Taylor & Francis
Taylor & Francis Group

Italian Journal of Animal Science

ISSN: (Print) 1828-051X (Online) Journal homepage: https://www.tandfonline.com/loi/tjas20

In vivo embryo production in buffalo: current situation and future perspectives

B. Gasparrini

To cite this article: B. Gasparrini (2007) *In vivo embryo* production in buffalo: current situation and future perspectives, Italian Journal of Animal Science, 6:sup2, 92-101

To link to this article: https://doi.org/10.4081/ijas.2007.s2.92

Copyright 2007 Taylor and Francis Group LLC

Published online: 15 Mar 2016.

Submit your article to this journal

Article views: 162

View related articles

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=tjas20

In vitro embryo production in buffalo: current situation and future perspectives

B. Gasparrini

Corresponding author: B. Gasparrini**.** DISC IZIA, Faculty of Veterinary Medicine, "Federico II" University, Naples, Italy **-** Tel. +39 0812536494 - Fax: +39 081 292981 - Email: bgasparr@unina.it

ABSTRACT: In the last few years, there has been an increasing interest in the in vitro embryo production technologies for faster propagation of superior germplasm in buffalo, due to the low efficiency of multiple ovulation and embryo transfer programs. Early attempts to produce buffalo embryos in vitro have been made by using procedures that were proven effective in cattle. However, the acquisition of more specific information on oocyte and embryo culture requirements in vitro in this species has resulted in an improved efficiency over the years. Although the IVEP efficiency has enhanced, as indicated by competitive embryo yields, pregnancy rate and development to term are still poor. Furthermore, the optimization of embryo cryopreservation methods in this species is critical for the diffusion of ET procedures in the field. The present review intends to describe the state of the art of IVEP in buffalo species, emphasizing the advances achieved and the limitations still to overcome.

INTRODUCTION - In the last few years interest in buffalo breeding has tremendously increased worldwide, due to the critical role that this species plays in many climatically disadvantaged agricultural systems. Successful buffalo breeding highly depends on the genetic improvement that can be achieved through the application of reproductive biotechnologies. As regards biotechnologies aimed to enhance genetic progress through the maternal contribution, in buffalo, due to the low and inconsistent response to multiple ovulation and embryo transfer (MOET) treatments (Zicarelli *et al*, 1997), there has been an increasing interest worldwide in the in vitro embryo production (IVEP) technology. The combined Ovum pick-up (OPU) and IVEP technology is currently the most promising tool for increasing the number of transferable embryos (TE) obtainable per donor over the long term in most species. In buffalo it has been demonstrated that this technology is even more competitive in terms of embryo yields compared to MOET (Gasparrini, 2002). Furthermore, a further increase of embryo yield (in our experience the best donor gave 37 TE in 6 months) may be achieved by selecting donors on the basis of their folliculogenetic potentials (Gasparrini, 2002). In addition to the limitations, in terms of embryo output, of MOET in this species (Zicarelli *et al*, 1997), it is worth emphasizing that OPU can be performed on a wider typology of donors such as non-cyclic animals, pregnant cows, subjects with patent oviducts or genital tract infections, and animals that are not responsive to hormonal stimulation, the last representing a high proportion in buffalo.

Although the buffalo IVEP system has greatly improved over the years, leading to high blastocyst yields (Gasparrini *et al.* 2006) and to the production of offspring (Neglia *et al.*, 2004; Hufana-Duran *et al.*, 2004; Sà Filho *et al.*, 2005; Huang *et al.*, 2005), this technology is still far from being commercially viable. The low IVEP efficiency recorded in buffalo compared

to cattle is in part due to peculiarities of the reproductive physiology of buffalo that are not easily modifiable, such as the low number of oocytes recruitable and their poor quality. Furthermore, the scarcity of experimental material in the majority of the countries where buffaloes are bred, together with the assumption that the reproductive biology in all ruminants is similar, led in the early attempts, to use the IVEP system in buffalo based solely on information acquired in cattle, with the consequent result of low IVEP efficiency. In fact, in the last decades improvements in IVEP have been achieved 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.*, 2006).

