Embryonic mortality in buffalo cows

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ABSTRACT: In buffalo species embryonic mortality is considered one of the major causes of fertility loss, especially in the animals that are not mated during their reproductive period. Embryonic loss in animals mated by artificial insemination (AI) is 20-40% during seasons characterized by high number of light hours. Also in buffalo naturally mated the incidence of embryonic mortality is about 20% and a higher incidence is observed between 28-60 days of gestation in buffaloes that conceive during increasing daylight length. A reduced capacity to secrete progesterone seems to explain in part this embryonic mortality but other as yet unidentified factors contribute between 40-50% to the embryonic losses. Treatments with hCG, GnRH agonist or progesterone on Days 5 after AI not always reduce embryonic mortality in buffalo species. Embryonic mortality in buffaloes appears to occur later (Day 25-40) than in cattle and P4 treatments should perhaps be applied later in buffaloes.

Key words: Embryonic mortality, Buffalo, Progesterone, GnRH, Hcg.

BUFFALO REPRODUCTIVE CHARACTERISTICS - Buffalo is an animal species that lives in regions found between 31°N parallel and 2°S. Currently, the distribution of the buffalo population covers the major climatic regions of the lower latitudes (Tropical zone) and middle latitudes (Temperate zone). This geographic origin and distribution logically suggests that buffaloes are adapted to hot, humid macro or microclimates (Shaffie, 1985). Buffalo is a photoperiodic species. Like sheep, buffaloes have to be considered a “short day” species. They have heats throughout the year but are more fertile when daylight hours decrease. According to Zicarelli (1995), this characteristic is due to their tropical origins; in fact, in these areas the availability of forage coincides with the period in which dark hours increase. Therefore, it has been supposed that animals which calve in the most suitable period for survival of the offspring were selected. It seems that they have retained this characteristic even when transferred to places where forage is always available (Zicarelli, 1995). In countries like Italy, where market demand requires a concentration of deliveries in the spring-summer period (not corresponding to buffalo reproductive activity) the out-of-season technique is widely applied. As a result, buffaloes which are less sensitive to photoperiodic effects have been selected. When the out-of-season technique has been applied for long periods a lower loss of fertility was observed (15% vs. 30%) compared to the farms in which it has been adopted for shorter periods (Campanile, 1997). These results are also due to the renewal of the herd that is very
frequent on farms that apply the out-of-breeding-season mating technique (Campanile, 1997). Moreover, the season-dependent reproduction phenomenon is more frequent in older buffaloes (Zicarelli et al., 1988a) that are more sensitive to the bull effect and show seasonal acycia more easily.

Buffalo reproduction is characterized by delayed puberty, silent oestrus, long post-partum ovarian inactivity, and, on the whole, poor fertility (Singh, 1988; Madan et al., 1994; Singla et al., 1996). Most of these problems result from the use of the “out of breeding season mating” technique (Zicarelli, 1997; Gasparrini B., 2002). In fact, if buffalo are bred without modification of their natural seasonality and without controlled breeding, an inter-calving period of less than 400 days and a culling rate of less than 12% has been observed in Italy, Brazil, Venezuela, and Argentina (Zicarelli et al., 1993). Poor fertility has also been observed when biotechnologies are applied to reproduction.

Immediately after parturition buffaloes show several physiological modifications, which are fundamental to sustain the new pregnancy. The first step is the resumption of ovarian cycle. This is blocked during pregnancy by progesterone, which avoids other ovulations and maintains hypotonic the uterus. In buffalo species, the resumption of ovarian activity is affected by the calving season. In fact, buffaloes that delivered during the spring period, showed an intercalving period on average longer by 30 or 70 days, respectively if they are pluriparous or primiparous (Zicarelli, 1994). This phenomenon is observed until the delivery happens in July-August period. Usually, after 90 days of lactation during the spring period, 44% of pluriparous and 80% of primiparous are acyclic (Zicarelli, 1994). The reproductive activity of Italian buffalo cows is also influenced by climatic variation. As regards spontaneous heats, temperatures lower than 8°C and continuous light for more than 11 hours cause a delay in ovulation, starting from the end of heats. This is probably a delay in the pituitary response to ovarian steroid secretion (Zicarelli et al., 1988a). Thermal excursions higher than 7°C and 9°C appreciably increase the incidence of double ovulation in both spontaneous oestrus and prostaglandin-induced oestrus (Zicarelli et al., 1988b). Such conditions limit the adoption of A.I. during specific periods of the year.

