, on the other hand is a disadvantage, since even after oestrous, the hormone continues to exert its action, causing a further growth of the non-ovulated follicles (Mapletoft and Bo, 2012). A high number of large follicles at the time of uterine flushing translates into both a lower number of embryos recovered and worse embryo quality (Gonzalez et al. 1994): the complexity in the embryo collection is mainly due to the presence of mucus in the cervix and in the uterus which obstructs the catheter during flushing. The worst quality of the embryos could be due to the oestrogen action, secreted by dominant follicles, that create an unsuitable oviductal and uterine environment. In our study anovulatory follicles were more in number and in size in Group B rather than in Group A, and this is perfectly in line with those reported in literature. In addition, the higher number of large follicles at oestrus and the higher ovulations recorded within 28 h did not result in an increased number of CL recorded at day 6 after oestrus, as well as the number of embryos recovered. In contrast, a higher number of ova was collected from buffaloes that underwent the conventional FSH protocol.

In conclusion, the replacement of the last four injections of FSH with a single administration of eCG within a superovulation protocol in buffalo allows a good superovulatory response, both in terms of number and size of follicles and in terms of ovulation, without improving the embryo recovery rate. The presence of a very high

number of large follicles six days after oestrus in the treated animals may account for the decreased ova recovery. This is likely due to the long half-life of eCG and hence an efficient alternative could be the use of anti-eCG antibody, in order to block the action of the hormone still circulating but further studies are needed to assess this hypothesis. However, what is overwhelming is the very poor recovery rate, i.e. the ova recovered in relation to the number of CL, recorded in both groups (on average < 20%). This suggests that, in order to improve the MOET efficiency in buffalo, more efforts should be made to better comprehend the mechanism and factors involved in the failure of ovum capture by the oviduct.

EXPERIMENTS 3 and 4

Analysis of factors involved in the failure of ovum capture in superovulated buffaloes

The poor number of embryos recovered per donor is undoubtedly the major constrain of MOET in buffalo. Several hypotheses have been formulated over the years to explain the low embryo recovery recorded in this species after MOET treatment. Several studies (Baruselli et al., 1999; Carvalho et al., 2002; Neglia et al., 2010) demonstrated that, despite a very low embryo/ova recovery rate (13-35%), superovulation results in the growth of a high number of ovulatory follicles, together with a relatively high ovulation rate (50-70%). Therefore, a limited capacity for follicular growth and ovulation, arising from the reduced number of primordial follicles typical of the species (Danell, 1987), cannot account for the poor embryo outcome recorded in superstimulated buffaloes. In superovulated buffaloes sacrificed at different days after AI the ratio of ova recovered in relation to the CLs after flushing of both oviducts and uteri was still low (Carvalho et al., 2006). As a consequence, the most likely hypothesis is that in superovulated buffaloes a failure in the ovum capture by the oviduct fimbria occurs. According to Hunter et al. (1988), the two main mechanisms involved in female gametes transport are the cilia beating of the oviduct epithelium and the smooth muscles contraction waves of myosalpinge. It has been shown that one of the factors involved into the regulation of oviductal contraction-relaxation and gamete/embryo transport is the active oviductal VEGF system during the peri-ovulatory period (Wijayagunawardane et al., 2005). Carvalho

(2006) found that buffalo's ovary adheres more firmly to the mesovarium than the cow's ovary. This feature, in association with hormonal changes observed during the superovulatory treatments (e.g. high oestradiol concentrations), could compromise the ovum capture and transport trough the oviduct. Indeed, since buffaloes have lower plasmatic levels of oestradiol (E2) than cows during the normal oestrous cycle (Batra and Pandey 1983) it is possible that they are more sensitive than cattle to the increase of plasmatic levels of 17β-oestradiol during the superovulatory treatments (Beg et al., 1997). Working with superovulated buffalos, Misra et al. (1998) speculated that high E2 level for long time, or a high E2/P4 ratio could increase the turgidity of the genital system and make more difficult the ovum capture by the oviduct fimbria. In fact, the imbalance between the levels of steroid hormones or the increased E2/P4 ratio may impair the interaction between the ovum and the ciliates cells of endosalpinge during the ovulations. Moreover, high concentrations of E2 found in superovulated small ruminants promote a reversal peristalsis of the oviduct, which may cause loss of ovum from the oviduct to the abdominal cavity (Bellve and McDonald 1970). Baruselli et al. (2002) speculated that a reduction of E2 plasmatic levels would decrease the turgidity of the oviduct providing greater mobility of the fimbria. However, exogenous P4 given between the administration of prostaglandin F2 alpha (PGF2α) and LH in superovulated buffaloes reduced E2 blood concentrations, without improving the embryo recovery rate.

Another critical point could be the binding of the newly ovulated COC to the ciliary tips of the epithelial cells lining the distal region of the oviduct. The degree of adhesion controls the pick-up and subsequent transport and compaction of the complex that allows its passage into the oviduct lumen, where it will contact the sperm that are swimming up the oviduct. Interestingly, a lower adhesion of cumulus

cells to the oocyte, as well as a reduced expansion of cumulus after maturation are observed in buffalo (Gasparrini, 2002). A reduced extracellular matrix resulting from inappropriate cumulus expansion may lead to a lower cohesion between the oviduct cilia and COCs and to a higher difficulty for COCs to move into the infundibulum.

A better comprehension of the mechanism and factors involved in the failure of ovum capture and transport is critical to develop corrective strategies in order to improve the efficiency of MOET in buffaloes. Therefore, the aim of our study was to evaluate at different levels the various factors involved in this phenomenon. Due to both the difficulty of heat detection in this species (Ohashi, 1994; Roy et Prakash, 2009) and to the high efficiency of synchronization (Neglia et al., 2003; Rossi et al., 2014; Campanile et al., 2016) we decided to use animals synchronized by Ovsynch as a control group. However, it is known that the response to Ovsynch program varies according to the presence of a dominant follicle and hence to the occurrence of ovulation after the first GnRH injection (around 60% Vasconcelos et al., 1999; Bello et al., 2006; Carvalho et al., 2015). This aspect has still not been truly investigated in buffalo. Therefore, in order to select the most reliable control group for the superovulation study, we carried out a preliminary experiment (Experiment 3) aimed to assess the relationship between the ovarian follicular response at the start of an Ovsynch program and the pregnancy outcome. The results of this experiment allowed us to better plan the experiment 4, by selecting the animals that ovulated after the 1st GnRH as a control group to compare with superovulated animals.

The aim of Experiment 4 was to compare the morphological parameters of the follicles and the steroid profile (progesterone and oestradiol) both in plasma and follicular fluid in the peri-ovulatory period in superovulated vs synchronized buffaloes. Furthermore, to investigate whether the failure is linked to lack of capture,

the oocyte morphology and expansion, as well as the mRNA expression of factors involved in cumulus expansion (*FSHr* and *LHr*) in granulosa cells were evaluated. In addition, the mRNA expression of substances playing a role on contraction-relaxation of the oviduct like *ER1, PGR, VEGF* and its receptors *FLK1 and FLT1* was analysed.

Experiment 3

Relationship between the ovarian follicular response at the start of an Ovsynch-TAI program and pregnancy outcome in the Mediterranean river buffalo

Materials and Method

The Ethical Animal Care and Use Committee of the Federico II University of Naples approved the experimental design and animal treatments.

Location and animals

The study was carried out on a commercial buffalo dairy located in southern Italy between 40.5°N and 41.5°N. Italian Mediterranean river buffaloes were maintained on cement pads that allowed 15 m^2 for each animal and 80 cm manger space. The buffaloes were milked twice daily and were fed a total mixed ration based on corn silage, wheat straw, soybean meal, barley meal, corn meal, wheat bran, beet pulp, hydrogenate vegetable fat, and vitamin and mineral supplement. A total of 116 pluriparous buffaloes (118 ± 65 days in milk) were chosen for the study after two clinical examinations 12 days apart (palpation of the ovaries for a follicle > 1.0 cm

and/or a CL to confirm cyclic ovarian activity; and palpation of the reproductive tract for any gross abnormalities such as uterine fluid).

Oestrus synchronization and artificial insemination

Buffaloes that were chosen for the study after clinical examination were synchronized by the Ovsynch-TAI program developed in cattle (Pursley et al., 1995) and previously applied in the Mediterranean river buffalo (Neglia et al., 2003, 2008). The program consists of an i.m. injection of GnRH agonist (buserelin acetate, 12 μg; Receptal®, Intervet, Milan, Italy) on day 0 (GnRH1), $PGF_{2\alpha}$ analogue (luprostiol, 15 mg; Prosolvin®, Intervet) on day 7, and GnRH agonist on day 9 (GnRH2). Fixed-time artificial insemination (TAI) was performed by the same operator in all buffaloes 20 hours after the second injection of GnRH. Because of the relatively low intensity of oestrous behaviour in buffaloes (Ohashi, 1994) animals were palpated per rectum and underwent ultrasound examination on day 10 to assess oestrous status. All animals with a follicle > 1.0 cm and a tonic uterus, with or without mucous vaginal discharge, were considered to be in oestrus. All buffaloes were given an injection of GnRH agonist on day 20 after TAI which is intended to reduce the incidence of late embryonic mortality (LEM) (Campanile et al., 2005, 2007, 2008; Rossi et al., 2014).