Oocyte source and quality

The major intrinsic limitation of IVEP technology in buffalo lies in the low number of immature oocytes that can be recovered per donor. In our experience controlled follicular aspiration of abattoir-collected ovaries allows the retrieval of 2.4 good quality oocytes per ovary on average (Gasparrini *et al.*, 2000) in comparison with 10 good quality oocytes recovered in cattle (Gordon, 1994). Similarly, a low number of oocytes is found when OPU is performed in buffalo compared to cattle (4.5 *vs* approximately 10 respectively; Galli *et al.*, 2000). This limitation is currently the most insurmountable impediment for the diffusion of IVEP in the field, arising from physiological peculiarities of the species, such as the low number of primordial (Danell, 1987) and antral (Kumar *et al.*, 1997) follicles present on the buffalo ovary, as well as the high incidence of follicular atresia (Palta *et al.*, 1998), and as such, it is not easily improvable. In short, biology can be manipulated for human purposes, but only to a limited extent. However, it has been shown that the number of competent oocytes may be increased by selecting donors on the basis of their follicular population (Sà Filho *et al.*, 2005). Furthermore, a pretreatment of buffalo donors with BST has been found (Sà Filho *et al.*, 2005) to promote follicular growth (12.2 *vs* 8.7 total follicles punctured; 9.1 *vs* 6.5 small follicles), without in parallel increasing the number of oocytes recovered (5.1 *vs* 4.5). These results are not conclusive because the study was only carried out on a limited number of donors and replicates, and it is known that the docility of an animal often affects recovery rate. Hence, it may be worth investigating different concentrations of BST or varying the administration protocol.

Oocyte quality, that is known to affect the IVEP efficiency in most species, plays a determining role in buffalo, further reducing the availability of the oocytes suitable for IVEP. It is worth pointing out that the percentage of good quality oocytes (Grade A and B according to the classification of Neglia *et al.*, 2003), is lower in this species compared to others, not exceeding, in our experience 50 % of the total oocytes recovered. An analysis of the data collected over a 4 year period in our lab showed that from a total number of 35.286 abattoir-derived oocytes (over 158 replicates) 47.8% were Grade A + B, 6.2 % were Grade C and 46.7 % were unsuitable for IVEP (unpublished data). A recent trial has shown an even lower proportion of good quality oocytes (33.7 % of Grade A+B), together with a higher incidence (37.9 %) of Grade C (Mishra *et al*, 2007a).

The oocyte quality may be affected by several factors, such as the aspiration pressure during collection, the source of gametes, the time between collection and processing, the temperature during transportation, season, etc. In our experience, the oocyte morphology varies with the source of gametes. Interestingly, despite their worse morphological appearance,

OPU-derived oocytes have a higher developmental competence compared to abattoir-derived ones (Neglia *et al.*, 2003). This is because OPU resets the follicular population, subsequently increasing the follicular wave frequency and, as a result, follicles are aspirated before they become atretic and hence oocyte quality is improved. Therefore, we speculate that technical factors during OPU, such as the length of the needle, as well as that of the line connected to the suction unit, may result in a greater loss of granulosa cells and, hence in an underestimated evaluation of their quality. Furthermore, when OPU is carried out in field conditions, with the donors often bred in farms distant from the laboratory, a significant improvement of blastocyst production can be achieved by reducing the time between oocyte collection and their maturation (Gasparrini, 2006). In order to do so, oocytes can be searched in the farm and transferred immediately in a hepes buffered in vitro maturation (IVM) medium in a portable incubator. It was speculated that the better developmental competence of OPU-derived *vs* abattoir-derived oocytes is related to the shorter exposure to environmental stress. Indeed, oocytes recovered from slaughterhouse ovaries undergo a longer time interval between excision of ovaries from the peritoneal cavity and laboratory processing. It follows that, in the latter case, another important factor to consider is the time interval between ovary collection and processing in the laboratory. In our setting the time lapse between collection of ovaries at slaughter and their arrival at the lab usually varies between 3 and 6 hours. A retrospective analysis of data collected over the last 4 years in our lab, however, showed that neither cleavage nor blastocyst rates are affected by extending the time interval up to 6 h (Di Francesco *et al.*, in press).
Buffalo oocytes are very sensitive to shock temperature so it is important to monitor the temperature carefully during collection as fluctuations can easily occur. It has been recently observed, that oocyte developmental competence is improved by lowering the temperature range during ovary transportation to 25-29.5°C (Di Francesco *et al.*, in press).
Finally, a preliminary analysis of data collected in our IVEP laboratory over the last 4 years has shown that the oocyte developmental competence is improved during the autumn months (unpublished data). This finding is in agreement with the seasonality pattern exhibited by the species at our latitudes, with the fertility improved during short-day months. It has been also recently reported (Mishra *et al.*, 2007b) that hot ambient temperature on day of slaughter negatively affects both cleavage (17.6% versus 36.6%, respectively for temperature > and < 40°C) and blastocyst development (0.0% *vs* 9.8%, respectively for temperature > and < 40°C) following IVF.