Moreover, Sastry and Georgie (1978) found a correlation between conceptions and temperature, relative humidity or rainfall. Specifically, a lower temperature and increased rainfall improve the conception rate. Obviously, rainfall during the three months previous to the conceptions improves the availability of herbage, meeting productive requirements. This represents an indirect effect of climate on buffalo reproduction; a hot climate, in fact, affects living and reproductive behaviour directly by its effect on their systemic functions and indirectly by governing the availability of nutrients.

**EMBRYO DEVELOPMENT** - A fundamental condition for the maintenance of a new gestation, is the presence of a suitable uterine environment, which is able to guarantee an adequate embryo development (Campanile, 2006). In bovine the first cleavage division is at around 30 hours after insemination and the second at around 48 hours. During the following 4-5 days, each blastomere undergoes subsequent divisions in the oviduct, yielding 4, 8, 16, 32 cells, tight morula and, finally, blastocyst (Senger, 2003). On day 6.5-7 the new embryo reaches the uterus and during the second week of gestation it begins to elongate and to send signals to the mother, in order to prevent luteolysis and maintain adequate progesterone circulating levels. Progesterone is important for allowing uterine secretions
and reducing myometrial tone, favouring embryo development and attachment. In buffalo species Karaivanov et al. (1987) observed that from the flushing of oviducts and uterine horns of slaughtered superovulated donors between 74 and 100 h, eggs were recovered only from the oviducts, while flushing conducted between 102 and 108 yielded eggs from both the oviducts and uterine horns. This observation suggests that embryo development is faster in buffalo than in bovine, as confirmed by several trials carried out in vitro, during which tight morulae and blastocysts were observed already on day 5-6 (Neglia, et al., 2001). Therefore, the maintenance of pregnancy is due to either the embryo capacity of signalling its presence and the mother capacity of recognizing these signals and maintaining an adequate uterine environment.

**EMBRYONIC MORTALITY** - Embryonic loss is increased when physiological regulation of oviductal and uterine function is inadequate or when the mother is exposed to one or more of the many stresses that can compromise embryonic survival (Hansen, 2002). Embryonic mortality usually happens during the first phases of gestation in various species: in cattle, for example, it is evident within 40 days of pregnancy. In particular, 30-40% of embryonic losses in bovine occurs between 7 and 17 days post fertilization and, in some cases, it can take place before embryo becomes foetus (Thatcher et al., 1995). Vasconcelos et al. (1997) recorded that during the subsequent phases of pregnancy (after 42 days), embryonic loss is a remote eventuality (around 10%).

In buffalo species embryonic mortality is considered one of the major causes of fertility loss, especially in the animals that are not mated during their reproductive period. In Italy, in fact, the application of the out of breeding season mating technique guarantees milk production in accordance with market requirements, but it forces the breeders to mate buffaloes during the less favourable periods. It was observed that embryonic loss in animals mated by artificial insemination (AI) is 20-40% during seasons characterized by high number of light hours (Campanile et al., 2005; Campanile et al., 2007a; Campanile et al., 2007b), whereas values of around 7% were recorded in Brazil during decreasing light days (Baruselli et al., 1997). In contrast to the previous work, an embryonic mortality rate of 20% was reported for buffaloes close to the equator (Vale et al., 1989). In any case, embryo mortality in buffalo occurs later than in bovine, usually between 25 and 40 days from AI (Campanile et al., 2005). In buffaloes naturally mated (Vecchio et al., 2007), independently from the conception period, 8.8% and 13.4% showed respectively embryonic mortality between 28-45 days (embryonic mortality-EM) days and between 46-90 days (foetal mortality – FM) of pregnancy. In this work no differences were found between the incidence of EM in relation to the conception period (Table 1), while a high incidence (P<0.01) of FM was found (Table 1) during a period of increasing daylight length (transitional period: December-March) compared to the April-July period. It is hypothesized that this condition is due to the presence, in the transitional period, of subjects that become pregnant, even if they have a lower function of the corpus luteum because they are going into anoestrus. In the subsequent months (April-July) an increased incidence of acyclic buffaloes is observed and, hence, only the subjects that are not sensitive to the photoperiod are cyclic and become pregnant. In fact, the incidence of FM is similar to that observed in the decreasing daylight length period (August-November), that is the favourable period for reproductive activity.
These data are in accordance with Baruselli (personal communication), that found an embryonic mortality (after 30 days from AI) of 13.2% and 7.0% respectively in decreasing and increasing daylight length period. In 1994, Zicarelli (1994) found an embryonic mortality of 21.8% in buffaloes naturally mated. The phenomenon was not correlated with the breeding season (spring vs. summer), but with the farm and the ovarian resumption after calving. In another trial (Zicarelli, unpublished data) performed on 3000 conceptions, a higher incidence of embryonic mortality was reported between 30 and 90 days in buffaloes that conceived during increasing daylight length (Figure 1). However, also in this case the phenomenon was affected by farm, management and environment. The incidence of embryonic mortality in Farm A was 5% vs. 14% observed in Farm B, but either these values were lower than those recorded in 1994.