Experimental design

All buffaloes underwent ovarian ultrasonography examination on days 0, 2, 7, 9, 10 and 11 of the Ovsynch-TAI program. Ultrasonography was carried out with a portable Sonoace Pico (Medison, Seoul, South Korea) equipped with a 7.5 MHz linear transducer adapted for trans rectal examination in large domestic animals. The same

experienced operator conducted all ultrasonography examinations. The ovarian features recorded were: (1) number of small, medium and large follicles; (2) diameter of the largest follicle and (3) presence of a CL and dimensions (length of the two axis). The Colour-Doppler mode was used to evaluate blood flow in the CL as previously reported (Russo et al., 2010; Neglia et al., 2014).

The animals were then classified according to their responses in the Ovsynch-TAI program:

- Response to GnRH1 (evaluated on day 2): positive response, ovulation of the follicle recorded on day 0; negative response, no ovulation.
- Response to $PGF_{2\alpha}$ (evaluated on day 10): positive response, regression of CL (CLs) recorded on day 7 (by assessing CL blood flow and ultrasound characteristics); negative response, presence of active and vascularized CL/CLs.
- Response to GnRH2 (evaluated on day 11): positive response, ovulation of the largest follicle recorded on day 10; negative response, no ovulation.

All animals underwent TAI on day 10 irrespective of the response to GnRH1, $PGF_{2\alpha}$ or GnRH2. Pregnancy diagnosis was carried out on day 27 after TAI using ultrasonography and pregnancy was confirmed on day 45 by rectal examination. Buffaloes pregnant on day 27 but not on day 45 were considered to have undergone LEM.

Progesterone assay

Blood concentrations of P4 were measured by RIA (Niswender, 1973; Skaggs et al., 1986) on day 0 and day 10 of the Ovsynch-TAI program and on day 10 after TAI.

Jugular blood samples were centrifuged at 800 x g for 15 min and blood was stored at -20 °C until analysed for P4. The minimum detectable amount of P4 was 2.1 ± 0.08 pg and the intra- and inter-assay coefficients of variation were 6.2% and 11.8%, respectively.

Statistical analysis

Statistical analysis was carried out by ANOVA. The factors considered in the analysis were: the number of follicles in each size category; the dimensions and area of large follicles; and the presence and area of the CL. Chi- square analysis was performed to compare pregnancy rates in animals categorized according to the response to different stages of the Ovsynch-TAI program. Logistic regression analysis models were used to evaluate the factors that affected the odds ratio for both the response to GnRH1 and pregnancy outcome. For the former (response GnRH1), the models incorporated the effects of all the parameters recorded on day 0: number of small, medium and large follicles, follicle area, CL presence and area, and P4 concentration. For pregnancy outcome, the response to GnRH1, and P4 concentrations on day 0 of the Ovsynch-TAI program and on day 10 after TAI, were considered. The number of small, medium and large follicles, follicle area, CL area, and P4 concentration on day 0 and day 10 of the Ovsynch-TAI program and on day 10 after TAI were used as continuous variables, whereas other factors were used as dummy variables.

Results

Response to the first GnRH (GnRH1) administered on day 0

The injection of GnRH on day 0 of the Ovsynch-TAI program induced ovulation in 70/116 (60.3%) buffaloes. Buffaloes that ovulated had a similar number of small, medium and large follicles as buffaloes that did not ovulate (7.99 ± 0.4 and 7.72 ± 0.6; 0.94 ± 0.1 and 0.84 ± 0.1; 1.17 ± 0.1 and 1.02 ± 0.1; respectively). Buffaloes that ovulated had a smaller ($P < 0.05$) area of the largest follicle than buffaloes that did not ovulate (Table 7). There was no difference in the occurrence of a follicle > 0.75 cm^2 between buffaloes that ovulated and those that did not ovulate (Table 7). Buffaloes that ovulated had a larger ($P < 0.05$) CL than buffaloes that did not ovulate (Table 7).

Table 7. Ovarian features recorded on day 0 of the Ovsynch-TAI Program and response to the first GnRH (GnRH1) evaluated on day 7 in river buffaloes undergoing an Ovsynch-TAI program. Results are presented as proportions or mean ± SE.

Response to GnRH1	**Number of buffaloes**	**Follicle diameter > 0.75 mm**	**Follicle area > 0.75 cm^2**	**N° of CL (%)**	**CL (area, cm^2)**
Ovulation	70/116 (60.3%)	60/70 (85.7%)	1.1 ± 0.1^a	54/70 (77.1%)	1.8 ± 0.1^a
No ovulation	46/116 (39.7%)	38/46 (82.6%)	1.3 ± 0.1^b	34/46 (73.9%)	1.5 ± 0.1^b

$^{a, b}$ values within columns with a different superscript differ (P < 0.05)

Response to $PGF_{2\alpha}$ administered on day 7 and status of the CL

At day 7 of the Ovsynch-TAI program, 101/116 (87.1%) buffaloes had a vascularized CL. A greater (P<0.01) proportion of buffaloes that ovulated after GnRH1 on day 0 had a vascularized (functional) CL on day 7 compared with buffaloes that did not ovulate (69/70=98.5% and 32/46 =69.6%, respectively). There was no difference in CL area at day 7 between buffaloes that ovulated and those that did not ovulate after GnRH1 (1.5 ± 0.1 cm^2 and 1.5 ± 0.1 cm^2, respectively). Despite of the number of buffaloes that showed CL on day 0 of the Ovsynch-TAI Program, the response to $PGF_{2\alpha}$ was evaluated on those that showed CL on day 7. At day 10, the number of buffaloes that had not undergone CL regression was less (P < 0.05) for buffaloes that ovulated after GnRH1 than for buffaloes that did not ovulate (1/69 =1.4% and 5/32 = 15.6%, respectively).

Response to the second GnRH (GnRH2) and AI

At day 10 of the Ovsynch-TAI program, 106/116 (91.4%) buffaloes were judged to be in oestrus based on the criteria of a tonic uterus and follicle > 1.0 cm. A higher ($P < 0.05$) proportion of buffaloes that ovulated after GnRH1 was in oestrus compared with buffaloes that did not ovulate (67/70 = 95.7 % and 39/46 = 84.8 %, respectively). The area of the preovulatory follicle was greater ($P < 0.01$) for buffaloes that ovulated after GnRH1 than buffaloes that did not ovulate (1.5 ± 0.1 cm^2 and 1.4 ± 0.1 cm^2, respectively). After GnRH2 on day 10, a higher ($P < 0.05$) ovulation rate was observed for buffaloes that had ovulated after GnRH1 than for buffaloes that did not ovulate (65/70 (92.8%) and 37/46 (80.4%), respectively).

Pregnancy to AI

The pregnancy to AI (P/AI) was greater ($P < 0.01$) at both day 27 and day 45 in buffaloes that ovulated after GnRH1 compared with buffaloes that did not ovulate (Table 8). There was also a tendency for lower LEM in buffaloes that ovulated after GnRH1 (Table 8) Regardless of the response to GNRH1, the area of the preovulatory follicle was greater ($P < 0.01$) in pregnant buffaloes at day 27 compared to non-pregnant. All buffaloes that failed to respond to $PGF_{2\alpha}$ on day 7 and/or GnRH on day 10 were not pregnant at day 27.

Table 8. Pregnancy to AI (P/AI) on day 27 and day 45 after TAI and occurrence of late embryonic mortality (LEM) in buffaloes that ovulated or did not ovulate after the first GnRH (GnRH1) administered on day 0 in an Ovsynch-TAI program.

Pregnancy to AI (P/AI)			
Response to GnRH1	**Pregnant day 27**	**Pregnant day 45**	**LEM**
Ovulation (70/116)	53/70 (75.7%) [a]	46/70 (65.7%) [a]	7/53 (13.2%)
No ovulation (46/116)	14/46 (30.4%) [b]	10/46 (21.7%) [b]	4/14 (28.6%)
Total (116/116)	67/116 (57.8%)	56/116 (48.3%)	11/67 (16.4%)

[a,b] values within columns with a different superscript differ ($P < 0.01$)

The logistic regression analysis showed that pregnancy outcome at day 27 was influenced by the number of small follicles at the time of GnRH1 (odds ratio = 1.248, $P < 0.01$), P4 concentrations on day 10 after TAI (odds ratio = 2.201, $P < 0.01$), and the response to GnRH1 (odds ratio = 7.564, $P < 0.001$). Pregnancy outcome at day 45 was also influenced by the number of small follicles at the time of GnRH1 (odds ratio = 1.305, $P < 0.01$), P4 concentrations on day 10 after TAI (odds ratio = 2.046, $P < 0.05$), and the response to GnRH1 (odds ratio = 9.037, $P < 0.001$). The response to GnRH1 was only influenced by P4 concentrations at day 0 (odds ratio = 1.543, $P < 0.05$).