In vitro maturation (IVM)

Buffalo oocytes can be matured in vitro in complex media**,** such as Tissue Culture Medium 199 (the most widely employed) and Ham's F-10, supplemented with sera, hormones and other additives, such as growth factors and/or follicular fluid (see for review Gasparrini 2002). It is known that the presence of cumulus cells is critical for the acquisition of developmental competence during IVM, as confirmed by the significantly reduced cleavage and embryo development of denuded *vs* cumulus-enclosed oocytes following IVF (Gasparrini *et al.*, 2007a). This is particularly important in buffalo because of the high proportion of totally or partially denuded oocytes usually recovered in this species. In order to rescue germinal material, poor quality oocytes can be matured on a cumulus cells monolayer, obtaining improved maturation and fertilization rates (Pawshe *et al.*,1993).

Based on the assumptions that buffalo oocytes and embryos are particularly sensitive to oxidative damages, due to their high lipid content (Boni *et al.*, 1992), the IVM medium has been enriched by thiol compounds, known to act as antioxidants factors, by stimulating glutathione (GSH) synthesis. It was previously demonstrated that cysteamine supplementation during IVM improves blastocyst yield in buffalo (Gasparrini *et al.*, 2000), by increasing intracytoplasmic GSH concentration (Gasparrini *et al.*, 2003), without nevertheless affecting cleavage rate. The addition of cystine, in the presence of cysteamine, to the IVM medium (Gasparrini *et al.*, 2006) has further increased the GSH reservoir of the oocytes and has significantly improved the proportion of oocytes showing normal synchronous pronuclei post fertilization (81 %), cleavage rate (78 %) and blastocyst yield (30 %).
Among factors affecting mammalian embryo development in vitro, the duration of IVM plays a critical role, since an inappropriate timing of maturation results in abnormal chromatin (Dominko and First,1997), oocyte aging (Hunter and Greve, 1997) and reduced development (Marston and Chang, 1964). Although large variations in the timing of oocyte maturation have been reported in buffalo, with the highest proportion of MII oocytes observed between 16 and 24 h ((Neglia *et al.*, 2001; Yadav *et al.*, 1997, Gasparrini *et al.*, 2007b), the majority of the authors inseminate buffalo oocytes in vitro 24 h after the start of IVM. The different oocyte maturation time-scale recorded among buffalo studies may be accounted for by different conditions of IVM and particularly by oocyte quality which, in this species, is also likely affected by seasonal factors. It has been recently demonstrated that the duration of IVM affects buffalo oocyte developmental competence, with a progressive decrease of fertilization capability and embryo development as the IVM duration increases from 18 to 30 h (Gasparrini *et al.*, 2007b). Therefore, the optimal time for IVF in buffalo appears to be at 18 h post-IVM or, in any case, not later than 24 h; in fact, delaying IVF over 24 h resulted in a significant deterioration of oocyte developmental competence. The importance of oocyte aging in this species is also confirmed by activation studies that showed, in contrast to most other species, a deterioration of post-parthenogenetic embryo development at increasing times post-maturation (Gasparrini *et al.*, 2004a).

In vitro fertilization (IVF)

Fertilization has often been considered the most critical step of the IVEP procedures in buffalo, as cleavage rates lower than those obtained in other domestic species have been widely reported (Neglia *et al.*, 2003, Gasparrini *et al.*, 2004b, Galli *et al.*, 2000). In an earlier work, the lower blastocyst yield recorded in buffalo compared to cattle (26 *vs* 34 %, respectively) was mainly related to the poor cleavage rate (65% *vs* 84%); in fact similar blastocyst yields were obtained in buffalo and cattle (40 %) when the percentages were calculated in relation to the zygotes (Neglia *et al.*, 2003).
Many factors may affect the in vitro fertilization efficiency, such as the sperm viability and capability, the adequate in vitro environment for gametes survival, the appropriate time of insemination, the duration of gametes co-incubation, the presence of cumulus cells and last but not least the acquisition of the oocyte developmental competence during the complex process of cytoplasmic maturation.
In earlier times the quality of the frozen semen was considered the major factor impairing IVF, based on the demonstration of several damages of the male gamete occurring following cryopreservation (Meur *et al.*, 1988), together with the drastic reduction of cleavage rate

reported with frozen compared to fresh semen (Totey *et al.*, 1992). Currently, the quality of frozen semen has improved, as indicated by similar fertility parameters, recorded for fresh compared to frozen semen (Wilding *et al.*, 2003), suggesting that other factors may negatively affect fertilization. However, the overall improvement of the quality of cryopreserved sperm has not eliminated another serious impediment, the so-called “bull effect”, consisting in the high degree of variation between buffalo bulls in the fertilizing capability in vitro (Totey *et al.* 1993).