The incidence of embryonic mortality found in Italy was higher between 28-60 days of gestation and lower after 71 days (Figure 2). This result is different from that reported in cattle (Silke et al., 2002), in which the embryonic loss from 28-87 days of gestation was similar.
Embryonic mortality, in buffalo species, was not affected by age, parity or lactation stage and infectious agents explained only about 2-8% of the cases (Campanile et al., 2005; Campanile et al., 2007a). Campanile et al. (2005) found a higher P₄ plasma levels in pregnant buffaloes than in buffaloes which showed embryonic mortality since day 10 after AI, whilst P₄ in non-pregnant buffaloes was intermediate. Pregnant buffaloes had also higher plasma P₄ on day 20 than both non-pregnant buffaloes and buffaloes that showed embryonic mortality. P₄ plasma concentration significantly decreased only in non-pregnant buffaloes between day 10 and 20. In a further trial, it was observed that pregnant buffaloes showed higher concentrations of P₄ milk whey than both animals showing embryonic mortality and non-pregnant buffaloes on day 20 and day 25 but only than non-pregnant buffaloes on Day 10 (Campanile et al., 2007b).

It may be hypothesised that embryonic mortality in buffalo species is primarily due to a reduced secretion of P₄ by corpus luteum. This conclusion would be consistent with several findings in cattle and sheep, where early embryonic mortality was associated with reduced circulating concentrations of P₄ (Garret et al., 1988; Mann and Lamming, 1999; Mann and Lamming, 2001). During a trial carried out in Italy in a period of increasing daylight length it was proposed that the relatively high incidence of buffaloes with low circulating concentrations of P₄ after oestrus synchronisation was reflective of a reduced activity of the reproductive endocrine system. As previously specified, buffaloes are seasonal animals, showing increased reproductive activity in response to decreasing daylight length (Zicarelli, 1997). Impaired P₄ secretion has been linked with a reduced capacity of the developing embryo to secrete interferon-tau (IFNτ) at threshold amounts necessary to prevent luteolysis (Wathes et al., 1998). In fact, as above mentioned, the maintenance of pregnancy is due either to the maternal recognition of pregnancy and to the embryo capability of blocking luteolysis since day 16 post-AI (Mann and Lamming, 1999). This process occurs by the production of bovine trophoblastic protein-1 (bTP-1), also called IFNτ (Roberts et al., 1992). This protein is able to avoid corpus luteum regression by two mechanisms: i) by inhibiting oxytocin receptors (OTR) development on endometrium (Robinson et al., 1997); ii) by activating a prostaglandin inhibitor (Thatcher et al., 1995).
It has been supposed that oestradiol is another factor involved in the luteolytic process, either by promoting OTR development and by stimulating prostaglandin secretion (Wathes et al., 1998). In fact, it has been demonstrated in ovine that the number of oestradiol receptors on endometrium is significantly lower in pregnant vs. not pregnant animals (Lamming et al., 1995; Spencer et al., 1995). However, in buffalo species oestradiol plasma levels do not differ between pregnant, not pregnant and buffaloes undergone embryonic mortality on day 0, 10, 20 and 25 after A.I. (Spagnuolo et al., 2007).

