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CICLO XXI

TESI DI DOTTORATO DI RICERCA:

**CAN EMBRYONIC MORTALITY IN THE DAIRY COW
BE RELATED TO OXIDATIVE STRESS?**

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SUMMARY

The present thesis aimed to investigate causes and factors involved in embryo mortality (EM) in the dairy cow, in particular the role played by oxidative stress (OS).

In Chapter 4 the pregnancy-associated glycoprotein (PAG) assay, a new method for bovine pregnancy/EM diagnosis never applied in Italy before, was tested in a commercial dairy farm. The PAG test turned out to be a reliable pregnancy/EM diagnosis method although some false positive or false negative were found. For instance, some cases of PAG detection in the maternal circulation after embryonic death occurrence were found, suggesting that PAG may be a better indicator of trophoblastic function rather than embryo viability. The relationship between progesterone (P4) and PAG production was investigated in three different experiments (chapter 4.3.6, chapter 5.3.3, and chapter 6.3.4). The results obtained showed a positive effect exerted by high P4 concentrations on PAG secretion, which is particularly evident when compared to the effect of P4 levels below the threshold of 6 ng/ml in these experiments.

The relationship between OS and EM was investigated in intensive dairy production system (chapter 5) and pasture-based system (chapter 6). Very few modifications of the OS indicators were observed in case of EM suggesting a weak involvement of OS in the aetiology of EM. It is interesting the relationship observed between advanced oxidation protein products (AOPP) levels and EM which suggests a greater degree of protein oxidation in case of EM. Since AOPP seem to be a by-product of neutrophil activation during infections, it is possible that the higher levels of AOPP observed in EM may reflect subclinical inflammatory events at uterine levels that can compromise the correct embryonic development. Therefore, inflammation and not OS might be the main responsible for embryo loss.

Thus, the main contribution of this PhD thesis was to provide new knowledge about the oxidative status of dairy cows. In particular, original contributions are in relation to OS and inflammation biomarkers, as AOPP, and their involvement in EM. As a result, new questions have arisen on this area that could constitute the objective of further research programs in the future.

RIASSUNTO

La presente tesi intende indagare le cause e i fattori implicati nella mortalità embrionale nella bovina da latte, in particolare il ruolo rivestito dallo stress ossidativo.

Nel capitolo 4, è stato testato in un'azienda di bovine da latte il dosaggio delle pregnancy-associated glycoproteins (PAG), un nuovo metodo per la diagnosi di gravidanza/morte embrionale mai applicato in Italia prima d'ora. Il PAG test si è dimostrato efficace nel diagnosticare gravidanze e morti embrionali, nonostante si siano riscontrati alcuni falsi positivi e falsi negativi. Ad esempio, in alcuni casi le PAG sono state rilevate nel sangue materno anche dopo la morte dell'embrione, suggerendo quindi che le PAG possano essere indicatori di funzionalità del trofoblasto anziché di vitalità dell'embrione.

Il rapporto tra i livelli di progesterone (P4) e la produzione di PAG è stato studiato in tre diversi esperimenti (capitoli 4.3.6; 5.3.3; e 6.3.4). I risultati ottenuti mostrano un effetto positivo esercitato da elevate concentrazioni di P4 sulla secrezione di PAG, che risulta evidente soprattutto quando confrontato con l'effetto di livelli di P4 inferiori alla concentrazione soglia di 6 ng/ml trovata in questi esperimenti.

Il rapporto tra lo stress ossidativo e la mortalità embrionale è stato valutato sia in un sistema di produzione intensivo (capitolo 5), che in un sistema estensivo basato sul pascolo (capitolo 6). Si sono osservate scarse modificazioni degli indicatori di stress ossidativo nei casi di mortalità embrionale, che suggeriscono quindi un suo debole coinvolgimento nell'eziologia di questa patologia. È comunque interessante considerare la relazione emersa tra i livelli di advanced oxidation protein products (AOPP) e la mortalità embrionale, la quale induce a ritenere che vi sia un maggior grado di ossidazione delle proteine in caso di morte dell'embrione. Poiché gli AOPP sembrano essere prodotti dall'attivazione dei neutrofili in corso di infezione, è possibile che gli elevati livelli di AOPP, osservati nei casi di morte embrionale, riflettano eventi infiammatori subclinici a livello uterino che potrebbero, a loro volta, compromettere il corretto sviluppo dell'embrione. Di conseguenza, l'infiammazione, e non lo stress ossidativo, potrebbe essere il principale responsabile della perdita dell'embrione.

Il contributo maggiore apportato dalla presente tesi è quello di fornire nuove conoscenze in merito allo stato ossidativo della bovina da latte. In particolare, si sono ottenute rilevanti informazioni riguardanti alcuni indicatori di stress ossidativo e di infiammazione, come le AOPP, e il loro coinvolgimento nella mortalità embrionale.

Alla luce delle argomentazioni trattate in questo lavoro di indagine scientifica, sono derivati nuovi interrogativi che interessano il complesso tema della fisiopatologia della

riproduzione della bovina da latte e che potrebbero costituire l'obiettivo di futuri programmi di ricerca.

Preface

The last few decades have seen a world-wide decline in reproductive efficiency of dairy cattle (for review see Lucy, 2007). Pregnancy loss is one of the major cause of infertility for the modern dairy system, having also an important impact on dairy economics (Royal *et al*, 2000; De Vries, 2006). Failure to establish pregnancy results from either fertilization failure or embryo death.

In order to investigate causes and mechanisms involved in embryo mortality (EM), a diagnostic method that discriminates between EM and AIs that do not results in pregnancy is an essential requirement. Proteins produced and secreted specifically by placenta early during pregnancy are thus obvious candidates for developing such a pregnancy test.

Pregnancy-associated glycoproteins (PAG) are a polymorphic family of proteins produced by the binucleated cells of the trophoctoderm of ruminants and can be detected in the maternal blood from 22-24 days after breeding until 80-100 days after delivering (Zoli *et al*, 1992). Once PAG are released in the maternal circulation, they can be used for pregnancy diagnosis, pregnancy follow-up, and for the monitoring of the trophoblastic function (Perényi *et al*, 2002b). PAG are also considered an indicator of the presence of a viable embryo (see Lopez-Gatius *et al*, 2007a).

Therefore, the first aim of this thesis was to verify whether PAG assay is a reliable method to perform pregnancy diagnosis in commercial dairy farms and whether it allows to differentiate between EM and AIs that do not result in pregnancy. In chapter 4, PAG assay was tested with satisfactory results allowing then the investigation of EM causes.

Oxidative stress (OS) may be involved in the aetiology of EM (Guerin *et al*, 2001). Several papers indicate reactive oxygen metabolites (ROMs) and oxidative stress (OS) having a role in the cause and progression of a number of pathological disorders related to reproduction in humans as well as in farm animals (for review see Agarwal *et al*, 2005; Fujii *et al*, 2005).

OS is a result of an imbalance between ROMs production and neutralising capacity of antioxidant mechanisms (Bernabucci *et al*, 2002; Castillo *et al*, 2005). Excessive production of ROMs during OS causes DNA damage, lipid and protein oxidation which, in turn, can lead to cell injuries, such as increased permeability of cell membranes, mutations, enzymes or ion transporters inactivation or even cell lysis (Imlay and Linn,

1988; Reilly *et al*, 1991, Dean *et al*, 1997; Takahashi *et al*, 2000). Several oxidation products are also highly immunogenic, therefore they may activate the immune reaction and be the target of autoantibodies (Shanti *et al*, 1999; Palinski and Witztum, 2000; Kurien *et al*, 2006). OS may also enhance inflammatory response since ROMs released by activated neutrophils during immune reactions contribute considerably to further generation of oxidants and tissue damage (Lykkesfeldt and Svendsen, 2007).

Inflammation, immune system activation and oxidative stress have been hypothesised to be part of the pathogenetic mechanism of several diseases, including EM. For instance, OS is responsible for numerous types of embryo damages which can often lead to embryonic death (Guerin *et al*, 2001; Agarwal *et al*, 2005). At the moment, the reciprocal role of OS, inflammation and immune system in respect to the others is far from being elucidated. Several pathways might be activated but a central role of oxidation products seems likely.

Therefore in this thesis, the hypothesis that oxidative status may affect AI outcome, EM in particular, was tested. It is important to consider that oxidative status of animals is regulated by the activity of antioxidants which, in turn, is influenced by nutrition (Andrés *et al*, 1997; Gatellier *et al*, 2004). For this reason, the relationship between OS and reproduction was investigated in two different rearing systems: intensive dairy production system (chapter 5) and pasture-based system (chapter 6).

Review of Literature

2.1 Establishment and maintenance of pregnancy

In ruminants, the successful course of gestation following a normal process of fertilization depends on complex interactions between the mother and conceptus (embryo and extra-embryonic membranes) within the female genital tract (López-Gatius *et al*, 2007a).