Although several agents have been proven to induce sperm capacitation in vitro, heparin is still the most efficient method in the majority of the domestic species. In order to investigate whether the capacitation process in vitro can be improved by agents different than heparin, buffalo sperm have been incubated under different conditions. It has been recently demonstrated that progesterone induces buffalo sperm capacitation in vitro and may be considered as an alternative capacitating agent for buffalo IVF (Boccia *et al.*, 2006a). Furthermore, it has been demonstrated that sperm treatment for 2 or 3 h with sodium nitroprusside, a well known generator of nitric oxide in vitro, improves the efficiency of buffalo sperm capacitation in vitro compared to heparin (Boccia *et al.*, 2007). The most promising results have been obtained by incubating buffalo sperm with biological fluids, such as buffalo estrus serum (BES) and the follicular fluid (FF) recovered from a pool of dominant follicles (Boccia *et al.*, 2005). In fact, significantly higher incidences of acrosome-reacted sperm (following incubation with lysophosphatidilcholine) were recorded following sperm treatment with both BES and FF than with heparin treatment (84.3, 94.5 *vs* 50.1 % respectively). These results show the possibility of significantly improving the efficiency of sperm capacitation in vitro in buffalo species and strongly suggest to investigate the effects of BES and FF also on the fertilizing capability of buffalo spermatozoa.

The media commonly utilized for buffalo IVF are Tyrode’s modified medium (TALP) and Brackett Oliphant (BO), supplemented by sperm motility inducing factors, such as combined hypotaurine-penicillamine or caffeine. However, significantly higher cleavage and blastocysts rates have been obtained, in a direct comparison trial, by using TALP medium, supplemented with heparin, hypotaurine and penicillamine (Gasparrini *et al.*, 2004b).

Another factor that may affect embryo development is the duration of gamete co-incubation during IVF. It has been recently demonstrated that the optimal sperm-oocyte co-incubation time for maximizing the blastocyst yield in buffalo is 16 h (Gasparrini *et al.*, 2007b). Interestingly, the lower blastocyst development recorded at the shorter durations of sperm-oocyte co-incubation tested were mainly due to the lower cleavage rates, as suggested by the fact that the oocytes that had cleaved developed further and as fast as those in the 16 h group. On the contrary, extending gamete co-incubation to 20 h was deleterious because, despite similar cleavage rates, the blastocyst production was reduced both when calculated in relation to COCs and to cleaved embryos. Furthermore, increasing the sperm-oocyte incubation time to 20 h was found to be correlated to a higher incidence of polyspermy.

As previously mentioned, the poor cleavage rate may also be due to the lack of oocyte developmental competence, normally acquired during the maturation process. In order to investigate this aspect activation studies were carried out. A significant improvement of cleavage (71 % *vs* 56 %, respectively) and blastocyst yield (33 *vs* 23 %, respectively) was obtained with ethanol-induced activation *vs* IVF indirectly suggesting that buffalo oocytes had acquired the developmental competence during IVM (Gasparrini 2004a). In any case, the im-

provement was not as such to make definite conclusions. It has more recently reported that activation with different methods give significantly higher cleavage and blastocyst rates compared to IVF, strongly suggesting that the problem has paternal rather than maternal origin (Mishra *et al.*, 2007a). However, we speculate that the evident difference shown by these authors may be due to serious problems with the quality of the sperm utilized in their system since very poor cleavage (< 37 %) and blastocyst rates (< 16 %) were obtained following IVF.
Finally, it is worth pointing out that, after many fruitless attempts to increase cleavage rate in this species, the fertilization efficiency has at last improved, reaching approximately 80 % of cleavage rate, by enriching the IVM medium with cystine and cysteamine. This improvement has been proven to be related to enhanced intracytoplasmic GSH levels (Gasparrini *et al.*, 2006). This interesting finding would indicate that the poor cleavage rate of this species so far recorded was likely related to an inappropriate maturation of the female gamete rather than to the deficiencies of the IVF system. It has been, in fact, suggested that the GSH production is critical for the acquisition of developmental competence of oocytes at a cytoplasmic level and that the measurement of GSH at the end of IVM can be a reliable indicator of the cytoplasmic maturation (de Matos *et al.* 1997).