Progesterone assay

Buffaloes that ovulated after GnRH1 had higher ($P < 0.05$) blood concentrations of P4 at the start of the Ovsynch-TAI program (day 0) than buffaloes which did not ovulate (Table 9). For all buffaloes, those pregnant at day 45 had higher ($P < 0.05$) blood concentration of P4 both at day 0 and at day 10 after TAI (Table 10).

Table 9. Blood progesterone concentrations in river buffaloes undergoing an Ovsynch-TAI program. Progesterone was determined at day 0 (first GnRH, GnRH1) and day 10 (TAI) of the program and at day 10 after TAI.

Progesterone (ng/mL)				
Response to GnRH1	**n**	**day 0 (GnRH1)**	**day 10 (TAI)**	**day 10 after TAI**
Ovulation	70/116 (60.3%)	1.8 ± 0.2^{a}	0.2 ± 0.0	2.1 ± 0.1
No ovulation	46/116 (39.7%)	1.3 ± 0.2^{b}	0.2 ± 0.0	1.8 ± 0.2
Total	116	1.6 ± 0.1	0.2 ± 0.0	2.0 ± 0.1

^{a,b} values within columns with a different superscript differ ($P < 0.05$)

Table 10. Blood progesterone concentrations at different stages of an Ovsynch-TAI program for river buffaloes that were pregnant and not pregnant at day 45 after TAI. Progesterone was determined at day 0 (first GnRH, GnRH1) and day 10 (TAI) of the program and on day 10 after TAI.

Progesterone (ng/mL)				
Pregnancy status at day 45	**n**	**day 0 (GnRH1)**	**day 10 (TAI)**	**day 10 after TAI**
Pregnant	56	1.9 ± 0.2^{a}	0.2 ± 0.1	2.2 ± 0.1^{a}
Not pregnant	60	1.4 ± 0.2^{b}	0.2 ± 0.1	1.8 ± 0.1^{b}

^{a,b} values within columns with a different superscript differ ($P < 0.05$)

Discussion

In the present study, only 10.3% of buffaloes did not ovulate after the second GnRH (GnRH2) and 90% were judged to be in oestrus at the time of TAI. These responses in the Ovsynch-TAI program were not, however, predictive of pregnancy to AI (P/AI). The most notable predictor of the likelihood of P/AI was whether buffaloes ovulated after the first GnRH (GnRH1) at the start of the program. The largest follicle in buffaloes that ovulated after GnRH1 was smaller than the largest follicle in buffaloes that did not ovulate. This finding was interpreted to suggest that buffaloes that

ovulated were at early stages of the oestrous cycle (day 5 to 9). Ovulation after GnRH1 was followed by the development of a CL that was responsive to $PGF_{2\alpha}$ on day 7, the selection and growth of a dominant follicle that ovulated after GnRH2 on day 10, and the release of a newly matured oocyte that was competent to undergo fertilization and embryonic development. The present findings in lactating river buffaloes are consistent with the relationship between the follicular response to GnRH1 and pregnancy outcome observed in lactating dairy cows (Vasconcelos et al., 1999; Bello et al., 2006; Carvalho et al., 2015) and dairy heifers (Moreira et al., 2000) subjected to the Ovsynch-TAI program. It can be concluded from the present findings in Mediterranean river buffaloes and from the studies in dairy cattle that pregnancy outcome in the Ovsynch-TAI program is optimized when treatment starts early in the oestrous cycle (day 5 to 9) and animals undergo ovulation in response to GnRH1.

The ovulation rate after GnRH1 observed in the present study was similar to that reported previously for the river buffalo (Baruselli et al., 2003, 2007; Campanile et al., 2008). The present study has extended these earlier reports by demonstrating the fundamental importance of ovulation to GnRH1 in the P/AI outcome in river buffaloes subjected to an Ovsynch-TAI program. As noted above, ovulation to GnRH1 would have been followed by the formation of a CL and the emergence of a new follicular wave (Baruselli et al., 2007) that optimized the subsequent responses to $PGF_{2\alpha}$ on day 7 and GnRH2 on day 10. Indeed, buffaloes that ovulated after GnRH1 had a higher CL regression rate after $PGF_{2\alpha}$ on day 7 and higher ovulation rate after GnRH2 on day 10 (Bello et al., 2006). In cattle, ovulation to GnRH1 reduces significantly the likelihood of a persistent dominant follicle that is associated with lower fertility (Tatcher et al., 1996; Austin et al., 1999; Wiltbank et al., 2011).

Persistent dominant follicles can enter atresia (Bello et al., 2006) and lose the ability to respond to GnRH2 in an Ovsynch-TAI program (Wiltbank et al., 2014).

In river buffaloes, follicular deviation occurs at about 7.2 mm and ovulatory capacity is acquired at around 8.0 to 8.5 mm (Campanile et al., 2008; Gimenes et al., 2011). Buffaloes that ovulated after GnRH1 in the present study tended to have a smaller follicle size than buffaloes that did not ovulate. This finding was interpreted to suggest that buffaloes which did not ovulate to GnRH1 were outside the day 5 to 9 window of the oestrous cycle and at later stages of follicular waves. This would be consistent with observations in dairy cows treated with the Ovsynch-TAI program (Pursley et al., 1995; Vasconcelos et al., 1999). The variability in stage of the oestrous cycle at the time of GnRH1 was the basis for the development of the Double-Ovsynch pre-synchronization treatment in dairy cows (Aires et al., 2013; Giordano et al., 2013).

Buffaloes pregnant at day 45 had higher blood P4 concentrations at the start of the Ovsynch-TAI program (day 0) and also at day 10 after TAI. Unexpectedly, the P4 levels on Day 0 of the Ovsynch-TAI Program did not influence the response to GnRH1. Higher concentrations of P4 at the start of the program would ensure an adequate environment for optimal follicular development and fertility (Bisinotto et al., 2010; Wiltbank et al., 2012; Pursley and Martins, 2012). Likewise, it has been shown in river buffaloes that P4 concentrations after TAI have an important bearing on early and late embryonic mortality (Campanile et al., 2005; Neglia et al., 2012; Balestrieri et al., 2013; Strazzullo et al., 2014). The positive relationship between P4 concentrations on day 0 and P/AI outcome in the present study was not consistent with observations in dairy cows subjected to the Ovsynch-TAI program. In lactating dairy cows, reduced P4 concentrations at day 0 are associated with a higher ovulation

rate to GnRH1 and increased P/AI (Carvalho et al., 2015; Giordano et al., 2012a, 2012b). It was reported that blood P4 concentrations > 3.0 ng/mL at day 0 suppressed the LH response to GnRH1 and lowered the ovulation rate in dairy cows (Giordano et al., 2012a). To investigate this further, cows were treated with a low dose of $PGF_{2\alpha}$ two days before the start of an Ovsynch-TAI program (Carvalho et al., 2015). Cows pre-treated with $PGF_{2\alpha}$ had lower P4 concentrations at day 0 and showed a higher ovulation rate to GnRH1 and higher P/AI (Carvalho et al., 2015). Although all buffaloes received GnRH on day 20 after TAI to increase P4 secretion (Campanile et al., 2010, 2016), a tendency to higher LEM was observed in buffaloes that did not ovulate after GnRH1. In lactating dairy cows, the beginning of Ovsynch-TAI program on days 5 to 9 of the oestrous cycle was associated with the highest P/AI and the lowest pregnancy loss (Vasconcelos et al., 1999). The common finding in river buffaloes and dairy cows of a direct relationship between ovulation after GnRH1 and pregnancy outcome strongly indicates that the new dominant follicle that ovulates after GnRH2 releases an oocyte that has optimal capacity to undergo fertilization and establish pregnancy.

In summary, the present study has provided conclusive evidence of the importance of ovulation after GnRH1 to the pregnancy outcome in Mediterranean river buffaloes undergone Ovsynch-TAI program. Ovulation after GnRH1 administered on day 0 optimizes the response of the CL to $PGF_{2\alpha}$ on day 7 and the response of the preovulatory follicle to GnRH2 on day 9, increasing the likelihood of pregnancy to TAI.

Experiment 4

Analysis of factors involved in the failure of ovum capture in superovulated buffaloes

Materials and Methods

The Ethical Animal Care and Use Committee of the Federico II University of Naples approved the experimental design and animal treatments.

Experimental design

The study was carried out in Southern Italy (latitude 40.5° - 41.5° N and longitude 13.5 - 15.5) during autumn, i.e. the breeding season, on 35 multiparous Italian Mediterranean Buffalo cows with a mean weight and age of 556.7 ± 13.8 kg and 5.6 ± 0.3 years, over four replicates. Animals were randomLy divided into two groups: a group (n = 25) was synchronized by Ovsynch (as described in details in Experiment 3) and another group (n=10) was superovulated as described below. Based on the results of Experiment 3, only synchronized animals that ovulated after the first GnRH in the Ovsynch protocol (n=14) were chosen as a control group and slaughtered 18 hours after the last GnRH, just before ovulation. According to Experiment 1, buffaloes with an antral follicle count ≥ 12 follicles, i.e. good donors (n =5), and < 12 follicles, i.e. bad donors (n = 5), were superovulated. In these subjects ovulation was also induced by administering GnRH at the end of the superovulatory treatment and animals were sacrificed in the peri-ovulatory period, i.e. 18 h after GnRH

administration. At slaughter, blood samples were taken for hormone analyses in both groups. All blood samples were separated using centrifugation at 4°C, 800 g for 10 min, and stored at −20°C until the analysis. Moreover, ovaries and oviducts of animals of both groups were recovered individually and transported to the laboratory in physiological saline supplemented with 150 mg/L kanamycin at 30-35°C.