Establishment of pregnancy involves maternal recognition of pregnancy and implantation. Maternal recognition of pregnancy is the physiological process whereby the conceptus signals its presence to the mother and prolongs the lifespan of the corpus luteum (CL) in order to maintain progesterone (P4) production (Spencer *et al*, 2004). P4 plays a vital role in early pregnancy since it stimulates and maintains endometrial functions necessary for conceptus growth, implantation, placentation and development to term (Goff, 2002; Spencer *et al*, 2004).

In the early stages of gestation, embryo development has to be synchronous with changes in uterine environment for the embryo to survive. In order to achieve it, the dialogue between the mother and conceptus needs to be finely regulated and P4 seems to play the major role (Goff, 2002). The most important condition required for embryo survival, hence, is a fully functional corpus luteum.

Thereby, before reviewing the establishment and maintenance of pregnancy, it is necessary to elucidate briefly CL physiology and P4 production.

2.1.1 Corpus luteum and progesterone production

Progesterone is the hormone of pregnancy and unequivocally required in all mammals for maternal support of conceptus survival and development. It is produced by corpus luteum, a temporary endocrine gland that develops from the follicular cells after ovulation. If a viable embryo is not present in the uterus by day 16 in the cow, corpus luteum undergoes a rapid regression after day 18 (Pineda, 2003).

As all steroids, progesterone is derived from the common precursor cholesterol. The major source of cholesterol for luteal cells is circulating lipoproteins, particularly high

density lipoprotein (HDL) and low density lipoprotein (LDL) (Diaz *et al*, 2002). The first step of steroidogenesis is the transport of cholesterol to the inner mitochondrial membrane mediated by the steroidogenic acute regulatory protein (StAR). Cholesterol is then converted to pregnenolone by P450 cholesterol side-chain cleavage enzyme (P450_{scc}). The subsequent conversion to progesterone is catalyzed by 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and it takes place out of the mitochondria, on the smooth endoplasmic reticulum. Progesterone then diffuses out of the luteal cell and into the bloodstream.

In ruminants, while initial luteinization, growth, and development of CL is strictly dependent on LH action, P4 production by CL is relatively independent of acute LH stimulation (Wiltbank, 1994). This may be due to distinct functional properties of the steroidogenic cell types in the corpus luteum.

In ruminants, indeed, CL contains two morphologically and functionally distinct steroidogenic cell types: the small and large luteal cells. The large luteal cells are derived from granulosa cells from the preovulatory follicle and represent approximately 40% of the volume of the CL. The small luteal cells are probably derived from luteinized thecal cells and represents approximately 20% of CL volume (Wiltbank, 1994; Diaz *et al*, 2002). The remaining 40% of CL volume is occupied by vascular space (20%), endothelial cells (13%) and fibroblast (6-9%) (Wiltbank, 1994). The concentrations of receptors vary in the two cell types: prostaglandin and oestradiol receptors are located primarily on the large luteal cells, while LH receptors are greater in small cells (Wiltbank, 1994). This different distribution of receptors determines important differences in regulation of P4 production, particularly regarding LH action. Indeed, small luteal cells respond to LH stimulation by activating the protein kinase A (PKA) stimulatory pathway to produce P4. In contrast, large luteal cells show a constitutive high basal production of P4 which represents at least 80% of the total luteal P4 and is independent from LH stimulation (Niswender *et al*, 1985). The underlying mechanisms responsible for this basal LH-independent secretion of P4 in large luteal cells are still not well defined. Likely, luteotropins other than LH tonically stimulate P4 production. Studies have shown stimulatory effects of PGE₂ (Fitz *et al*, 1984), insulin (Wiltbank *et al*, 1992), and a number of growth factors (Miyamoto *et al*, 1992) on large luteal cells. Also lipoproteins are stimulatory to P4 production. Indeed, cholesterol substrate from lipoproteins is critical for P4 production since *de novo* cholesterol production in luteal cells is not enough to support the high P4 synthesis rate by CL (Diaz *et al*, 2002). Nevertheless, the major key rate limiting step in luteal P4 production seems to be the transport of cholesterol from the outer to the inner mitochondrial membrane, rather than cholesterol availability itself. This movement is mediated by StAR which

expression is very elevated in large luteal cells (Diaz *et al*, 2002). This may partly explain the high constitutive P4 production in the large luteal cells which is essential to maintain relatively constant circulating P4 concentrations. StAR expression is stimulated by many factors like insulin, LH, and estradiol (see Diaz *et al*, 2002) but the precise mechanism whereby they support StAR biosynthesis is still poorly understood.

2.1.2 Luteolytic mechanism

In ruminants, the oestrus cycle is dependent on the uterus as the pulsatile secretion of prostaglandin $F_{2\alpha}$ (PGF_{2 α}) from the endometrium results in functional and structural regression of the ovarian CL, termed luteolysis. PGF_{2 α} is transferred locally to the ovary by a countercurrent mechanism and acts on the CL causing its destruction and a decline in P4 secretion (McCracken *et al*, 1999).

In the cow the luteolytic pulses of PGF_{2 α} are produced by the endometrial epithelial cells on day 16-17 in response to oxytocin secreted by the pituitary gland and CL. PGF_{2 α} acts to induce luteolysis in the ovary and, at the same time, stimulates further secretion of oxytocin from the luteal cells, thus creating a powerful positive feedback loop (Horn *et al*, 1998).

Luteolysis is thus initiated by an increase in the number of oxytocin receptors (OTR) in the endometrium on day 15-17 (Jenner *et al*, 1991; Robinson *et al*, 1999) and it requires sequential effects of progesterone, oestrogen and oxytocin acting through their respective receptors (Goff, 2002; Spencer *et al*, 2004). How exactly this mechanism is regulated is not entirely clear.

Studies in sheep (see Robinson *et al*, 1999) have shown that OTR number is regulated during the oestrus cycle by P4 and oestradiol: oestradiol seems to up-regulate endometrial oxytocin receptors (Hixon and Flint, 1987), whereas P4 has an inhibitory action on both oxytocin and oestrogen receptor gene expression during the early and mid luteal phase of the oestrus cycle (Wathes and Hamon, 1993). The effects of P4 on OTR gene expression may be indirect through suppression of oestradiol receptors although the precise molecular mechanism whereby P4 suppresses oestradiol receptor gene transcription is still unknown (Spencer *et al*, 2004). However, the effect of P4 is temporary and after 8-10 days of continuous exposure of the uterus to P4, P4 itself down-regulates its own endometrial receptors thus allowing a rapid increase in expression of oestradiol receptor. Oestradiol then raises endometrial oxytocin receptors and initiates luteolysis

(Spencer *et al*, 1995). Thus, P4 is involved both in suppressing and then inducing the endometrial luteolytic mechanism during the oestrus cycle (Spencer *et al*, 2004).

The situation, however, may be more complex than this. Some evidences suggest that oestradiol is not essential for the initial development of oxytocin receptors in the luminal epithelium as their up-regulation occurs before any detectable change in oestrogen receptor concentration in sheep (Whates and Hamon, 1993). Also Robinson *et al*. (2001) observed that OTR up-regulation in luminal epithelium seems to be independent of any change in P4 or oestradiol plasma concentration in the cow. Furthermore, oxytocin receptors develop spontaneously in explants of endometrial tissue collected from both ewes and cows in absence of any added steroid (Sheldrick and Flick-Smith, 1993; Horn *et al*, 1998). An hypothesis arising from this findings is that oestradiol is not essential for the initial development of oxytocin receptor in the luminal epithelium, but it may just speed up the process (Leung and Whates, 2000).

What is evident from all these studies is that OTR development is crucial in determining the beginning of luteolysis, but the exact mechanism of its up-regulation is still unknown and further investigations are required, particularly in cattle.

2.1.3 Inhibition of luteolysis

Successful pregnancy establishment requires CL lifespan to be prolonged to maintain P4 production, which is essential for embryo survival and development (Mann and Lamming, 2001; Inskeep, 2004; Spencer *et al*, 2004). Likely, this is achieved either by suppressing luteolysis (antiluteolytic effects) or protecting the CL against the luteolytic action of PGF_{2α} (luteoprotective effects) (McCracken *et al*, 1999). Since the difference between cycling and pregnant cows is the presence of a conceptus in uterus, luteal maintenance probably reflects a response of CL to physiological conditions initiated by the conceptus and its products within the uterine lumen (Thatcher *et al*, 1984). Indeed, early studies in sheep (Moor, 1968) and cattle (Northey and French, 1980) indicated that proteins secreted by the conceptus, tentatively termed “trophoblastin”, appeared to be necessary components for CL maintenance as their intrauterine infusion in non-pregnant animals extended luteal function.

More recent studies have better understood the mechanisms involved in determining CL lifespan and how they are regulated.