In vitro culture (IVC)

The in vitro culture system developments for buffalo embryos have imitated those for other ruminant species. Buffalo embryos have been co-cultured with cumulus and oviductal cells (Totey *et al.*, 1992; Madan *et al.*, 1994) or with established cell lines such as BRL (Boni *et al.*, 1999). Although many authors still prefer the co-culture system for embryo production in this species, the utilization of defined media for embryo culture has become necessary to comprehend the requirements of buffalo embryos in vitro which, in turn, would allow the formulation of an optimal species-specific culture system. A well known defined medium, such as the Synthetic Oviduct Fluid (SOF) has been utilized for embryo culture in this species since 1999 (Boni *et al.*, 1999). Subsequently, buffalo zygotes/embryos have been successfully cultured either in SOF and in another defined cell-free system, known as Potassium Simplex Optimized Medium (KSOM) with similar embryo development (Caracciolo di Brienza *et al.*, 2001). The great improvement of blastocyst yields (35-40%) achieved in the following years is, according to our experience, due to the optimization of the IVM and, in part of the IVF systems rather than to modifications applied to the IVC system. In fact, despite attempts to modify its original composition, at present the original version of SOF remains the most suitable medium for embryo culture in buffalo.
It has been demonstrated (Monaco *et al.*, 2006) that, in contrast to sheep and cattle, the presence of glucose is absolutely required for in vitro culture of buffalo embryos, particularly during the early embryonic development (up to Day 4).
In order to reduce the accumulation of free radicals, ammonium and other catabolites that may affect embryo development, it has been suggested to use the easy expedient to change the medium more times during culture. However, no significant differences in buffalo embryo development have been recorded by changing the IVC medium 3 (Day 1, 3 and 5) or 2 (Day 1 and 5) times during culture, with a tendency of improvement in the latter case (Boccia *et al.*, 2006b) Therefore, in contrast to other species, the addition of fresh medium on Day 3 of culture in buffalo does not exert any positive influence. It is likely that this is

related to the higher sensitivity of buffalo embryos to fluctuations of temperature and/or pH that normally occur during a culture change even if to limited extents; it results that it is advisable “not to disturb” buffalo embryos during culture.
Buffalo embryos in vitro develop approximately 12-24 h earlier than cattle embryos (Galli *et al.*, 2000) and this pattern of development reflects that observed in vivo, with most of the blastocysts collected by uterine flushing in the hatched stage at 6.5 days after the onset of oestrus (Drost and Elsden, 1985). On Day 6 (Day = IVF) it is possible to find embryos in advanced stages of development, including hatched blastocysts but most embryos reach the blastocyst stage on Day 7. A small proportion of embryos are delayed, reaching the blastocyst stage on Day 8 but their quality and viability is poor, as demonstrated by their lower resistance to cryopreservation (Gasparrini *et al.*, 2001).

Embryo cryopreservation

Embryo cryopreservation is the best tool to overcome the major problem affecting the commercial application of embryo transfer (ET) procedures, i.e. the limited number of suitable recipients, that is particularly accentuated in buffalo because of the lower response to hormonal stimulation and hence to synchronization treatments. Furthermore, because of the seasonality of the species, it is advisable to carry out the transfers in the most favourable period for reproductive activity. Unfortunately, buffalo IVP embryos seem very sensitive to cryopreservation, probably due to their high lipid content. The tolerance to cryoprotectants may be increased by in vivo culture of the cleaved embryos in surrogate sheep oviducts (Galli *et al.*, 1998), as demonstrated by improved development to term following transfers of embryos, which were frozen in 10 % glycerol with the slow-freezing method. Nevertheless, although this system yields embryos of quality comparable to MOET, it requires appropriate facilities and is more unpractical and expensive.
Buffalo embryos that were entirely produced in vitro have been successfully cryopreserved by vitrification, as demonstrated by their survival following in vitro culture (Gasparrini *et al.*, 2001) and development to term after ET (Neglia *et al.*,2004, Sà Filho *et al.*, 2005). Although development to term has been obtained, efficiency still needs to be improved for the diffusion of OPU-IVEP technologies in the field. A significant improvement of embryo survival rate following IVC of vitrified-warmed buffalo IVP embryos has been recently obtained by using minimum volume vitrification methods, such as Open Pulled Straw (De Rosa *et al.*, 2006) and Cryotop (De Rosa *et al.*, 2007) vitrification. It has also been demonstrated that the stage of development affects freezability of IVP embryos, with increased in vitro survival for the advanced embryo stages (De Rosa 2006, 2007), such as the expanded blastocysts, which are, in any case, better quality embryos since they develop faster in vitro. Unfortunately no data are as yet available regarding the pregnancy rate and the development to term following ET of embryos vitrified in such ways.