Multiple ovulation schedule

According to the results of the experiment 2, the animals were synchronized by a double injection of 0.524 mg of PGF2a analogue (Cloprostenol, Estrumate®, Schering-Plough Animal Health, Milan, Italy) 12 days apart. Ovulation was induced by administering GnRH analogue (buserelin acetate,12 mg; Receptal; Intervet, Milan, Italy) 2 days after the last PGF2a and the following day, at the presumptive time of ovulation, a progesterone releasing intravaginal device (PRID®, Ceva Vetem SpA, Milan, Italy) was inserted (day 0). Then, superovulation treatment, consisting in 400 mg FSH (Folltropin®, Bio98, Milan, Italy) given in decreasing dosages for 4 consecutive days started on day 8. Briefly, the scheme was: 80 mg on day 8, 60 mg on day 9, 40 mg on day 10 and 20 mg on day 11, given twice daily. On day 10 (72 and 84 h after initiating gonadotrophin treatment) buffaloes were administered 0.524 mg PGF2a twice (12 h apart) for inducing luteolysis and PRID was removed. On day 12 animals received GnRH analogue to induce ovulation and on day 13, precisely at 18 h after GnRH, were sacrificed at a local abattoir.

Collection of follicular fluid and granulosa cells

At the laboratory, the presumptive dominant follicles (diameter > 0.7 cm) in both synchronized and superovulated animals were measured: follicle size was assessed by measurement of the two perpendicular diameters with a millimetre scale. The FF of the dominant follicle was aspirated by a syringe with a 21-gauge needle, poured into a petri dish for a quick search of the oocyte, recovered in a vial and centrifuged at 600 X g for 10 min to separate the follicular fluid and the GCs. After centrifugation, the FF recovered was directly snap frozen and stored at - 80°C until hormonal assay determination, while the GCs-containing pellet was previously washed with PBS+PVA (0.1%), centrifuged again at 600 X g for 10 min, then snap frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Evaluation of oocyte quality and maturation status

The FF recovered from the dominant follicles of synchronized and superovulated buffaloes was inspected under a microscope for the presence or absence of the COC. For each run, the recovery rate, i.e. the percentage of COCs in relation to the aspirated follicles, and the percentage of oocytes showing an appropriate cumulus expansion were recorded. The cumulus-oocyte complexes recovered were scored for quality on the basis of morphological features according to our classification (Di Francesco et al., 2011). Oocytes were stripped of the cumulus cells by gentle pipetting, allocated on a clean slide and fixed in absolute ethanol overnight. Then oocytes were stained with DAPI to evaluate the nuclear status using a Nikon Diaphot 300 inverted microscope equipped with fluorescence filters.

Collection of oviduct samples

At the laboratory, both oviducts of each synchronized and superovulated animal were trimmed free of tissue. According to anatomy, the oviduct was divided into four parts: fimbriae, infundibulum, ampulla and isthmus. Tissues from fimbriae were directly frozen into liquid nitrogen and stored at −80°C until RNA isolation. The infundibulum, ampulla and isthmus of both oviducts were placed into a Petri dish, washed three times with physiological saline and then opened longitudinally. Once opened they were gently scraped with a blade and flushed with 500 μL PBS+PVA (0.1%), to recover epithelial cells. Cells were centrifuged twice at 600 X g for 10 min, PBS was removed and the pellet was snap frozen and stored at −80°C until RNA isolation.

Hormonal assay

Oestradiol and progesterone were determined by a solid-phase microtiter RIA. In brief, a 96-well microtitre plate (OptiPlate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with goat anti-rabbit γ-globulin serum (diluted 1:1000 in 0.15 mM sodium acetate buffer at pH 9) and incubated overnight at 4°C. The plate was then washed twice with RIA buffer (pH 7.4) and incubated overnight at 4°C with 200 μL of the antibody serum diluted 1:8.000 for P4 and 1:80.000 for 17-β-oestradiol. After washing the plate with RIA buffer, standards (5–200 pg/well), the quality control extract, the test extracts and the tracer (progesterone [1,2,6,7-3H (N)] or 17-β-oestradiol [2,4,6,7,16,17-3H (N)]; PerkinElmer Life Sciences Inc., Boston, MA, USA) were added, and the plate was incubated overnight at 4°C. Bound hormone was separated from free hormone by decanting and washing the wells in RIA buffer. After

the addition of 200 μL/well scintillation cocktail (Microscint 20, Perkin- Elmer Life Sciences), the plate was counted using a beta-counter (Top-Count, Perkin-Elmer Life Sciences).

The cross-reactivity of the anti-progesterone antibody with other steroids was as follows: 11 β-OH-progesterone, 46%; 17 α-OH-progesterone, 0.4%; 20 α-OH-progesterone, 0.04%; testosterone, 0.08%; cortisol, <0.01%; estradiol-17-β, <0.01%; estradiol-17-α, <0.01%; and oestrone, <0.01%. The cross-reactivity of the anti-17-β-oestradiol antibody with other steroids was as follows: 17-α-oestradiol, 0.62%; oestrone, 1.5%; estrone-3-sulphate, 0.3%, estriol, 0.8%; estriol-3-sulphate. 0.03%.

P4 Intra- and inter-assay coefficients of variation were 7.3% and 12%, respectively. The minimum detectable amount of P4 was 0,8 pg. Intra and inter-assay coefficients of variation for 17-β-oestradiol were 7.0 % and 11.8%, respectively. The minimum detectable amount of P4 was 0.91 pg.

RNA Isolation and detection

Total RNA was purified for each sample (for fimbriae 5 mg of tissue were used) by using RNeasy Mini Kit (Qiagen, 74104, Germany). The protocol was performed according to the manufacturer's instructions. The final elution step was repeated and the purified total RNA was subsequently stored at -80°C. Quantity of the total RNA samples was evaluated by Thermo Scientific™ NanoDrop™ 2000 spectrophotometers. The level of degradation was analysed using agarose electrophoresis. Samples with Clear 28S and 18S ribosomal RNA bands and band intensities of 28S:18S close to two were chosen in this study.

cDNA synthesis

One µg of detected RNA was used to synthesize first-strand cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen, 205311, Germany) according to the manufacturer's protocol. To eliminate genomic DNA contamination in RNA samples, the gDNA elimination reaction was performed using 2 µL of gDNA wipe-out buffer, 1 µg template RNA and RNase-free water to adjust the final volume to 14 µL at 42°C for 2 min. Reverse transcription was carried out in a total of 20 µL reaction containing 1 µL Quantiscript Reverse Transcriptase, 4 µL Quantiscript RT buffer with Mg2+ and dNTPs, 1 µL RT Primer Mix and 14 µL Template RNA (Entire genomic DNA elimination reaction) at 42°C for 15 min and 95°C for 3 min. All steps were performed on ice. The cDNA was stored at -20°C until real-time PCR analysis.

Real-Time PCR analysis

Real-time quantitative PCR reactions were run using QuantiFast® SYBR® Green PCR Kit (Qiagen, 204054, Germany) on BIORAD CFX Manager Machine. The *ER1*, *PGR*, *VEGF*, *FLK1* and *LHR*, *FSHR*, as well as *ACTB* primers (shown in Table 11) were designed by Primer Premier 5 software and synthesized by Eurofins Genomics. Samples were prepared according to the manufacturer's instructions. A total reaction volume of 20 µL contained 10 µL Master Mix, 2 µL primers and 8 µL diluted cDNA (1:30). The samples were then centrifuged at 1000 X g at 4°C for 1 min.

All cDNA samples were amplified under the following conditions: 95°C for 5 min, 40 cycles at 95°C for 10 s and 60°C for 30 s for *LHR* and *FSHR*, 95°C for 5 min, 40 cycles at 95°C for 10 s and 64°C for 30 s for *PGR, FLK1* and *FLT1* and 95°C for 5 min, 35 cycles at 95°C for 10 s and 64°C for 30 s for *VEGF* and *ER1*. The data were

subsequently quantified according to the comparative cycle threshold (CT) method and expression levels were presented as 2-ΔCT normalized to the *ACTB* housekeeping gene.

Table 11. Primers used in this study for Real time PCR.