To prevent CL destruction, luteolytic pulses of PGF_{2α} must be suppressed and the main factor responsible for this is a protein secreted by the expanding trophoblast, named

interferon tau (IFN τ) (Asselin *et al*, 1997; McCracken *et al*, 1999; Spencer *et al*, 2004). IFN τ is an unusual Type I interferon which has been found in ruminants only (Goff, 2002). It is secreted in large quantities by the mononucleate cells of the trophectoderm as the blastocyst begins to elongate at about days 13 in cattle with a peak production when the conceptus reaches its maximal size (Guillomot, 1995). It acts locally within the uterus to inhibit luteolytic PGF $_{2\alpha}$ secretion through both the induction of a prostaglandin synthesis inhibitor (Thatcher *et al*, 1995) and the regulation of oestrogen and oxytocin receptor expression. It has been hypothesized by Spencer and Bazer (1995) that, in the sheep, the inhibitory action of IFN τ on OTR up-regulation in the endometrium is achieved by suppressing the increase in oestrogen receptor expression. However this hypothesis is not supported by the data in cows reported by Robinson *et al*. (1999 and 2001). The presence of an embryo down-regulated oestrogen receptor but not until days 16-18 of pregnancy, whereas OTR expression started to increase on day 14 in non pregnant cows, indicating that the oestrogen receptor down-regulation did not precede the oxytocin receptor up-regulation. Bathgate *et al*. (1998) found an interferon response element in the oxytocin receptor gene promoter region in cows. Furthermore, in bovine uterine explants, IFN τ can suppress endometrial OTR expression in the absence of oestradiol *in vitro* (Leung *et al*, 2001). All these findings suggest that, in cows, IFN τ may suppress expression of the oxytocin receptor gene directly (Robinson *et al*, 2001).

In addition to the inhibition of pulsatile PGF $_{2\alpha}$ secretion, it seems that in early pregnancy CL is more resistant to the luteolytic action of PGF $_{2\alpha}$ (see McCracken *et al*, 1999). PGF $_{2\alpha}$ injected into follicles of early pregnant sheep caused luteolysis in only 17% of the animals as compared with 83% in non pregnant sheep undergoing the same treatment (Inskeep *et al*, 1975). Furthermore, the amount of PGF $_{2\alpha}$ required to induce luteolysis in pregnant sheep was more than that required in non-pregnant animals (Silvia and Niswender, 1986). It is possible that luteotrophic factors (e.g. PGE $_2$) secreted by the blastocyst may overcome the effects of luteolysins. In support to this view, Reynolds *et al*. (1983) found that PGE $_2$ infusion into non-pregnant cows uterus delayed luteolysis.

Thus, PGE $_2$ may be one of the factors that protect CL from luteolysis even though the precise mechanism whereby this is achieved is still unknown. It has been proposed by Asselin *et al*. (1997) that in the cow IFN τ may interfere directly with endometrial production of prostaglandins. They found that, *in vitro*, IFN τ was able to abolish the oxytocin effect on luteolysis not by inhibiting the response of the endometrial epithelial cells but changing the major prostaglandin produced in response to oxytocin from the luteolytic PGF $_{2\alpha}$ to the luteotrophic PGE $_2$. This finding seems to be in contrast with some of the studies reported above about luteolytic mechanism regulation (Spencer and Bazer,

1995; Robinson *et al*, 1999 and 2001; Spencer *et al*, 2004) thus indicating that more investigations are required, particularly in the cow.

2.1.4 Maternal recognition of pregnancy

IFN τ is the main and most important antiluteolytic and luteoprotective signal and its production is essential for pregnancy establishment, representing the first step of the maternal recognition of pregnancy process.

Numerous studies (see Stronge *et al*, 2005) have investigated the relationship between maternal hormonal environment and embryo development during early pregnancy, as the degree of embryo development seems to be closely dependent on an appropriate maternal hormonal pattern. At this stage complex interactions between the endometrium, ovary and embryo begin to develop (figure 2.1). On one hand, the conceptus is able to interfere with maternal physiology very early in pregnancy by producing growth factors, steroids, prostaglandins and cytokines. On the other hand, secretions from endometrial glands induced by ovarian P4 are essential for an adequate embryo development and consequently producing IFN τ in sufficient quantities (Goff, 2002).

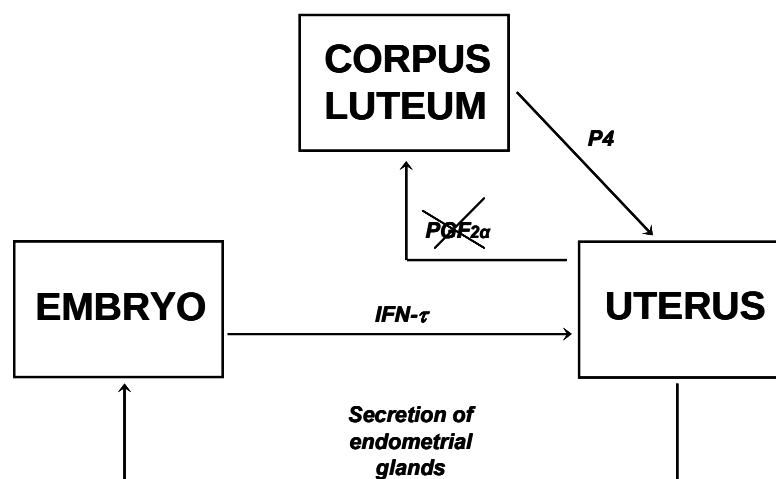


Figure 2.1 Establishment and maintenance of pregnancy model. P4 secreted by CL acts upon the uterus to stimulate the secretion of endometrial glands, necessary for embryo survival and development. Embryo signals its presence to the mother by the secretion of IFN τ which acts within the uterus to inhibit PGF $_{2\alpha}$ production. Luteolysis is thus prevented and P4 production maintained.

During the pre-attachment period, the development of the conceptus has to be synchronous with the changes in uterine environment, in particular with changes in uterine secretions that are critical for embryo survival. Several embryo transfer experiments have indicated that the uterus must be approximately in phase with the embryo for the transfer to be successful: development of embryos is retarded when transferred to uteri that are in earlier stages of the oestrus cycle, whereas development is accelerated when embryos are transferred to “older” uteri (Moore and Shelton, 1964; Lawson *et al*, 1983).

Progesterone is the main factor involved in maintaining synchrony between the conceptus and the endometrium (Mann and Lamming, 2001). The time of the post-ovulatory rise in plasma P4 and its concentration in early luteal phase are responsible for both the changes in uterine secretions (Lawson and Chaill, 1983) and IFN τ secretion (Mann and Lamming, 2001). In this latter study, cows with poorly developed embryos on day 16 produced little or no IFN τ and exhibited both a delayed increase in P4 concentration after ovulation and a lower luteal phase plateau than did cows with well developed embryos. Also Kerbler *et al*. (1997) and Whates *et al*. (2003) found a strong positive correlation between P4 concentration and synthesis of IFN τ by conceptus which suggests that increasing P4 concentration in the cow is beneficial to embryonic development. However, also high P4 concentration in early luteal phase (day 4) seems detrimental to embryo survival, likely because it creates an advanced or asynchronous uterine environment (Starbuck *et al*, 1999; Stronge *et al*, 2005). Probably both sub- and supra-optimal concentrations of P4 in early luteal phase impair embryo survival.

In addition to the antiluteolytic and luteotrophic actions, IFN τ aids to embryo survival through its antiviral, immunosuppressive and antiproliferative properties. Its antiviral activity could potentially serve to protect the peri-implantation conceptus from local viral infection (Pontzer *et al*, 1988). IFN τ also suppresses the maternal immunoresponse to embryo antigens thus allowing conceptus survival (Pontzer *et al*, 1991). The antiproliferative effects may function in the early pregnant uterus to slow growth of the endometrium during the period when the conceptus is elongating in order to establish contact with a high percentage of the uterine luminal epithelium (Pontzer *et al*, 1991). The more the conceptus stays in contact with the endometrium the best it develops. A rapid elongation of the trophoderm is in turn associated with increased secretion of IFN τ (Goff, 2002). IFN τ also stimulates proteins secretion from the endometrium (Emond *et al*, 2000) which in turn can increase IFN τ secretion from the embryo (Imakawa *et al*, 1997).

Also P4 plays other important roles that allow embryo survival in early stage of pregnancy. For many years now, P4 action in suppressing the local maternal immune response

against paternal antigens present on the trophoblast is well established (Siiteri and Stites, 1982; Hansen, 1998). Blood flow to uterine horn containing the conceptus increases two to three times at approximately Days 16-17 and either maternal or embryonic P4 seems to play a major role in regulating this process (Thatcher *et al*, 1984). This indicates once again that the conceptus is physiologically active in signalling its presence to the mother and P4 acts by connecting this two interdependent systems.

In conclusion, the successful recognition of pregnancy in the cow relies on an adequate embryo development and IFN τ production which, in turn, is dependent on an appropriate pattern of P4 secretion after ovulation (Mann and Lamming, 2001).