RESULTS AND CONCLUSIONS - The acquisition of more specific information on buffalo oocytes and embryo requirements in vitro, has led to a significant improvement of the IVEP efficiency in this species. However, although blastocyst yield has greatly increased, pregnancy rates are still low and only few calves have been produced after transfer of cryopreserved embryos from live animals. In addition to the low number of oocytes recovered, that is a feature intrinsic to the species, an important limiting factor is the low resistance

to cryopreservation of buffalo in vitro derived embryos that can be also considered a result of poor viability due to suboptimal culture conditions. In future perspectives, the first limitation may be in part overcome by selecting donors on the basis of their folliculogenetic potentials and, in part by optimizing stimulation protocols for promoting follicular growth and hence, oocyte recovery. Furthermore, in order to improve the embryo cryopreservation efficiency in this species, future studies are needed to optimize both the in vitro culture system and the cryopreservation protocols, with particular attention to minimum volume vitrification methods that gave promising results in vitro. The improved freezability of buffalo IVEP embryos will result in advanced reproductive strategies to become a routine procedure in buffalo breeding.

REFERENCES - Boccia L., De Rosa A., Attanasio L., Di Palo R., Zicarelli L., Gasparrini B., 2005. Capacitation of buffalo spermatozoa in vitro. Reprod. Fertil. Dev. 17 (1,2): 270. **Boccia** L. Attanasio L., Monaco E., De Rosa A., Di Palo R., Gasparrini B., 2006a. Effect of progesterone on capacitation of buffalo (Bubalus bubalis) spermatozoa in vitro. Reprod. Dom. Anim. 41 (4): 311. **Boccia** L., Monaco E., Attanasio L., De Rosa A., Gasparrini B., 2006b. Influenza del cambio di coltura (doppio *vs* singolo) sullo sviluppo di embrioni bufalini in vitro. "XII Giornate scientifiche", Università degli Studi di Napoli Federico II,, Polo delle Scienze e delle Tecnologie per la Vita; 15-16 Giugno, Facoltà di Medicina e Chirurgia, Napoli: 171. **Boccia** L., Attanasio L., De Rosa A., Pellerano G., Di Palo R., Gasparrini B., 2007. Effect of sodium nitroprusside on buffalo sperm capacitation in vitro. Reprod. Fertil. Dev. 19: 276. **Boni** R., Santella L., Dale B., Roviello S., Di Palo R., Barbieri V., 1992. An ultrastructural study of maturation in buffalo oocytes. Acta Medica Veterinaria 38: 153-l 61. **Boni** R., Roviello S., Gasparrini B., Langella M., Zicarelli L., 1999. *In vitro* production of buffalo embryos in chemically defined medium. Buffalo Journal. 1: 115-120. **Caracciolo di Brienza** V., Neglia G., Masola N., Gasparrini B., Di Palo R., Campanile G., 2001. In vitro embryo production in chemically defined media. Atti 1° Congresso Nazionale sull'Allevamento del Bufalo, 3-5 ottobre, Eboli (SA), Italy: 341-344. **Danell** B., 1987. Oestrus behaviour, ovarian morphology and cyclic variations in follicular system and endocrine pattern in water buffalo heifers. PhD thesis, Uppsala, Sweden: Sveriges Lantbruksuniversiter. **de Matos** D.G., Furnus C.C., Moses D.F., 1997. Glutathione synthesis during in vitro maturation of bovine oocytes: role of cumulus cells. Biol Reprod 57: 1420-1425. **De Rosa** A., Di Palo R., Attanasio L., Monaco E., Campanile G., Gasparrini B., 2006. Open pulled straw vitrification for in vitro produced buffalo (Bubalus bubalis) embryos. Reprod. Fertil. Dev. 18 (1,2): 153. **De Rosa** A., Attanasio L., Boccia L., Pellerano G., Campanile G., Gasparrini B., 2007. Cryotop vitrification for in vitro produced buffalo (Bubalus bubalis) embryos. Reprod. Fertil. Dev. 19: 174. **Di Francesco S.,** Boccia L., Di Palo R., Attanasio L., De Rosa A., Gasparrini B., in press. Influence of temperature (and time for) during ovary transportation on in vitro embryo production efficiency in the buffalo species (Bubalus bubalis). Proc. VII World Buffalo Congress. **Dominko** T., First N.L., 1997. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. Mol. Reprod. Dev. 47: 456-67. **Drost** M., Elsden R.P., 1985. Blastocyst development in water buffalo (Bubalus bubalis). Theriogenology 23: 191. **Galli** C., Duchi R., Crotti G., Lazzari G.,1998. Embryo production by Ovum Pick-up in Water Buffalo. Theriogenology 50: 259. **Galli** C., Crotti G., Notari C., Turini P., Duchi R.,Lazzari G.,2000. Embryo production by ovum pick-up from live donors. Theriogenology