Gene		Sequence (5'to3')	Accession No.	Product size (bp)
VEGF	F	aaaacacagactcgcgttgc	NM_001316956.1	248
	R	gcctcctcttccttcatgtca		
FLK1	F	gcggccaatgtgtctgcttt	XM_006058316.1	140
	R	acattctcctgctcggtggg		
FLT1	F	cacctgccttctccgaggac	XM_006078571.1	250
	R	agtgaggccttgggtttgct		
PGR	F	gatgctatattttgcgcctga	XM_006057027.1	266
	R	ctccttttgcctcaaacca		
ER1	F	aatctgccaaggagactcgc	XM_006080203.1	240
	R	tccgtatcccgcctttcatc		
FSHR	F	aaacgtgttctccaacctgc	NM_001290962.1	157
	R	cttgtgaacagctggcaagt		
LHR	F	gctctacctgctgctcattg	XM_006078413.1	216
	R	ttcagtcgcagcttttggtc		
ACTB	F	atgatgatattgccgcgctc	NM_001290932.1	216
	R	cgtgctcaatggggtacttg		

Statistical analysis

Differences in morphological follicular parameters, as well as in the concentration of progesterone and oestradiol both in plasma and in follicular fluid were analysed by ANOVA, using Least Significant Difference (LSD) as a post-hoc test. Differences in the recovery rate and in the percentage of oocytes showing cumulus expansion were analysed by Chi Square Test.

The follicular parameters and steroid profile in FF of follicles containing a properly expanded oocyte (good follicles) vs those with an unexpanded oocyte (bad follicles) in synchronized and superovulated animals were compared. In particular, as most of the follicles of synchronized animals contained an expanded oocyte, the comparison was carried out among the good follicles of synchronized animals and good and bad follicles of the superovulated counterparts, by ANOVA, using Least Significant Difference as a post-hoc test.

Pearson correlation analysis was carried out to evaluate the relationship between steroid profile (both in FF and plasma) and the characteristics of the follicles. Furthermore, to evaluate the correlation between E2 and P4 concentration and gene expression, samples from 10 animals were used. Differences in gene expression levels between superovulated and synchronized animals were analysed by Student's t test.

Results

Response to synchronization

Buffaloes that ovulated after the first GnRH were 14/25 (56%) and only these animals were chosen for the comparison with the superovulated counterparts. Interestingly, a high incidence of animals showed the preovulatory follicle on the right ovary (71.4%).

Phenotypical evaluation of superovulated buffaloes

The first important information that arose from this study is the high variation in the superovulatory response of the animals. In table 11 the average, as well as minimum and maximum values for the number and size of preovulatory follicles and steroid concentrations in plasma and follicular fluid (FF) in superovulated animals are reported. The proportion of preovulatory follicles was similar in left and right ovary (47.9% vs 52.1% respectively). However, in 9 out of 10 superovulated animals a higher percentage was recorded on the right ovary (55.5 vs 44.5; $P<0.05$). A high individual variability was also observed in the number of preovulatory follicles, that ranged from 3 to 29, and in the recovery rate (from 0 to 76.9%), as shown in Table 11. Moreover, the percentage of COCs with cumulus expansion was very low, but ranged from 0 to 100% (Table 12).

Table 12. Average, minimum and maximum values for the number and size of preovulatory follicles and steroid concentrations in plasma and follicular fluid (FF) in superovulated animals.

	Mean ± SE	**Min**	**Max**
n. of preovulatory follicles	14.4 ± 2.7	3	29
Min. follicle Diameter (cm)	0.75 ± 0.05	0.6	1.0
Max. follicle Diameter (cm)	1.10 ± 0.06	0.9	1.4
Mean follicle Diameter (cm)	0.9 ± 0.05	0.8	1.2
FF volume (mL)	0.6 ± 0.1	0.3	1.3
E2 concentration (ng/mL) in FF	30.3 ± 5.9	9.1	47.7
P4 concentration (ng/mL) in FF	71.6 ± 8.5	29.2	104.5
E2:P4 ratio in FF	0.6 ± 0.1	0.3	1.1
E2 in plasma (ng/mL)	15.4 ± 0.9	11.6	17.4
P4 in plasma (ng/mL)	0.4 ± 0.04	0.3	0.5
E2:P4 ratio in plasma	0.04 ± 0.01	0.03	0.06
Recovery rate (%)	55.0 ± 5.6	33.3	76.9
Oocytes with cumulus expansion (%)	44.2 ± 14.8	0.0	100.0

Based on the results of experiment 1, we choose good (≥ 12 follicles before the treatment; n=5) and bad donors (< 12 follicles before the treatment; n=5) according to the AFC, synchronizing the animals before starting the superovulation protocol. It is interesting to provide a description of the parameters recorded in superovulated animals divided in relation to the number of preovulatory follicles (Table 13).

Interestingly, the animals with a low response in terms of the number of preovulatory follicles had a higher (P<0.05) proportion of oocytes exhibiting a proper expansion of the cumulus cells, whereas no other differences were recorded.

Table 13. Follicular parameters and intrafollicular steroid profile in superovulated animals in relation to number of preovulatory follicles, classified as good (>12 follicles) and bad (<12 follicles) donors.

Follicular parameters	**good (n=5)**	**bad (n=5)**
	Mean ± SE	**Mean ± SE**
N. preovulatory follicles	20.8 ± 3.3^{A}	4.5 ± 1.5^{B}
Minimum follicle Diameter (cm)	0.8 ± 0.1	0.7 ± 0.0
Maximum follicle Diameter (cm)	1.1 ± 0.1	1.1 ±0.03
Mean follicle Diameter (cm)	0.9 ± 0.1	0.9 ±0.0
Follicular fluid (FF) volume (mL)	0.5 ± 0.1	0.9 ±0.4
Oestradiol (E2) concentration (ng/mL) in FF	28.1 ± 8.0	35.9 ± 7.3
Progesteron (P4) concentration (ng/mL) in FF	63.1 ± 8.7	92.7 ± 11.7
E2:P4 ratio in FF	0.6 ± 0.2	0.6 ± 0.1
Oestradiol (E2) in plasma (pg/mL)	15.6 ± 1.3	15.1 ± 0.3
Progesteron (P4) in plasma (ng/mL)	0.4 ± 0.1	0.3 ± 0.1
E2:P4 ratio in plasma	0.04 ±0.0	0.05 ± 0.0
	N (%)	N (%)
Recovery rate (%)	59/104 (56.7)	5/9 (55.6)
Oocytes with cumulus expansion (%)	$15/59\ (25.4)^{a}$	$4/5\ (80.0)^{b}$

^{A,B} Values with different superscripts are significantly different; P<0.01

^{a,b} Values with different superscripts are significantly different; P<0.05

Differences between synchronized vs superovulated animals

Results regarding the morphological parameters of the follicles, the hormonal concentration of progesterone and oestradiol both in plasma and in follicular fluid, as well as the recovery rate and the proportion of oocyte with cumulus expansion in

synchronized and superovulated buffaloes are summarized in Table 14. No differences were observed between groups in both the diameter and the volume of the follicular fluid. However, in superovulated animals the E2 and P4 concentrations in FF were lower ($P<0.05$) than in synchronized animals, but the oestradiol: progesterone ratio in FF was not affected. Despite the decreased concentration of steroids in the FF, the plasmatic levels of both oestradiol and progesterone, as well as the oestradiol: progesterone ratio, were similar in the two groups. Interestingly, both the recovery rate ($P<0.05$) and the percentage of oocytes exhibiting proper cumulus expansion ($P<0.01$) decreased in the superovulated animals. The majority of oocytes showing cumulus expansion reached the metaphase II stage in both groups (100% and 94.4%, respectively in synchronized and superovulated buffaloes).

The follicular parameters and steroid profile in FF of follicles containing a properly expanded oocyte (good follicles) vs those with an unexpanded oocyte (bad follicles) in synchronized and superovulated animals are shown in Table 15. Unexpectedly, no differences were recorded between good and bad follicles within superovulated animals. However, a tendency to lower P4 values in bad follicles vs good follicles of SO group was observed but, due to the high SE, the difference was not significant. A decreased concentration of E2 and P4 was observed in both good and bad follicles of the SO group compared to the good follicles of the synchronized group, confirming the previous analysis. However, the difference found in FF P4 level between synchronized and superovulated animals was higher ($P<0.01$) when compared to the bad follicles rather than to good follicles ($P<0.05$) of SO animals. In addition, although the diameter of the follicles was similar in the three groups, the FF volume was higher ($P<0.05$) in the Synchronized group rather than in both SO groups.

Table 14. Morphological parameters of the follicles, hormonal concentration of progesterone and oestradiol both in plasma than in follicular fluid and recovery rate in Synchronized vs Superovulated animals. Data are presented as mean ± SE.