2.1.5 Establishment of pregnancy

2.1.5.1 Implantation

Implantation is a critical step in pregnancy establishment as the conceptus has to acquire a fixed position within the uterus thus allowing the development of the placental structures. This process involves structural and functional modifications in both the uterine endometrium and trophoblast so that to ensure a close cell adhesion between the two tissues throughout pregnancy (Guillomot, 1995).

According to Guillomot (1995), in ruminants, implantation is characterized by three main steps: a long pre-attachment period, an apposition stage and an adhesion stage (figure 2.2). In cows, the pre-attachment period lasts 2-3 weeks during which the conceptus sheds of the zona pellucida (day 10), elongates (by day 13) and orients itself within the uterine lumen.

During the apposition stage, which begins by day 19-20, contacts between trophoblastic cell membrane and the tips of the microvilli of the uterine cells are established. In this phase, both trophoblast cells surrounding the embryo and endometrial tissue undergo profound morphological and functional changes. At endometrial level, increase of vascular permeability, stromal oedema and crumpling of luminal surface of caruncles occur, while on the trophoblast apposed to the intercaruncular endometrium small papillae develop and project into the openings of the uterine glands (Guillomot and Guay, 1982). These processes facilitate immobilization and subsequent apposition between the trophoblast and the uterine epithelium (Guillomot, 1995).

At day 21-22 the contact between trophoblast and endometrium is reinforced by the interdigitation of uterine microvilli within cytoplasmic projections of trophoectoderm cells. As the adhesion proceeds, formation of syncytium occurs as a consequence of fusion of the maternal columnar epithelial cells and the conceptus trophoblastic binucleate cells (Whates and Wooding, 1980). It represents the most invasive phase of implantation in ruminants. This is the adhesion stage which ends the implantation process and gives rise to the cellular structure of an epithelio-chorial placenta.

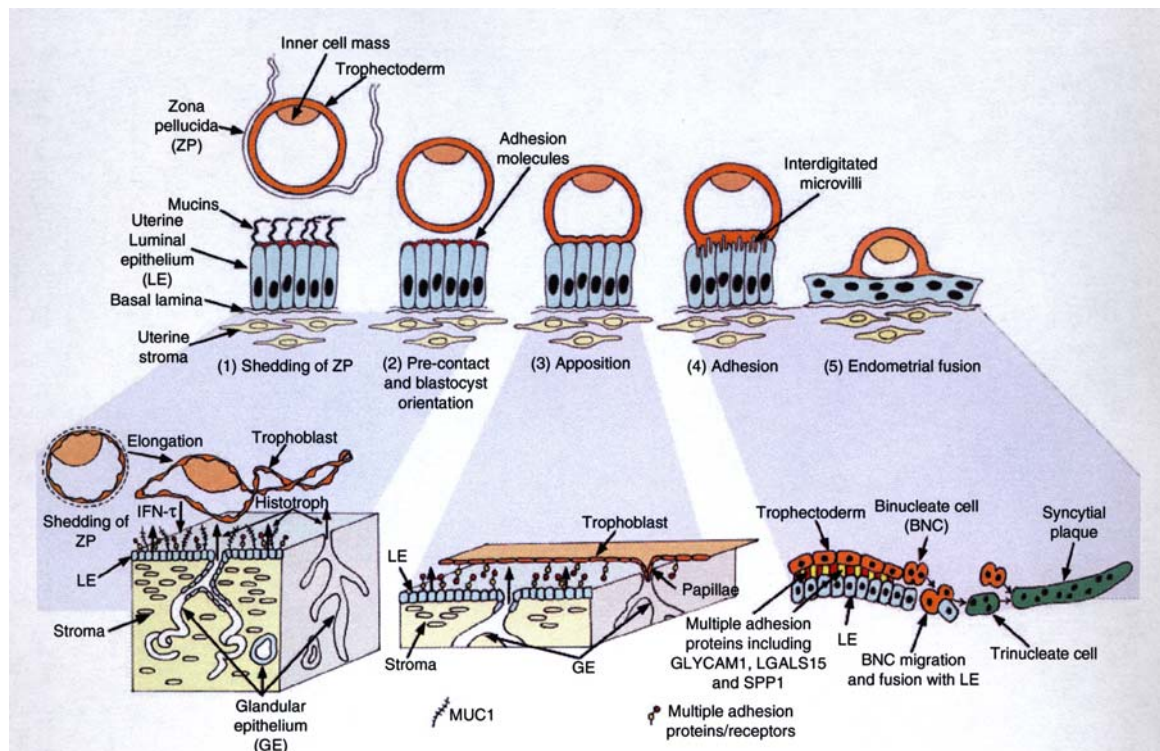


Figure 2.2 Phases of blastocyst implantation in ruminants. From Spencer *et al*, 2007.

2.1.5.2 Placenta

The ruminant placenta is classified as cotyledonary and synepitheliochorial on the basis of its gross anatomical features and histological characteristics, respectively (Wooding, 1992). “Synepitheliochorial” describes the foetal-maternal syncytium formed by the fusion of trophoblast binucleate cells and uterine epithelial cells, whereas “cotyledonary” refers to the gross structures of placenta, the placentomes, generated by fused foetal (cotyledons) and maternal (caruncles) tissue.

Ruminants endometrium consists both of aglandular caruncular areas, which are the sites of superficial implantation and placentation, and glandular intercaruncular areas which

glands synthesize and secrete a complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins, and other substances, termed histotroph or “uterine milk” (Amoroso, 1952; Bazer, 1975; Ing *et al*, 1989; Gray *et al*, 2001a).

During the blastocyst stage, the embryo develops a fluid-filled cavity, the blastocoel, which is surrounded by a single layer of cells known as the trophoectoderm (Igwebuiké, 2006). Two morphologically and functionally distinct cell types have been recognized in the trophoectoderm of ruminant placenta: the mononucleate trophoblast cells and the binucleate trophoblast cells (Wimsatt, 1951).

The mononucleate trophoblast cells represent about four-fifths of the trophoblast population and they are primarily involved in maternal recognition of pregnancy and nutrient exchange (Igwebuiké, 2006). Indeed, they are responsible for IFN τ production (Roberts *et al*, 1996) and the presence of microvilli on their apical membranes provides an increased surface area for absorption of histotroph.

The binucleate trophoblast cells represent about 15-20% of trophoectodermal cells and they are thought to arise from mononucleate trophoblast cells by consecutive nuclear divisions, the second without subsequent cytokinesis (Wooding, 1992). The great majority of binucleate cells contain two nuclei, but mononuclear and trinuclear forms have been reported (Klisch *et al*, 1999) so that sometimes they are referred as “Trophoblast Giant Cells” (TGC).

The binucleate trophoblast cells have two main functions: formation of syncytia during the adhesion phase of implantation and synthesis and secretion of protein and steroid hormones. The binucleate cells migrate to the foeto-maternal interface and fuse with uterine epithelium cells to form trinucleate foeto-maternal hybrid cells (Wooding, 1992). Continued binucleate cells migration and fusion with trinucleate cells between day 19 and 28 lead to formation of multinucleate syncytial plaques, also known as multinucleate giant cells. In the cow, these syncytia are transient structure as they are present at implantation only becoming displaced by overgrowth of mononuclear uterine epithelial cells (Whites and Wooding, 1980). No structural function has been ascribed to the syncytial plaques, but they are thought to be important in reducing the distance between maternal and foetal blood capillaries allowing a better delivery of conceptus binucleate cells products to maternal circulation (Wooding, 1984). The main binucleate cells products are placental lactogen (PL), steroid hormones (P4 and oestradiol), prolactin-related protein and pregnancy associated glycoproteins (PAG). These hormones are important factors controlling uterine gland morphogenesis, differentiation and functions, necessary for embryo nutrition and survival in utero (Igwebuiké, 2006).

2.1.5.3 Regulation of implantation and placenta formation

Establishment and maintenance of pregnancy require reciprocal communication between the mother and embryo by means of integration of endocrine and paracrine signals from the ovary, conceptus and uterus (Spencer *et al*, 2004).

As implantation progresses, important morphological and functional modifications in uterine environment occur. The uterus grows and remodels considerably to accommodate conceptus development and growth (Spencer *et al*, 2004). In particular, endometrial glands undergo extensive hyperplasia and hypertrophy in the pre-attachment period (Gray *et al*, 2001a). At this stage, indeed, embryo survival and nourishment are strictly dependent on uterine secretions, so an adequate endometrial adenogenesis is necessary (Gray *et al*, 2001b).