55:1341-1357. **Gasparrini** B., Neglia G., Di Palo R., Campanile G., Zicarelli L., 2000. Effect of cysteamine during in vitro maturation on buffalo embryo development. Theriogenology 54: 1537-1542. **Gasparrini** B., Neglia G., Caracciolo di Brienza V., Campanile G., Di Palo R., Zicarelli L., 2001. Preliminary analysis of vitrified in vitro produced embryos. Theriogenology 55: 307. **Gasparrini** B.,2002. In vitro embryo production in buffalo species: state of the art. Theriogenology 57 (1): 237-256. **Gasparrini** B., Sayoud H., Neglia G., de Matos D., Donnay I. , Zicarelli L.,2003. Glutathione synthesis during in vitro maturation of buffalo (Bubalus bubalis) oocytes: effects of cysteamine on embryo development. Theriogenology 60: 943-952. **Gasparrini** B., Boccia L., De Rosa A., Di Palo R., Campanile G., Zicarelli L., 2004a. Chemical activation of buffalo (Bubalus bubalis) oocytes by different methods: effects of aging on post-parthenogenetic development. Theriogenology 62 (9): 1627-1637. **Gasparrini** B., Boccia L., De Rosa A., Vecchio D., Di Palo R., Zicarelli L., 2004b. In vitro fertilization of buffalo (Bubalus bubalis) oocytes: effects of media and sperm motility inducing agents. Reprod. Fertil. Dev. 16: 255. **Gasparrini** B., Boccia L., Marchandise J., Di Palo R., George F., Donnay I., Zicarelli L., 2006. Enrichment of in vitro maturation medium for buffalo (Bubalus bubalis) oocytes with thiol compounds: Effects of cystine on glutathione synthesis and embryo development. Theriogenology 65(2): 275-287. **Gasparrini** B., 2006. Advances on in vitro embryo production in water buffalo. Proc of the 5th Asian Buffalo Congress on "Social economic contribution of buffalo to rural areas", April 18-22, Nanning, China Vol 1: 15-21. **Gasparrini** B., Attanasio L., De Rosa A., Monaco E., Di Palo R., Campanile G., 2007a. Cryopreservation of in vitro matured buffalo (Bubalus bubalis) oocytes by minimum volumes vitrification methods. Anim. Reprod. Sci. 98 (3-4): 335-42. **Gasparrini** B., De Rosa A., Attanasio L., Boccia L., Di Palo R., Campanile G., Zicarelli L., 2007b. Influence of the duration of in vitro maturation and gamete co-incubation on the efficiency of in vitro embryo development in Italian Mediterranean Buffalo (Bubalus bubalis). Anim. Reprod. Sci. (in press) PMID: 17481834. **Gordon** I., 1994. Aspiration techniques: Old and new. In Laboratory Production of Cattle Embryos. Wallingford, UK: CAB International, 71-72. **Huang** Y., Zhuang X., Gasparrini B., Presicce G.A., 2005. Oocyte recovery by ovum pick-up and embryo production in Murrah and Nili-Ravi buffaloes (Bubalus bubalis) imported in China. Reprod. Fertil. Dev. 17 (1,2): 273. **Hufana-Duran** D., Pedro P.B., Venturina H.V., Hufana R.D., Salazar A.L., Duran P.G., Cruz L.C., 2004. Post-warming hatching and birth of live calves following transfer of in vitro-derived water buffalo (Bubalus bubalis) embryos. Theriogenology 61: 1429-1439. **Hunter** R.H.F., Greve T.,1997. Could artificial insemination of cattle be more fruitful? Penalties associated with aging eggs. Reprod. Domest. Anim. 32: 137-42. **Kumar** A., Solanki V.S., Jindal S.K., Tripathi V.N., Jain G.C., 1997. Oocytes retrieval and histological studies of follicular population in buffalo ovaries. Anim. Reprod. Sci. 47: 189-195. **Madan** M.L., Singla S.K., Chauhan M.S., Manik R.S., 1994. In vitro production and transfer of embryos in buffaloes. Theriogenology 41: 139–143. **Marston** J.H., Chang M.C.,1964. The fertilizable life of ova and their morphology following delayed insemination in mature and immature mice. J. Exp. Zool. 15: 237-51. **Meur** S.K., Roy S.B., Mohan G., Dhoble R.I., 1988. Cryogenic changes in seminal proteins of cattle and buffalo. Theriogenology 30:1005-1010. **Mishra** V., Misra A.K., Sharma R.,2007a. A comparative study of parthenogenetic activation and in vitro fertilization of bubaline oocytes. Anim. Reprod. Sci., doi:10.1016/j.anireprosci.2006.12.019. **Mishra** V., Misra A.K., Sharma R., 2007b. Effect of ambient temperature on in vitro fertilization of Bubaline oocyte. Anim. Reprod. Sci. doi:10.1016/j.anirepro-