Gametes quality is one of the main factors involved in the phenomenon of embryonic mortality in domestic animals. Oocyte quality is able to affect embryo development and interfere with the following gestation. In buffalo species this phenomenon may be more frequent during the seasonal anoestrus, which coincides with day length increase (Campanile et al., 2005) and, consequently, with the resumption of sexual promiscuity in the farms in which the out of breeding season mating technique is applied. Campanile et al. (2005) demonstrated that 51% of buffaloes which showed embryonic mortality had P4 concentrations on days 10 and 20 similar to those of animals which maintained pregnancy. Therefore, it is possible that other factors, rather than reduced circulating P4 concentrations, also contributed to embryonic mortality. With this regard, it was reported that oocyte quality, judged as the capacity to result in embryonic development and pregnancy, is worse in buffaloes during the anoestrous period (Abdoon et al., 2001), occurring when daylight length increases (Zicarelli, 1997). Furthermore, the incidence of embryonic mortality between 40th and 60th day post AI is three times higher in buffaloes that are acyclic 70 days post partum (Zicarelli, 1994), compared to those that are cyclic. It is known that in buffalo species high incidence of atresia is present and the mean recovery of good quality oocytes per ovary is low? (Gasparriini, 2002). The maturation and the quality of oocyte depend on the function of the granulosa cells that are sensitive to oxidative stress (Dharmarajan et al., 1999). It is worth mentioning that the antioxidant defence system plays a key role in preventing apoptosis and atresia, thus preserving steroidogenic function of granulosa cells (Cassano et al., 1999). Spagnuolo et al. (2007) found no significant differences in redox status between pregnant, not pregnant and cows with embryonic mortality.

**TREATMENTS FOR PREVENTING EMBRYONIC MORTALITY IN BUFFALO SPECIES** - The importance of progesterone (P4) concentration during the first weeks of pregnancy for reducing embryonic mortality has been demonstrated in cattle (Mann and Lamming; 1999 and 2001). According to some reports the presence of an early P4 peak (within 5 days after mating or AI) facilitates the elongation of the conceptus and, consequently, the secretion of adequate interferon-tau (Starbuck et al., 1999; Mann, 2002). In cattle, interferon-tau extends the lifespan of the corpus luteum (Plante et al., 1989) by suppressing estradiol receptor and oxytocin receptor genes (Spencer and Bazer; 1996) and by attenuating the endometrial secretion of PGF2 (Helmer et al., 1989a). It has also been shown that interferon-tau reduces PGF2 secretion by bovine endometrial explants (Helmer et al., 1989b) and endometrial epithelial cells (Danet-Desnoyers et al., 1994). Several approaches have been used to increase P4 concentration in blood in order to reduce the occurrence of embryonic mortality. Increased plasma P4 concentrations were achieved either by inducing increased endogenous secretion or by administering exogenous P4 (Mann and Lamming; 1999). Studies have shown that administration of natural sequence GnRH,
GnRH agonists or hCG after AI can stimulate corpus luteum function, induce accessory corpus luteum formation, increase P₄, and reduce estradiol production, with a consequent positive effect on embryonic survival (Kerbler et al., 1997; Thatcher et al., 2003; Bartolome et al., 2005). In buffalo species there are some controversial results, regarding the best moment for hormonal treatment. Campanile et al. (2007a) reported that treatment with exogenous P₄ (PRID®, Vetem) on day 5 after A.I gave the lowest pregnancy rate and highest incidence of embryonic mortality, suggesting that exogenous P₄ can have had a detrimental effect on conception. It is possible that exogenous P₄ may contribute to the regulation of LH and reduce the capacity of the preformed corpus luteum to increase P₄ synthesis and release. After removal of the exogenous source of P₄ the corpus luteum may not be able to secrete P₄ in the amount required to maintain pregnancy. Furthermore, the injection of 12.6 µg GnRH agonist (buserelin) or 1500 I.U. of hCG on Day 5 after A.I. increased P₄ concentrations without reducing the incidence of embryonic mortality. It should be noted, however, that P₄ in buffaloes treated with buserelin and hCG was significantly different to control buffaloes only on Day 15 after AI. It is therefore possible that P₄ was not elevated for a sufficient time in the period after AI to have a major effect on uterine function and embryomaternal interactions (Campanile et al., 2007a). The present findings are in contrast with Kumar et al. (2003) who reported an increase in conception rate in buffaloes treated with 125 mg of 17-α hydroxyprogesterone caproate s.c. on Day 4 after AI. It is possible that the type and mode of exogenous P₄ treatment may influence the response in buffaloes. With this regard, 341 mg of 17-α hydroxyprogesterone caproate administered i.m. 3 times, at 4-day intervals, starting on Day 25 after AI, reduced the incidence of embryonic mortality in a buffalo herd characterised by a high incidence of embryonic mortality (Campanile et al., 2007b). Treatment with buserelin or hCG on Day 25 after A.I. in pregnant buffaloes also reduced the incidence of embryonic mortality in buffaloes bred in a farm characterized by high incidence of embryonic mortality (Campanile et al. 2007b).