A servomechanism has been proposed to regulate endometrial gland development and function which involves sequential actions of ovarian steroid hormones, pregnancy recognition signals, and lactogenic hormones from the pituitary or placenta (Chilton *et al*, 1988). For example in the sheep, PL secreted by binucleate trophoblast cells seems to play an important role in stimulating endometrial gland morphogenesis as its receptors are expressed exclusively by uterine glandular epithelium and increase throughout gestation (Stewart *et al*, 2000). Moreover, the coordinated actions of PL and P4 stimulate production of uterine milk proteins and osteopontin (OPN) (Spencer *et al*, 2004). Uterine milk proteins, as major part of the histotroph, have an unequivocal role in embryo nourishment (Ing *et al*, 1989) while OPN is a glycoprotein which is essential for implantation and placentation, since it promotes cell adhesion, spreading and migration by binding integrins expressed on endometrial luminal epithelium and trophoblast (Johnson *et al*, 2003). OPN is also involved in signal transduction at the uterine-placental interface, it stimulates immunoglobulin production by B cells and promotes migration of immune cells (Johnson *et al*, 2003).

Both uterine milk proteins (Ing *et al*, 1989) and OPN (see Johnson *et al*, 2003) productions are tightly regulated by P4. A recent study showed that OPN expression required an interaction between P4 and its receptors (PR) even though P4 induced OPN expression only in those cells that lacked PR (Johnson *et al*, 2000). This result suggests that epithelial PR down-regulation in uterine luminal and glandular epithelium just prior implantation is required for up-regulation of endometrial gland secretory function. Since PR continues to be expressed in stroma and myometrium, it has been suggested that P4 may regulate OPN expression through both the down-regulation of its own epithelial receptors and regulation of growth factor(s) produced by the stroma or myometrium

(progesterone) that mediates P4 effects on glandular epithelium (Johnson *et al*, 2000). Nevertheless, how does this mechanism work is still not entirely clear and further investigations, particularly in the cow, are required.

Collectively, these studies show how establishment and maintenance of pregnancy are complex processes that consist of important morphological and functional modifications of both the endometrium and conceptus. These modifications need to be finely regulated in order to maintain synchrony between uterine environment and conceptus development. Although by now it is well established that P4 plays a pivotal role in the regulation of luteolytic signals, maternal recognition of pregnancy mechanisms, endometrial morphogenesis, uterine gland development and secretions, and conceptus growth and development, many of these events are still poorly understood. Therefore, investigations aimed to elucidate how the most critical steps of early pregnancy work may help to prevent pregnancy losses.

2.2 Embryo mortality

2.2.1 Definition and incidence of embryo mortality

The last few decades have seen a world-wide decline in reproductive efficiency of dairy cattle (for review see Lucy, 2007). Infertility can be broadly defined as any condition leading to failure to establish a pregnancy following completion of uterine involution at 40-50 days postpartum (Royal *et al*, 2000). Failure to establish a pregnancy at the expected time may reflect a number of pathological conditions affecting cow endocrine system or conceptus development (Royal *et al*, 2000). This has a major impact on dairy economics, largely due to an extended calving interval, lost income from milk sales, feed and semen costs and increased culling (Royal *et al*, 2000; De Vries, 2006).

Pregnancy loss is probably the major source of economic wastage for the modern dairy system. It has been estimated that, in the USA, the average cost of a pregnancy loss is \$ 555 with a maximum of \$1,373, depending on the stage of lactation and the relative milk yield (De Vries, 2006). For this reason, it is not surprising that, over the past few years, many researches have been trying to investigate pattern, extent, causes, and mechanisms of embryonic mortality (EM).

In order to properly standardize bovine EM terms, the Committee on Bovine Reproductive Nomenclature (1972) established that the embryonic period of gestation extends from conception to the end of the differentiation stage, i.e. approximately at day 42 of gestation, while the foetal period extends from day 42 to the end of gestation. This allows to differentiate between embryo mortality (within day 42 of gestation) and foetal loss (after day 42 of gestation), but the difficulty to differentiate, on a scientific basis, between early and late EM still remains. An attempt has been made by Humblot (2001) who has classified pregnancy losses prior to day 24 as early embryonic mortality (EEM) and those between days 24 and 42 as late embryonic mortality (LEM) on the basis of results obtained by experiments aimed to investigate CL function and conceptus survival. The pattern of P4 in relation to induced embryo death at the time of corpus luteum maintenance (i.e. around day 16) suggests that when embryonic death precedes luteolysis, luteal regression is delayed and interestrus interval is extended by at least three days (i.e. 24 days). On the other hand, when luteolysis precedes, and probably causes embryonic death, returns to oestrus are likely to occur prior to day 24. Thus, based on these findings, luteolysis and return to oestrus prior to day 24 may be considered as linked to EEM, whereas corpus luteum maintenance and return to oestrus beyond day 24 could be associated to a LEM occurring at more than 16 days after AI.

However, as previously mentioned, this criterion is just an attempt and, in fact, other authors consider EEM those pregnancy losses that occur before day 21 (Goff, 2002; Green *et al*, 2005b) or day 28 (Silke *et al*, 2002; Diskin *et al*, 2006).

This lack of conformity about an unequivocal definition of EEM, LEM, or foetal loss, to some extent, makes it difficult to analyse the problem, firstly in terms of incidence assessment.

In the last few decades, indeed, a wealth of literature about incidence of embryo loss in modern dairy industry has been generated, but there is still no agreement about its real extent. Moreover, a number of studies on EM confounded the interpretation of their findings by not differentiating embryonic loss from losses due to fertilization failure (Zavy, 1994).

However, it is widely accepted that most pregnancy losses occur during the embryonic period. According to many authors (Sreenan and Diskin, 1986; Silke *et al*, 2002; McNeill *et al*, 2006) up to 80% of the total pregnancy losses occurs by about day 16 after insemination.

Collecting results from numerous studies, Humblot (2001) observed that the frequency of EEM represented 20,5 to 43,6% (average 31,6%), whereas the frequency of LEM was noticeably lower and ranged between 8 and 17,5% (average 14,7%). According to some

authors, indeed, LEM seems less likely to occur, as reported by Silke *et al.* (2002), who found an incidence of embryonic death between days 28 and 84 of gestation of about 3,2%. On the contrary, Grimard *et al.* (2006) observed a high incidence of LEM, since more than 25% of pregnant cows on days 21-24 lost their conceptus later. Even higher LEM incidence (42,7%) was found by Cartmill *et al.* (2001) in their study of high producing cows under heat stress.

In an effort to reconcile these differences, Santos *et al.* (2004) reviewed quite a number of studies regarding late (after day 27) embryo mortality in dairy cattle and they came to the conclusion that the average rate of late pregnancy loss on modern dairy industry can be considered of 12,8% (ranging from 3,2% to up to 42,7%).

What appears clear looking at all these data is that one of the main barrier for a correct EM incidence assessment is the time when pregnancy diagnosis is performed. Indeed, we can talk about embryo loss when, after a first positive pregnancy diagnosis, the cow is diagnosed not pregnant at subsequent examinations. Therefore, timing and accuracy of pregnancy diagnosis are to be considered the key steps for a correct assessment of EM.

In the last two decades early examination for pregnancy by means of proper methods has improved so much the approach to EM that it is impossible now to compare historical data on embryonic loss with recent estimates. Most of recent estimates, indeed, are based on early pregnancy diagnosis performed mainly by ultrasound technology, which was not routinely used in cattle before 1980s (Lucy, 2001). The result are important differences in terms of EM assessment: a 10% estimated only by nonreturn to oestrus and cyclic milk P4 pattern (Kummerfeld *et al.*, 1978) can hardly be compared to the current 30-40%, as reported above.

2.2.2 Causes of embryo mortality

Pregnancy is not a completely harmonious relationship, in which the conceptus and the uterus function together to achieve a common target, that is the successful outcome of the pregnancy. The mother, in fact, provides barriers that the embryo must surmount for the pregnancy to continue. The embryo has to demonstrate to the mother to be capable of executing its developmental program since in the early stages of pregnancy: resources are not to be risked on progeny that are likely to be genetically flawed or otherwise unfit (Roberts *et al.*, 1996). Moreover, because of its paternal as well as maternal genes, the conceptus is an intruder in maternal genital tract, recognized as foreign by the mother and so as a target for her immune system.

It appears, thus, that establishment of pregnancy is a conflict between mother and conceptus and factors affecting either embryonic and maternal functions or the way they communicate to each other may lead to pregnancy loss.

2.2.2.1 Embryo-dependent early pregnancy loss

Oocyte quality

Embryo's fate is dictated also by events before fertilization: factors affecting the health of the gametes may ultimately determine the ability of an embryo to survive and grow. Indeed, embryos formed from incompetent oocytes have a lower probability of successful development than embryos developing from normal oocytes.

The term *oocyte competence* refers to the potential for an oocyte to give rise to a normally developing embryo following fertilization (Hansen, 2002). Oocyte competence can be compromised by alterations in follicular dynamics. Oocytes belonging to persistent dominant follicles exhibit various morphological abnormalities (Mihm *et al*, 1999), after fertilization they give rise to zygotes which development is retarded and early embryonic death usually occurs before the 16-cells stage (Ahmad *et al*, 1995).