sci.2006.10.020. **Monaco** E., De Rosa A., Attanasio L., Boccia L., Zicarelli L., Gasparrini B., 2006. In vitro culture of buffalo (Bubalus bubalis) embryos in the presence or absence of glucose. Reprod. Domest. Anim. 41 (4): 332. **Neglia** G., Marino M., Di Palo R., Wilding M., Caracciolo di Brienza V., Dale B., Gasparrini B., Zicarelli L., 2001. A comparison of in vitro maturation in buffalo (Bubalus Bubalis) and bovine oocytes using confocal microscopy. Theriogenology 55: 488. **Neglia** G., Gasparrini B., Caracciolo di Brienza V., Di Palo R., Campanile G., Presicce G.A., Zicarelli L., 2003. Bovine and buffalo in vitro embryo production using oocytes derived from abattoir ovaries or collected by transvaginal follicle aspiration. Theriogenology 59: 1123-1130. **Neglia** G., Gasparrini B., Caracciolo di Brienza V., Di Palo R., Zicarelli L., 2004. First pregnancies to term after transfer of buffalo vitrified embryos entirely produced in vitro. Vet. Res. Commun. (Special Issue) 28: 233–236. **Palta** P., Banzai N., Prakash B.S., Manik R.S., Madan M.L.,1998. Endocrinological observation of atresia in individual buffalo ovarian follicles. Ind. J. Anim. Sci. 68: 444-447. **Pawshe** C.H., Totey S.M., 1993. Effects of cumulus cells monolayer on in vitro maturation of denuded oocytes of buffalo (Bubalus Bubalis). Theriogenology 39: 281. **Sá Filho** M.F., Carvalho N.A.T., Gimenes L.U., Torres-Junior J.R., Ferriera C.R., Perecin F., Perini A.P., Tetzner T.A.D., Vantini R., Soria G.F., Garcia J.M., Tonhati H., Zicarelli L., Gasparrini B., Baruselli P.S., 2005. Birth of the first buffalo calves after transfer of vitrified embryos produced in vitro in America. Atti del 3° Congresso Nazionale sull'Allevamento del Bufalo- 1° Buffalo Symposium of Europe and the Americas; October 12-15, Capaccio-Paestum (SA), Italy: 250. **Totey** S.M., Singh G., Taneja M., Pawshe C.H., Talwar G.P., 1992. In vitro maturation, fertilization and development of follicular oocytes from buffalo (Bubalus Bubalis). J. Reprod. Ferti1. 95: 597-607. **Totey** S.M., Pawshe C.H., Singh G.P., 1993. In vitro maturation and fertilization of buffalo oocytes (Bubalus Bubalis): Effects of media, hormones and sera. Theriogenology 39: 1153-1171. **Wilding** M., Gasparrini B., Neglia G., Dale B., Zicarelli L., 2003. Mitochondrial activity and fertilization potential of fresh and cryopreserved buffalo sperm. Theriogenology 59: 466. **Yadav** B.R., Katiyar P.K., Chauhan M.S., Madan M.L., 1997. Chromosome configuration during in vitro maturation of goat, sheep and buffalo oocytes. Theriogenology 47: 943-51. **Zicarelli** L., 1997. Superovulatory response in buffaloes bred in Italy. Third Course on Biotechnology of Reproduction in buffaloes, Caserta, Italy, 167-188.