Follicular parameters	**Synchronized (control) n = 14**	**Superovulated* n = 10**
	Mean ± SE	**Mean ± SE**
Minimum follicle Diameter (cm)	0.8 ± 0.04	0.8 ± 0.05
Maximum follicle Diameter (cm)	1.2 ± 0.1	1.1 ± 0.1
Mean follicle Diameter (cm)	1.0 ± 0.1	0.9 ± 0.05
Follicular fluid (FF) volume (mL)	0.9 ± 0.1	0.6 ± 0.1
Oestradiol (E2) concentration (ng/mL) in FF	63.2 ± 10.6[a]	30.3 ± 5.9[b]
Progesteron (P4) concentration (ng/mL) in FF	130.1 ± 19.8[a]	71.6 ± 8.5[b]
E2:P4 ratio in FF	0.65 ± 0.2	0.63 ± 0.1
Oestradiol (E2) in plasma (pg/mL)	15.8 ± 1.0	15.4 ± 0.9
Progesteron (P4) in plasma (ng/mL)	0.4 ± 0.1	0.4 ± 0.04
E2:P4 ratio in plasma	0.05 ± 0.0	0.04 ± 0.0
	N (%)	N (%)
Recovery rate (%)	12/14 (85.7)[a]	64/113 (56.6)[b]
Oocytes with cumulus expansion (%)	9/12 (75.0)[A]	18/64 (28.1)[B]

*Mean values were used for superovulated animals

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

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

Table 15. Follicular parameters and steroid concentration in follicular fluid (FF) of follicles containing a properly expanded oocyte (good follicles) vs those with an unexpanded oocyte (bad follicles) in synchronized and superovulated animals.

Follicular parameter	Synchronized (control)	Superovulated*	
	Good follicles (n=9)	Good follicles (n=18)	bad follicles (n=46)
	Mean ± SE	Mean ± SE	Mean ± SE
Minimum follicle Diameter (cm)	0.9 ± 0.04	0.8 ± 0.1	0.7 ± 0.03
Maximum follicle Diameter (cm)	1.3 ± 0.1	1.1 ± 0.1	1.0 ± 0.04
Mean follicle Diameter (cm)	1.1 ± 0.05	0.9 ± 0.1	0.9 ± 0.03
Follicular fluid (FF) volume (mL)	0.9 ± 0.1^{a}	0.5 ± 0.05^{b}	0.4 ± 0.1^{b}
Oestradiol (E2) concentration (ng/mL) in FF	63.6 ± 12.9^{A}	30.3 ± 3.9^{B}	27.2 ± 3.0^{B}
Progesteron (P4) concentration (ng/mL) in FF	139.2 ± 25.7^{Aa}	78.1 ± 9.6^{bc}	62.4 ± 7.3^{Bc}
E2:P4 ratio in FF	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1

*Mean values were used for superovulated animals

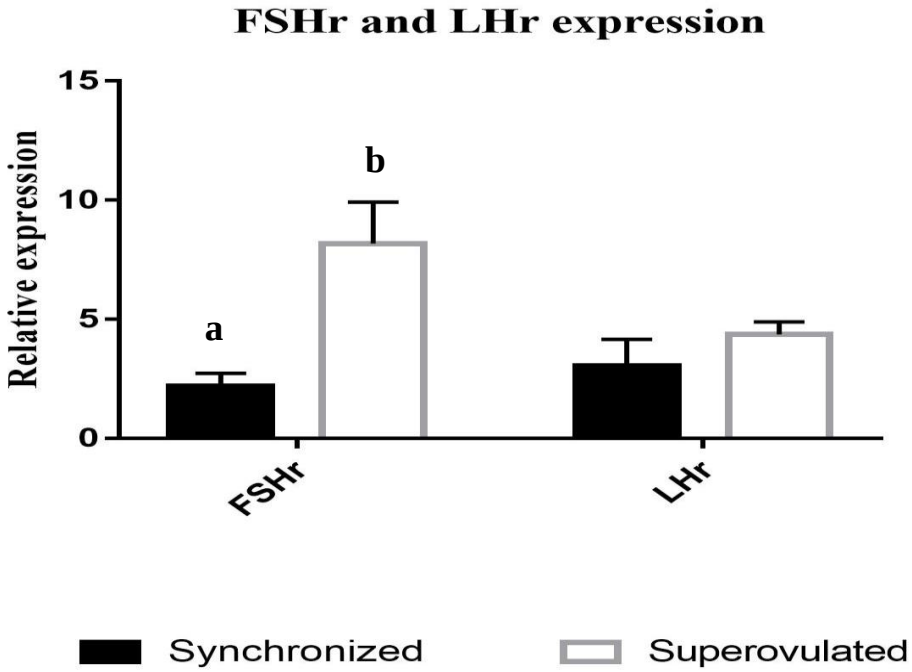
$^{A, B}$ Values with different superscripts are significantly different; P<0.01

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

Gene expression

Regarding the granulosa cells, as shown in Figure 11, mRNA expression of *FSHR* was higher ($P < 0.05$) in superovulated group than in synchronized group, whereas no differences were found in *LHr* expression between groups.

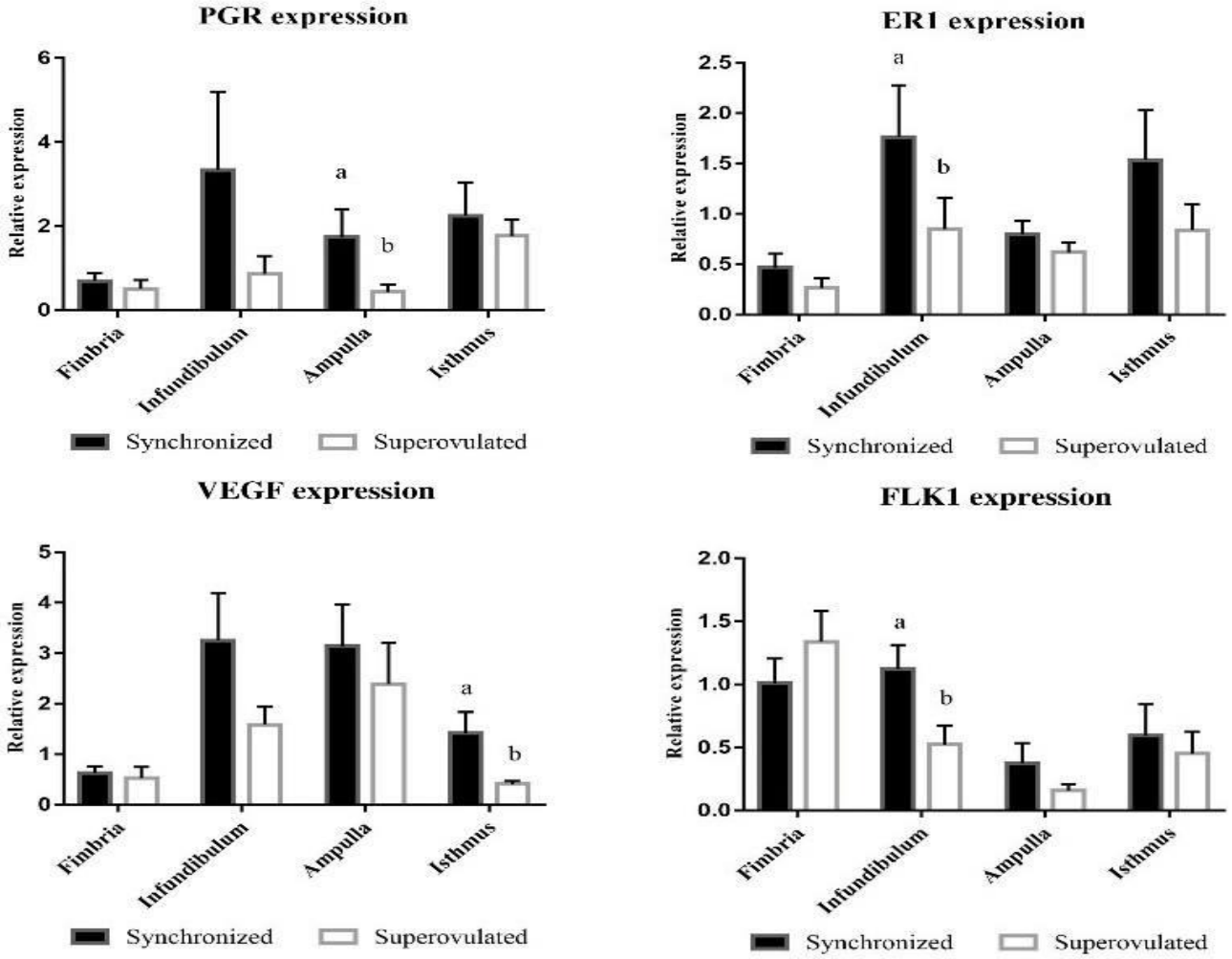
Figure 11. Relative mRNA expression of *FSHr* and *LHr* in Synchronized animals vs Superovulated animals



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

With regard to the oviduct, decreased ($P < 0.05$) mRNA expression of *PGR and ER1* was observed in superovulated compared to synchronized animals, at the level of ampulla and infundibulum respectively, as shown in Figure 12. Moreover, the mRNA expression of the *VEGF* and the VEGF receptor *FLK1*, was also lower ($P<0.05$) in superovulated animals, respectively in the isthmus and ampulla (Figure 12). No mRNA expression of *FLT1* was found in any tracts of the oviduct in both groups.