Cows with persistent pre-ovulatory follicles show low fertility, as reported in the review by Hansen (2002), and have altered protein secretory patterns in the oviduct (Binelli *et al*, 1999). These events may be due to the larger size and the longer lifespan of a persistent follicle. Oocytes belonging to persistent follicles are at a too advanced stage of maturation at the time of fertilization and this increases the probability to have poor quality zygotes (Mihm *et al*, 1999). Persistent follicles also produce greater quantities of estrogens than normal follicles with detrimental effects on embryo survival (Revah and Butler, 1996). This abnormal follicular dynamic may be explained by a failure of normal luteal function that affects P4 production and its regulation of LH secretion. Indeed, as reviewed by Inskeep (2004), the sequential relationship of lowP4, increased frequency of LH pulses, a persistent dominant follicle, and increased secretion of estradiolo-17 β is widely accepted as one of the causes of early embryonic death. However, neither the exact timing of an oestrogen effect nor the mechanism by which oestrogen may interfere with the developing embryo have been established.

Also inadequate follicular development reduces the ability of fertilized oocytes to develop to the blastocyst stage. Perry *et al*. (2002) observed low pregnancy rate and high embryo losses in cows induced to ovulate prematurely, likely because P4concentration rose at a

slower rate than in cows induced to ovulate later and this could have led to poorly developed embryos.

Role of spermatozoa in embryonic mortality

Investigations about pregnancy loss tend to focus on the cow and ignore the importance of the bull for the success of reproduction. Although, like the oocyte, the male gamete influences fertility by imparting characteristics to the embryo that influence its ability to proceed through development (Hansen, 2002).

The main male-derived causes of embryo loss are chromosomal abnormalities, such as nondisjunction in spermatogenesis (Logue and Harvey, 1978), 1/29 Robertsonian translocation (Dyrendahl and Gustavsson, 1979), or defective chromatin structure (Ballachey *et al*, 1988).

Intrinsic embryo factors: the heat stress example

Whether or not an embryo can survive the early stages of pregnancy depends, at least in part, on the degree to which the embryo can adjust its own physiology (Hansen, 2002). The pre-implantation embryo is most susceptible to certain types of stresses very early in its development because its genome is largely repressed. However, the embryo possesses cellular adaptive mechanisms to limit adverse effects of stress, as it occurs in the case for heat stress.

There is abundant evidence that heat stress is one of the cause that lead to EM (for review see Hansen, 2002). Heat shock induces the disruption of embryonic development by influencing several of the key events leading to successful pregnancy. First, follicular dynamics are altered during heat stress: persistent pre-ovulatory follicles (Wolfenson *et al*, 1995), lower steroidogenic capacity (Wolfenson *et al*, 1997), and a decline in oocyte competence (Al-Katanani *et al*, 2002) have been found in cows exposed to high environmental temperature. Moreover, under heat stress conditions proteins synthesis is reduced (Edwards and Hansen, 1996) and free radicals metabolism is increased (Aréchiga *et al*, 1995).

However, as development proceeds, the embryo acquires mechanisms to become resistant to elevated temperature which involve heat shock protein 70 expression (Edwards and Hansen, 1996), antioxidant systems activation (Aréchiga *et al*, 1995), and the ability to undergo apoptosis (Paula-Lopes and Hansen, 2002). Failure of any of these mechanisms to develop may lead to embryo death.

Immune system

Contributing to the high rate of embryonic mortality may be the maternal attack against conceptus antigens of paternal origin (Thatcher, *et al*, 1984). The conceptus, indeed, is recognized as an intruder by the mother but, normally, the “foreign” antigens are not a target for her cytotoxic T-lymphocytes (Roberts *et al*, 1996; Davies, 2007). How it is possible is still under investigation. In early studies, it has been proposed that trophoblast cells may either synthesize substances that act locally as an immunoregulatory “buffer zone” between the functional maternal immune system and the allogenic conceptus graft (Staples *et al*, 1983) or not express a normal complement of histocompatibility antigens on their surface (Croy, 1994). Over the past few years, the major histocompatibility complex (MHC) has been extensively studied since its downregulation on the trophoblast cells in early pregnancy seems to be essential to protect the conceptus from attack by the maternal immune system (Davies, 2007). There are two types of MHC: class I and class II. MHC class II proteins are in charge of presenting peptides derived from extracellular pathogens to CD4⁺, helper T lymphocytes. They are never expressed on trophoblast cells. MHC class I is composed of classical and nonclassical genes (Kelley *et al*, 2005). The classical MHC class I molecules generally present peptides derived from intracellular pathogens to CD8⁺, cytotoxic T lymphocytes. The nonclassical MHC class I proteins act as ligands for inhibitory leucocytes receptors. Indeed, their expression by trophoblast cells is believed to inhibit activation of uterine leukocytes in order to protect the conceptus from immune-mediated rejection (Hunt *et al*, 2005). In contrast, allografts expressing classical MHC class I proteins do not survive in uterus (Hansen *et al*, 1986) likely because these proteins have the potential to trigger foetal rejection (Davies, 2007). Interestingly, it is normal for bovine trophoblast cells to express classical MHC class I proteins during the last third of pregnancy (Davies *et al*, 2000). This unique characteristic is probably beneficial to the positive outcome of pregnancy since classical class I proteins cause a uterine inflammatory response at parturition, which contributes to placental separation (Davies, 2007).

Also PAG have been suggested to play a role in minimizing maternal recognition of paternal and foetal immune antigens thus preventing T-cells activation (Roberts *et al*, 1996).

Failing the ability of the conceptus to develop any of these defence mechanisms, an immunocompetent uterus will suppress the embryo.

2.2.2.2 Impairment of maternal-foetal interactions

Asynchrony

According to many authors (Roberts *et al*, 1996; Goff, 2002; Johnson *et al*, 2003; Green *et al*, 2005b; Stronge *et al*, 2005), one of the most important causes of EM is the asynchrony between the embryo and the mother. In the early stages of gestation, embryo development has to be synchronous with changes in uterine environment to prevent abnormalities and failure to implant. Natural asynchrony between the embryo and the mother can arise from a late onset of the first meiotic division which leads to delay in oocyte maturation (Roberts *et al*, 1996). Also delayed fertilization may result in asynchrony but, undoubtedly, the main cause of asynchrony is an altered secretory pattern of P4 (Lawson and Cahill, 1983; Mann and Lamming, 2001; Goff, 2002).

Signaling between conceptus and mother

The major cause of pre-implantation EM is thought to be difficulties in signaling between the conceptus and mother (Goff, 2002; Green *et al*, 2005b).

Failure of the conceptus to communicate its presence at the appropriate time by secreting adequate concentrations of IFN τ leads to pregnancy loss (Hansen, 2002; Igwebuike; 2006). Inadequate IFN τ production may be due to intrinsic defects of embryo (e.g. delayed or retarded trophoblast development) or adverse alterations in maternal environment (e.g. low P4 concentration in uterus) (Hansen, 2002).

Since 1986, Thatcher *et al*. hypothesized that some of the embryonic losses in cows could be due to the inability of the embryo to suppress the luteolytic cascade, likely because of a failure of the conceptus to produce luteotropic signals, or perhaps failure of the CL to respond to luteotropins. More recently, Mann and Lamming (2001) demonstrated that, indeed, inadequate IFN τ production leads to failure of luteolysis inhibition.

Also a failure of the embryo to produce steroids may compromise its own survival. It seems that steroids produced by the embryo may exert local control upon the endometrium to modify nutrient flux in order to provide for its own nutritional requirements during early pregnancy. Moreover, steroids of conceptus origin could have local effect on the endometrium to stimulate production of histotrophe or factor(s) necessary for the apposition and attachment phases of implantation (Zavy, 1994). Chiappe *et al*. (2002), using the key steroidogenic enzyme 3 β -HSD as an indicator of the embryonic steroidogenic ability, observed that only 50% of the pre-blastocyst stages showed 3 β -HSD activity, while all of the blastocysts were positive. It could be speculated that a process of selection takes place during the transformation from morula to blastocyst stage and the

acquisition of steroidogenic ability may positively influence the embryo development potential (Chiappe *et al*, 2002). Embryos that do not acquire the steroidogenic capacity do not develop and die.

Embryo mortality results also from failure of the mother to respond appropriately to embryo signals (Goff, 2002; Hansen, 2002; Green *et al*, 2005b). Embryos direct massive changes in endocrine and endometrial function of the mother in order to improve certain inadequate maternal environment (Hansen, 2002). Nevertheless, sometimes the mother fails to respond properly since, for example, luteolysis can occur in spite of the presence of a normal embryo (Lafrance *et al*, 1989).