In cattle, treatment with hCG on Day 5 or Day 7 after AI increases P₄ concentrations by enhancing secretion from the existing corpus luteum and also by inducing ovulation and formation of an accessory corpus luteum (Kerbler et al., 1997; Schmitt et al., 1996; Santos et al., 2001). In buffalo species, P₄ increased on Day 10 after injection of 1.500 I.U. of hCG. It is speculated that in buffaloes hCG may not increase the P₄ secreting capacity of the existing corpus luteum, but can induce ovulation and formation of an accessory corpus luteum which leads to increased P₄ some time later. It is known that hCG administered at Day 25 after AI induces ovulation in around 57% of buffaloes (Campanile et al., 2007c) and that there is a similar response to GnRH agonists, using which ovulation rates of 62% (Campanile et al., 2007d) and 68.6% (Campanile et al., 2007c) are observed, respectively after administration on day 5 or 25 post AI. The mean follicular diameter which resulted sensitive to the hormonal treatment was about 8.9 mm in both treatments, varying between 4.2 and 13.0 mm (Campanile et al., 2007c; Campanile et al., 2007d). It is worth pointing out that the dimensions of the follicles recorded in buffaloes responsive to the treatments were similar to those of buffaloes in which ovulation did not occur. These data are in accordance with those reported in bibliography in cattle (Martinez et al., 1999), regarding the incidence of subjects responsive to the treatment with GnRH and the dimensions of responsive follicles. Buffaloes that ovulated in response to the treatment with a GnRH agonist showed a progressive increase in milk whey progesterone concentrations on Days 10, 15 and 20, while progester-
one levels remained relatively constant for buffaloes that did not ovulate. The injection of a GnRH agonist on Day 5 after AI increased milk whey progesterone concentrations in 97% of buffaloes subsequently pregnant on Day 40, compared to 68% in the non-pregnant buffaloes (P<0.01). A greater (P<0.05) proportion of the buffaloes that ovulated (96.7%), compared to buffalo that did not ovulate (68.4%) recorded a gestational chamber on Day 40 after AI and were judged to be pregnant (Campanile et al., 2007d). Ovulation also increased milk whey progesterone levels and reduced embryonic mortality in buffalo cows treated with 1500 IU of hCG or 12.6 µg of GnRH agonist on Day 25 after A.I. (Campanile et al. 2007c).

**CONCLUSIONS** - In buffalo species embryonic mortality is one of the major causes of fertility loss, above all in those animals that are not mated during their reproductive period. Buffaloes that undergo oestrus synchronisation and AI during a period of increasing day length have a relatively high occurrence of embryonic mortality. In naturally mated buffalo embryonic loss is about 20% and a higher incidence is reported between 28-60 days of gestation in buffaloes that conceive during increasing daylight length. This phenomenon is probably affected by farm, management and environment. A reduced capacity to secrete progesterone seems to explain in part this embryonic mortality but other as yet unidentified factors contribute between 40-50% to the embryonic losses. It will be important to further elucidate the factors that can contribute to embryonic mortality in buffaloes so that strategies can be developed to optimise fertility after synchronisation and AI during periods of reduced reproductive activity.

The treatments to increase P₄ early in pregnancy, that have proven successful in increasing pregnancy rates in cattle, are not necessarily applicable to buffaloes. It is however acknowledged that whilst P₄ tended to be elevated on Day 10 in buffaloes treated with buserelin and hCG, concentrations were significantly different to controls only on Day 15. As noted above, this finding was interpreted to suggest that hCG and buserelin did not stimulate increased P₄ secretion from the existing corpus luteum but rather induced ovulation and formation of an accessory corpus luteum, at least in a proportion of buffaloes. Embryonic mortality in buffaloes mated by AI in mid-winter appears to occur later than in cattle and hence P₄ treatments applied on day 25 after A.I. reduce embryo mortality in farms in which embryo loss is highly present.

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