Figure 12. Relative mRNA expression of *ER1*, *FLK1*, *PGR* and *VEGF* in the oviduct of Synchronized animals vs Superovulated animals.



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

Correlation analysis

Regardless of the treatment, the intrafollicular E2 concentration was positively correlated with the follicular volume ($r = 0.563$; $P<0.01$) and with the intrafollicular P4 concentration ($r = 0.551$; $P<0.01$). As expected, the blood E2/P4 ratio was positively correlated with blood E2 concentration ($r = 0.595$; $P<0.01$) and negatively correlated with blood P4 concentration ($r=0.537$; $P<0.01$). In contrast, the intrafollicular E2/P4 ratio was only negatively correlated with the intrafollicular P4 concentration ($r=-0.525$; $P<0.01$). Within superovulated animals, the number of preovulatory follicles was not correlated to steroids concentration both in the FF and plasma. Interestingly, the percentage of oocytes showing cumulus expansion and the recovery rate were respectively positively and negatively correlated with the intrafollicular P4 concentration ($r = 0.829$ and $r = - 0.760$; $P<0.05$).

Regardless of the treatment, the intrafollicular E2 concentration was positively correlated with *PGR* expression in the fimbria ($r=0.734$; $P<0.05$), whereas the intrafollicular P4 concentration was positively correlated with *PGR* expression in fimbria, infundibulum and ampulla ($r = 0.818$ and $r = 0.790$, $P <0.01$; $r = 0.642$, $P <0.05$, respectively). The E2/P4 blood ratio was also positively correlated with *PGR* expression in fimbria, infundibulum and ampulla ($r=0.740$, $P<0.05$; $r=0.808$ and $r=935$, $P<0.01$, respectively). Regarding the gene expression within the oviduct a positive correlation between *PGR* and *ER1* was found in infundibulum, ampulla and isthmus ($r=0.851$, $P<0.01$; $r = 730$, $P<0.05$; $r = 0.675$; $P<0.05$, respectively). Finally, no correlation was found within groups, probably due to the lower number of the samples.

Discussion

The aim of this experiment was to investigate the different factors affecting the embryo recovery in superovulated buffaloes, and particularly to verify the hypothesis that in animals undergoing multiple ovulation programs a failure of ovum capture occurs. This hypothesis arises from the observations that the SO treatment promotes the growth of multiple follicles and their ovulation, as indicated by high number of CL at the moment of flushing, while the embryo recovery remains very poor (Zicarelli, 1997b; Baruselli et al., 1999; Carvalho et al., 2002; Misra and Tyagi, 2007; Neglia et al., 2010). In addition, the flushing of oviducts and uteri of superovulated buffaloes sacrificed at different days after AI also resulted in a low number of embryos recovered (Carvalho et al., 2002), suggesting that the ovum transport to the uterus is not impaired, and hence leaving the failure in ovum capture the most likely hypothesis. Therefore, the question arises about which factors ensure a proper ovum pick-up by the oviduct. It is known that COCs, consisting of oocytes surrounded by the zona pellucida and their cumulus investment, are ovulated into the peritoneal or bursal cavity, where they are picked up by the fimbria. It follows that the appropriate epithelial cilia movement and contraction-relaxation of the oviduct, that are under the control of ovarian steroids (Wijayagunawardane et al., 1996), are fundamental on one side. On the other side, the cumulus expansion occurring during the oocyte maturation process and the consequent proper development of the extracellular matrix play a pivotal role. The study was designed to compare the morphological features of preovulatory follicles and oocytes, the steroid profile both in the FF and plasma, as well as the expression of genes involved in the cumulus expansion in granulosa cells and that of genes involved in contraction-relaxation of the oviduct between superovulated and synchronized buffaloes. It is worth reminding that, based

on the results of Experiment 3, the synchronized animals that ovulated after the 1st GnRH administration were chosen as control. Within the superovulation group, a high individual variability was recorded in the number of preovulatory follicles (from 3 to 29), as well as in the recovery rate (33 to 77%) and the percentage of oocytes showing cumulus expansion (0 to 100%). Interestingly, the animals with a low response in terms of the number of preovulatory follicles (on average 4.5) had a higher proportion of oocytes exhibiting a proper expansion of the cumulus cells compared to animals with high (on average 20.8) number of preovulatory follicles.

The most interesting findings derive from the comparison between superovulated and synchronized animals. Unexpectedly, the size of the preovulatory follicle, in terms of minimum, maximum and mean diameter, as well as the volume of FF, were not different between the two groups. This is in contrast with a previous study where non stimulated bovine heifers had larger preovulatory follicles than superovulated ones (Assey et al., 1994); this could be probably due to the different species. More importantly, the intrafollicular steroid profile was altered in superovulated buffaloes, that showed a significant reduction of both oestradiol and progesterone. However, as both steroids decreased, the E2:P4 ratio in FF was not affected. The steroids concentration, and particularly P4 in the FF was higher than in plasma, as previously reported in other species (Aller et al., 2013). These results are in agreement with earlier studies in cattle, where the concentration of both oestradiol and progesterone in superovulated follicles was also lower than that recorded in the preovulatory unstimulated follicles (Assey et al., 1994; Aller et al., 2013; Fortune and Hansel, 1985). It is speculated that the preovulatory follicles of stimulated animals may be in early atresia and the low levels of E2 in FF may reflect their lack of aromatase activity (Aller et al., 2013). In contrast, higher levels of both E2 and P4 were reported in superovulated sheep but at a different collection time, i.e. before the LH surge (o' Callaghan et al., 2000). Unexpectedly,

the decreased concentration of E2 and P4 in the FF did not result in a reduction of the steroids in plasma. It was previously speculated that the failure in the ovum pick-up by the fimbria in buffalo may be due to high levels of E2 and E2:P4 ratio, as well as to prolonged rise of E2, enhancing the turgidity of the genital tract (Misra et al., 1998). In fact, a poorer recovery of ova was recorded in animals showing increased and prolonged E2 in blood. In contrast, in our study the E2 and E2: P4 ratio in plasma of superovulated buffaloes were similar to those recorded in synchronized buffaloes and no variation was observed in relation to the number of preovulatory follicles. However, as hormones were measured in the peri-ovulatory period, after the LH surge induced by GnRH, it is not possible to rule out that E2 levels were higher at earlier times in FSH-stimulated buffaloes. Nevertheless, the decreased concentration of steroids in the FF in the peri-ovulatory period may impair the process of ovum capture by the oviduct. Interestingly, a reduced mRNA expression of the steroid receptors *PGR* and *ER1* in the oviduct of superovulated buffaloes was also observed. Our results are in disagreement with those reported in an earlier trial in cattle, in which superovulation, associated with higher circulating steroids, did not influence the *PGR* and *ER1* pattern within the oviduct (Valle et al., 2007). The decreased expression of *ER1* in the oviduct may be accounted for by the lower E2 concentration found in the FF. In fact, it is known that under natural oestrous cycles E2 stimulates the production of both *PGR* and *ER1* in cattle (Ulbrich et al., 2003). However, P4 is known to inhibit its own receptor; indeed, negative correlations were reported between *PGR* and circulating P4 in cattle (Valle et al., 2007). In contrast, in the present study the intrafollicular P4 concentration was positively correlated with *PGR* expression in fimbria, infundibulum and ampulla, whereas no relationship was observed with plasma P4 levels. In addition, the expression of *PGR* was positively associated with that of *ER1* in infundibulum, ampulla and isthmus.

Furthermore, in the present study we investigated the VEGF system, that is under the control of ovarian steroids (Wijayagunawardane et al., 2005), known to play a role in the relaxation-contraction of the oviduct. Interestingly, the mRNA expression of both the *VEGF* and its receptor *FLK1* significantly decreased in the oviduct of superovulated buffaloes. Unexpectedly, the expression of VEGF receptor *FLT1* was not found in any tract of the oviduct in both groups. This could be due to the use of primers designed for bovine, as the buffalo genome has still not been completely sequenced. In the female reproductive tract, *VEGF* is involved in follicular development and steroids synthesis (Gordon et al., 1996), CL formation and maintenance (Berisha et al., 2000), CL vascularisation (Neglia et al. 2015) endometrial vascular growth and permeability (Meduri et al., 2000), and foetal development and placental function (Cheung, 1997). In addition, the higher expression of the VEGF system in human and bovine oviducts (Gordon et al., 1996; Lam et al., 2003, 2004; Gabler et al., 1999) during the peri-ovulatory period suggests a critical role also during early reproductive events, such as the ovum capture and transport within the oviduct. It is known that the maximum contractile activity of the bovine oviduct (Ruckebusch and Bayard, 1975; Bennett et al., 1988), as well as the highest VEGF content and expression *of VEGF* receptors (Gabler et al., 1999) are recorded during the peri-ovulatory period. Wijayagunawardane et al (2005), using cultured bovine oviductal cells as a model, demonstrated that VEGF stimulates the synthesis of contraction-relaxation related factors, such as *PGE2*, *PGF2a* and *ET-1*. Moreover, a combined treatment of VEGF with LH, E2 and P4 increased the mRNA expression of *VEGF* and its receptors. It is therefore likely that the decreased expression of *VEGF* and *FLK1* in the oviduct of superovulated buffaloes results from the reduced steroid levels found in the FF. It has been hypothesized that in the peri-ovulatory period the LH surge, the increased E2 levels and basal P4 levels up-regulate the VEGF system that in turn enhances the production of contraction-relaxation related factors. This may allow the ovum

pick-up and a rapid transport to the fertilization site. After ovulation *VEGF* switches over its own production, ensuring the embryo to reach the uterus at the proper time. Our results taken together suggest that in superovulated buffaloes the contraction-relaxation of the oviduct may be impaired, and that the lower contractility may interfere with the ovum capture.