Role of P4 in embryo mortality

The role of P4 in embryonic loss has been extensively explored and it is now widely accepted its pivotal role in establishment of pregnancy and embryo survival and development. However, the degree and timing of association between EM and P4 concentration during the early luteal phase have not been clearly established yet.

Stronge *et al*. (2005) and McNeill *et al*. (2006) found a positive relationship between the probability of embryo survival and milk P4 concentrations on day 5-7 and day 4-6, respectively. Likewise, Franco *et al*. (1987) observed that cows undergoing pregnancy loss had lower milk P4 concentration. Starbuck *et al*. (2004) found that pregnancy loss before day 45 was greater in cows with the lowest plasma concentration of P4 at day 28 to 37 of gestation.

However, an optimum or threshold blood P4 concentrations below which embryo mortality may occur are not known. It is possible that, as P4 is transferred locally from the ovary to the uterus (Weems *et al*, 1989), peripheral concentrations of P4 are less relevant for embryo survival than local (uterine tissue and lumen) concentrations (Lucy, 2001; Chagas e Silva and Lopes da Costa, 2005).

There are many potential mechanisms by which low P4 concentrations may compromise embryo survival since it is essential to regulate the most critical steps of pregnancy establishment and embryo development, as previously described in this review. Low P4 may lead to asynchronous uterine environment (Mann and Lamming, 2001), lower uterine secretions (Goff, 2002), failure to inhibit uterine immune response against the conceptus (Hansen, 1998), excessive concentrations of other hormones that may affect either oocyte quality or embryonic development (Inskeep, 2004), lower antiluteolytic signal (Mann and Lamming, 2001).

Reviewing the causes of reduced P4 concentration in the cow is beyond the scope of the present thesis. However, it can be hypothesised that low P4 concentrations can be due to

reduce production and secretion by CL or to increased metabolism and clearance of secreted P4 by the liver (Lucy, 2001; Inskeep, 2004). Indeed, Sansritavong *et al.* (2002) have clearly demonstrated that peripheral concentrations of P4 can be lowered by increased liver blood flow as a result of high feed intake in high producing dairy cows.

2.2.2.3 Mother-dependent early pregnancy loss

Uterine environment

Defects in endometrial adenogenesis or function during the peri-implantation period may be a cause of EM (Gray *et al.*, 2001a). Unequivocal role of secretions of endometrial glands as primary regulators of conceptus survival and development, onset of pregnancy recognition signals, and implantation is well established (Amoroso, 1952; Bazer, 1975; Gray *et al.*, 2001b). Uterine secretions are thought to be particularly important for conceptus survival and development in ruminants because of the prolonged period of preimplantation conceptus development that precedes superficial attachment and placentation (Gray *et al.*, 2001a). Therefore, it appears clear that a failure of the endometrial glands development or the servomechanism supposed to regulate endometrial function in early stages of pregnancy can lead to EM.

Also uterine pH is thought to play an important role in determining whether the embryo might survive and develop or not. There is abundance of evidence that low pH in the uterine luminal fluid has detrimental effects on embryo survival and reproduction in general (Elrod and Butler, 1993; McEvoy *et al.*, 1997; Rohads *et al.*, 2004; Meza-Herrera *et al.*, 2006). It has been suggested that the decrease of uterine pH caused by an excess of protein supplementation may influence endometrial secretory activity, creating a hostile or suboptimal environment for embryo development and, thus, compromising the establishment of pregnancy (Elrod and Butler, 1993; Meza-Herrera *et al.*, 2006).

Nutrition and metabolic status

Although the relationship between nutrition and reproduction is complex and responses are often quite variable and inconsistent, it is widely accepted that nutritional status of the animal affects reproductive function in dairy cattle. In particular, loss of body condition that occurs in the early post-partum seems to affect embryonic and foetal survival. Silke *et al.* (2002) noticed that cows that lost body condition during days 28-56 of gestation had a higher rate of embryonic loss (11.6%) compared with cows that either maintained (4.7%)

or gained (5.7%) body condition during the same period. Likewise, López-Gatius *et al.* (2002) observed that a 1 unit decrease in body condition score (BCS; 1-5 scale) from calving to 30 days postpartum resulted in a 2.4 fold increase in pregnancy loss. They have hypothesized that the excessive loss of body condition during early lactation may impair uterine involution with negative consequences on placentation. Another hypothesis is that an abrupt loss of nutritional status may act indirectly by increasing the odds ratio for metabolic and infectious diseases with consequent detrimental effects on embryo survival (Heuer *et al.*, 1999).

Also an excess of energy intake may lead to an increase of embryo mortality. Excess rumen degradable protein elevates urea concentration both in plasma and uterus with detrimental effects on oocyte quality (Armstrong *et al.*, 2001) and embryo survival (McEvoy *et al.*, 1997). High urea concentration in uterus, indeed, contributes to decrease uterine luminal pH thus creating a uterine environment that is hostile to the embryo.

In conclusion, although it is widely accepted that nutritional status can affect embryo survival in the early stages of development (see Boland and Lonergan, 2003), the mechanisms by which nutrition impairs early embryonic development remain to be established. Indeed, it is not clear if nutrition affects embryo quality through changing the developmental capacity of the oocyte or through changes occurring during embryo development.

Milk yield

Role of milk yield on EM is still controversial. According to Diskin *et al.* (2006), a high proportion of embryo recovered from high producing dairy cows can be classified as low quality grade or abnormal or “retarded” embryos. Also Vasconcelos *et al.* (1997) and Grimard *et al.* (2006) found that the incidence of LEM was higher in high than in low yielding cows.

In contrast, López-Gatius *et al.* (2002) and Chebel *et al.* (2004) found no significant relationships between milk production and LEM. The same result has been observed by Silke *et al.* (2002) who failed to detect any significant association among various measures of milk production or milk composition and LEM.

However, most of these studies refers to late embryonic death suggesting that the effects of milk production on embryo survival, if any, are more likely to affect the early embryo rather than the late embryo stage.

Pathological conditions

Bacterial, viral, and mycotic infections can cause EM, as exhaustively reviewed by Zavy (1994) to whom the reader is referred for details since every single infectious agent may act by different pathogenetic mechanisms and the description of the role of infectious diseases in causing embryonic losses is beyond the scope of this thesis.

The inflammatory status that characterises disease occurrence is thought to affect embryo wellbeing through several putative mechanisms. As inflammation is associated with oxidative stress, a thorough discussion of the effects of inflammation on embryonic death will be done in chapter 2.4.4.

2.2.3 Diagnosis of embryo mortality

There are several techniques to perform pregnancy diagnosis in dairy cattle, some of them are not ordinarily used because outdated or unable to give an early diagnosis (e.g. estrone sulphate which is not reliably detectable until around day 80 of gestation (Heap and Hamon, 1979)).

The ideal pregnancy test should be 1) sensitive (i.e. correctly identify pregnant animals), 2) specific (i.e. correctly identify non-pregnant animals), 3) inexpensive, 4) simple to perform under field condition, and 5) able to determine pregnancy status without the need to physically handle the animal (Fricke, 2008).

Although inaccurate diagnosis of pregnancy (i.e. false positive) is undesirable, its consequences are less severe compared with inaccurate diagnosis of non-pregnancy (i.e. false negative), since it increases the rate of iatrogenic embryonic death when PGF_{2α}, or one of its analogue, is administered to induce oestrus to reduce the interval to the next AI service (Romano *et al*, 2006).

The last part of this chapter briefly describes some of the methods for pregnancy diagnosis in dairy cattle that are currently available or under development and their efficacy to determine EM.

2.2.3.1 Return to oestrus

Widely used for a long time (Nebel, 1988), return to oestrus from 18 to 24 days after AI is still often considered by farmers the easiest and least costly method for determining non-pregnancy in dairy cattle. However, this method has many limitations.

First of all, oestrus detection efficiency has decreased noticeably and it has been estimated to be less than 50% (Senger, 1994). This is likely a result of increased herd size (Senger, 1994) and shorter and poorly displayed oestrus behaviour (Dransfield *et al.*, 1998). Moreover, oestrus cycle duration varies widely among lactating dairy cows making it difficult to target the detection of return to oestrus for groups of animals receiving AI on the same day (Sartori *et al.*, 2004). This may be due, at least in part, to LEM which may increase the interval from insemination to return to oestrus.

2.2.3.2. *Palpation per rectum*

Palpation per rectum is still now the most widely used methods for pregnancy diagnosis in dairy cattle. Nevertheless, its use to perform pregnancy diagnosis is still controversial. On one hand, its widespread use, high accuracy, and relatively low cost per animal make it the method of choice for pregnancy diagnosis (Fricke, 2008). On the other hand, evidences have proved that palpation per rectum can be a iatrogenic cause of EM, even though controversy still exists regarding the extent of iatrogenic EM induced by transrectal palpation. In an early study, Franco *et al.* (1987) found that pregnancy diagnosis by transrectal palpation from days 42 to 46 of gestation caused an increase in foetal deaths ranging from 7,5% to 11,4%. Similar trend was observed by Oltenacu *et al.* (1990) with an increase of EM from a baseline value of 10% to 12% as a result of uterine palpation at day 35 of gestation.