Nevertheless, the decreased percentage of oocytes with cumulus expansion recorded in the superovulated buffaloes indicates that an inappropriate oocyte maturation may also play a critical role. Indeed, in case of failure of cumulus expansion a less developed extracellular matrix could not ensure a perfect adhesion with the oviduct. If there is not a correct adhesion degree between the matrix and the cilia, the COC cannot enter the oviduct (Lam et al., 2000). Normally, the COC is too large to pass through the oviduct ostium (Talbot et al., 2003) thus, to move into the infundibulum, it would, by the action of the cilia, churns for several minutes, to compact the extracellular matrix and consequently reduce its diameter (Talbot et al., 1999). Using an in vitro model for assessing COC pick-up in the hamster it was demonstrated that expanded COCs are picked up at the normal rate, whereas unexpanded COCs, which had cumulus cells but not cumulus matrix, failed to adhere to the infundibulum and were not picked up at all (Huang et al., 1997). Interestingly, when unexpanded COCs were placed in the ostium, they remained in place and were not picked up even though cilia were beating normally. It is known that the both the adhesion of cumulus cells to the oocyte and the degree of cumulus expansion following maturation are lower in buffalo than in cattle (Gasparrini 2000). It follows that the extracellular matrix in this species is also less developed. However, in the present study most of the COCs recovered in superovulated animals were unexpanded and at the GV stage, in contrast to what observed in synchronized buffaloes. It was reported in cattle that multiple ovulation treatment shortens the follicular phase of the oestrous cycle, due to the decreased interval between prostaglandin administration and LH surge (Dieleman and Bevers 1987, Merton et al., 2003). Likewise, superovulated buffaloes undergo oestrus

earlier than unstimulated buffaloes (Beg et al., 1997). A shortened follicular phase may result in inappropriate time for the oocyte to complete the maturation process, accounting for the lower proportion of expanded oocytes found in superovulated buffaloes. A high degree of asynchrony between the oocyte and follicular status shortly before expected time of ovulation (60 h after PG) was previously reported in superovulated cattle (Merton et al., 2003). The proportion of oocyte lacking an expanded cumulus varied between 16% (when COCs were collected by ovariectomy) and 40% (when COCs were recovered by OPU), and only 3% of unexpanded COCs developed into embryos compared to 42% of the expanded COCs (Merton et al., 2003). In our work the percentage of unexpanded COCs was much higher in the superovulatory group than in their synchronized counterpart, confirming the occurrence of asynchrony between the oocyte and the follicular maturation status. Unexpectedly, when follicles with expanded or unexpanded oocytes from superovulated buffaloes were compared, no differences were recorded both in terms of follicular size and intrafollicular steroid profile. However, the intrafollicular P4 concentration within superovulated buffaloes was positively correlated with the percentage of oocytes with cumulus expansion and negatively correlated with the recovery rate. It is therefore hypothesized that high intrafollicular P4 levels results in a greater expansion of cumulus cells and a greater viscosity of the FF, reducing the recovery rate.

In the present study the mRNA expression of *LHR* in granulosa cells was similar in synchronized and superovulated buffaloes. In contrast, the mRNA expression of *FSHR* in granulosa cells was higher in superovulated buffaloes, despite the lower oestrogen levels in FF. It is known that oestrogen increases follicular expression of both FSH and LH receptors in rat granulosa cells (Richards et al., 1976, 1979). It was hypothesized that oestrogen synergizes with FSH and exogenous cAMP to increase the number of FSH receptors. However, other studies reported that oestrogen by itself has no effect on the distribution or

number of FSHRs or affinity of FSH binding to granulosa cells (Richards, 1980; Mineghisi et al., 1996). It is worth pointing out that, as the time of collection in our study was at the peri-ovulatory period, we could not detect an earlier increase of oestradiol levels probably due to the gonadotrophin treatment. It is likely that the superovulation treatment with exogenous FSH resulted in the up-regulation of *FSHR* that persists until the peri-ovulatory period. In line with this hypothesis, effects of FSH have been observed on its own receptor mRNA in the mouse ovary. Treatment of mice with *in vivo* eCG stimulation increased the transcription (Yaron et al., 1998) of all four FSHR transcripts and this is associated with an increase in FSH binding (LaPolt et al., 1992). Mineghisi et al (1996), using an *in vitro* model with granulosa cells from oestrogen-treated immature rats, reported a transient decrease after 2-6 h followed by a recovery of *FSHR* mRNA after FSH treatment. After 12-60 h from the FSH treatment the *FSHR* mRNA levels were similar to the pre-treatment levels, preventing the decline observed in controls not receiving FSH. Nowadays it is still uncertain how FSH regulates its own receptors, with earlier reports of both up- and down-regulation of ligand binding after treatment with FSH (Richards, 1980). The influence of FSH is indeed dose and time dependent and may also depend on the species, the model used and the stage of folliculogenesis.

In summary, the results of this experiment demonstrated that the intrafollicular concentration of both oestradiol and progesterone in the preovulatory follicles decreases in superovulated compared to the synchronized buffaloes. In addition, the recovery rate and the percentage of oocytes exhibiting an appropriate cumulus expansion were reduced in stimulated animals. Finally, in superovulated buffaloes a decreased expression of both *ER1* and the VEGF receptor *FLK1* in the infundibulum and an increased expression of FSHR in granulosa cells were observed. In conclusion, the exogenous FSH treatment increased the expression of *FSHR* in granulosa cells that was though not followed by an increase of oestradiol synthesis,

suggesting an impairment of granulosa cell function. It results an altered steroid profile affecting in turn both the expansion of cumulus cells during maturation, and the contraction-relaxation of the infundibulum, confirming the hypothesis of a failure in ovum capture in superovulated buffaloes.

Conclusions

In conclusion, the results of this thesis taken together highlight the limitations of MOET in buffalo, suggesting that at present the only way to improve the intensity of genetic selection, through the maternal lineage, in this species is the OPU-IVEP technology.

Indeed, buffaloes do respond to the gonadotropin treatment with multiple ovulations but the ova capture is severely impaired, resulting in poor embryo recovery/corpora lutea ratio after uterine flushing, compared to their bovine counterparts. Nevertheless, the outputs of this thesis led to an improved knowledge and understanding of the limiting factors of the superovulation technique in this species. Beyond the high inter-individual variability, also described in other species, the major limiting factor in buffaloes is the low recovery rate likely caused by an impaired capture of the oocyte, both due to an altered extracellular matrix development, indicated by decreased cumulus expansion, and to an altered contraction-relaxation of the infundibulum, suggested by the modified expressions of key genes. Both phenomena are known to be under the control of the ovarian steroids that are reduced during the peri-ovulatory period in the FF of superovulated animals. Furthermore, it is not possible to preliminarily select the donors to be enrolled in MOET programs in a practical way. In fact, a reliable method to measure plasmatic AMH levels still needs to be developed in this species, leaving as a possible option the assessment of the AFC or the less practical measurement of intrafollicular AMH. To complicate the scenario, the selection of animals with a higher number of follicles does not seem to be advisable because in these subjects the percentage of oocytes exhibiting a proper expansion of the cumulus cells was decreased. Moreover, the replacement of the last four injections of FSH with a single administration of eCG within a superovulation protocol in buffalo failed to improve the embryo recovery rate.

Therefore, more research in this area is required to improve especially the embryo recovery following MOET in buffalo for this technology to be feasible in the field. In particular, it is essential to modify the superovulatory treatment in this species in order to avoid the alterations of steroid profile in the follicular fluid that do not appear in other species and to improve the expansion of cumulus cells during maturation, as well as the oviduct vascularization and motility. Therefore, in future perspective it will be worth evaluating whether the use of recombinant FSH may improve the steroid synthesis, likely affected by the LH contamination of pituitary extracts, and hence, the cumulus expansion and the contraction of the infundibulum, increasing the embryo recovery. Another possible strategy to improve ova pick-up and hence embryo recovery rate may be to evaluate different schedule of PGF2α treatment around ovulation, with the attempt to increase the VEGF and contractility of the oviduct, possibly combined with treatments aimed to improve the cumulus expansion. Undoubtedly, new advances in molecular biology might highlight other factors influencing the recovery rate or the FF steroid profile, and the identification of genes that play important roles in oocyte cumulus expansion might lay the basis for strategies aimed to influence gene expression or their products to increase cumulus expansion and hence the recovery rate.

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