This is thought to be due to the physical handling of embryonic structures, as investigated by Vaillancourt *et al.* (1979). They observed that EM rate increased the more the embryonic structures were touched: 3,8% in pregnancy diagnosis performed by means of palpation of foetal fluid fluctuation only, 6% by palpation of foetal fluid fluctuation and of the amniotic vesicle, and 9,1% by palpation of foetal fluid fluctuation and membrane slip. However, since the risk of EM is high during the period of gestation when cows are diagnosed pregnant by transrectal palpation, it is hard to distinguish between iatrogenic losses occurring due to transrectal palpation and spontaneous losses that would normally have occurred in these cows (Fricke, 2008).

.2.3.3 Transrectal ultrasonography

Transrectal ultrasonography offers several potential advantages over palpation per rectum, first of all an earlier and more accurate diagnosis of pregnancy.

As reported by Fricke (2002), ultrasounds allow rapid and accurate diagnosis as early as 26 days post AI with a sensitivity and specificity that reach 97,7% and 87,7% respectively, when conducted between 26 and 33 days post AI. Some authors, however, disagree about the accuracy that can be reached at this time since a high rate of false negative has been found in many studies (from 2,3 to 31,2%; see Romano *et al*, 2006). Also false positives have been reported using ultrasounds but in most cases they were due to embryonic mortality rather than to low specificity of the method (Romano *et al*, 2006).

Ultrasonography is also a less invasive technique for early pregnancy diagnosis than is transrectal palpation. It has been reported as not affecting embryonic viability probably because the direct physical manipulation of the gravid reproductive tract is not necessary (Baxter and Ward, 1997).

Besides accuracy, precocity, rapidity, and the immediate outcome of the test at the time the test is conducted, one of the most important applications of ultrasonography in cattle is the possibility to evaluate, at least in part, embryo/foetal wellbeing. Ultrasound imaging of bovine embryos allows reliable detection of a heartbeat as early as d 22 after breeding which, in turn, allows an assessment of embryo viability (Beal *et al*, 1992). However, on the field the 22 day interval is considered very brief to properly perform a pregnancy diagnosis. Kastelic *et al*. (1988) observed an apparent reduction in embryonic heart rate prior to embryonic death in heifers and, thus, suggested that embryo heartbeat could be considered a good indicator of embryonic distress.

2.2.3.4 Milk and plasma progesterone profile

Determination of milk P4 concentration is widely accepted as a reliable method of pregnancy diagnosis at 21 to 24 days post AI in dairy cows (Franco *et al*, 1987). The main advantages are: i) milk or plasma P4 determination is easy to perform either by radioimmunoassay (RIA) in specialized laboratories or by enzyme-linked immunosorbent assay (ELISA) which can be easily performed on-farm using commercial kits. ii) according to Oltenacu *et al*. (1990), on-farm milk P4 at d 19 is economically the most profitable method for pregnancy diagnosis; iii) milk P4 is the earliest proven method for identifying

non-pregnant animals post breeding with a specificity reported to be around 98% at 18 to 24 days after breeding (Fricke, 2008).

The main disadvantage of P4 assay for pregnancy diagnosis is its low sensitivity, which varies from 60 to 96% (Franco *et al*, 1987; Nebel, 1988). High P4 concentrations during the period when the test is normally performed (i.e. between day 18 and 24 post breeding) is not a specific indicator of pregnancy due to variation among cows in oestrus cycle duration (Sartori *et al*, 2004), or pathological conditions that prolonged CL lifespan in the absence of pregnancy, e.g. luteal cyst, pyometra, and EEM (Nebel, 1988; Fricke, 2008)

Franco *et al*. (1987) attributed the 20% false positive diagnosis they obtained by milk P4 assay to early embryonic death and piometra.

It can be concluded, as proposed by Nebel (1988), that milk or blood P4 is a better indicator of non-pregnancy and ovarian activity than of pregnancy status.

2.2.3.5 Pregnancy associated proteins

Proteins produced and secreted specifically by placenta early during pregnancy are obvious candidates for development of an early pregnancy test.

Trophoblastic proteins originating from bovine binucleated cells, such as pregnancy-specific protein B (PSPB) (Butler *et al*, 1982) and bovine pregnancy associated glycoprotein (bPAG) (Zoli *et al*, 1992), thus represent the ideal antigen for an indirect early pregnancy test in dairy cattle.

Development of a specific RIA for PSPB (Sasser *et al*, 1986) and for bPAG (Zoli *et al*, 1992) offered the possibility to study their secretory patterns during pregnancy or in case of EM. In pregnant cows, mean PSPB concentrations begin to increase from 15 to 35 days after breeding and rise continuously as pregnancy advances. However, very important individual variation exists, especially when the assay is performed at the beginning of pregnancy leading to observe this rise only by day 30 in some cases (Humblot, 2001). Sensitivity has been reported as ranging between 90 and 92% and specificity between 82,6% and 99,5% (Szenci *et al*, 1998; Humblot, 2001).

Similar profiles have been described for bPAG-1 which can be easily detected in maternal serum as early as day 29 to 30 onward with a sensitivity and specificity of 95,2 to 100% and 56,7% to 79%, respectively (Szenci *et al*, 1998). At 35 days after insemination the overall accuracy of bPAG-1 as pregnancy test has been reported being 94,65% (Zoli *et al*, 1992).

Although their function is not completely clear, PAG can be considered good indicators of embryo/foetal wellbeing as, in case of LEM, their production and secretion start to decrease steadily within 24 hours after embryo death, reaching non-pregnant levels within 15 (Humblot, 2001) or 6 days (Szenci *et al*, 2003).

Although pregnancy can be detected early after breeding, the presence of PAG in maternal serum for nearly 100 days postpartum constitutes a problem for diagnosis of pregnancy during the subsequent lactation whether rebreeding occurs at less than 80 days postpartum (Zoli *et al*, 1992; Mialon *et al*, 1993).

For this reason, research has been focusing towards new pregnancy test that measures PAG in milk, since the decrease of PAG concentration after parturition is reported to be faster and more accentuated in milk than in blood (Metelo *et al*, 2004). In dairy goats, for example, PAG milk-test provides an accurate pregnancy diagnosis from day 32 after breeding and it would be suitable under farm conditions due to facility of milk sampling, its ability to diagnose early pregnancy and to evaluate foeto-placental wellbeing (González *et al*, 2001). However, studies in this regard in dairy cows are still lacking.

2.3 Pregnancy-associated glycoproteins (PAG) in cattle

2.3.1 Origin and characteristics of PAG

Ruminant placenta synthesises substantial amounts of proteins related to pregnancy. Among these, a polymorphic family of pregnancy-associated glycoproteins (PAG) has been discovered in the last 25 years (Butler *et al*, 1982; Sasser *et al*, 1986).

PAG are also known under a variety of other names including pregnancy-specific protein B (PSPB), and pregnancy-specific protein 60 (PSP-60) since they have been reported to have similar, if not identical, amino-terminal amino acid sequences (Xie *et al*, 1991).

Pregnancy-specific protein B (PSPB) was characterized and partially purified for the first time in 1982 from bovine placental tissue (Butler *et al*, 1982). Although initially it was believed that there was only a single PAG isoform, it has become increasingly clear that there are possibly more than 100 PAG genes in ruminants encoding for several proteins (Green *et al*, 1998). Indeed, PAG are a complex family with cDNA coding for 22 distinct bovine PAG molecules: boPAG-1 to boPAG-22 (PAG are numbered in the order they were discovered) (Xie *et al*, 1997; Green *et al*, 2000). Even PSPB is thought to represent

a mixture of related proteins since multiple molecular weight forms (47000-90000) and isoelectric variants (pI 3.7-4.4) have been reported (Butler *et al*, 1982; Sasser *et al*, 1989). Many distinct PAG have been identified so far, all belonging to a family of proteolytic enzymes known as aspartic proteinases (Xie *et al*, 1997). Indeed, they have more than 50% amino acid sequence identity with pepsin and cathepsin D and E (Sousa *et al*, 2006) although they are probably not proteolytically active (Xie *et al*, 1991).

PAG are synthesised by the mono- and binucleate trophoblastic cells and some of them are secreted in maternal blood from the moment when the conceptus becomes more closely attached to the uterine wall and formation of placentomes begins (Wooding, 1992). They can be separated empirically into two main groups: one evolutionary more ancient (PAG-2 subgroup), largely localised at the placental foeto-maternal interface in all cotyledonary trophoblast (i.e. both mono- and binucleate cells), and one produced by a more recent series of genes (PAG-1 subgroup), expressed primarily in trophoblastic binucleate cells (Green *et al*, 2000).

2.3.2 PAG profile during gestation

In most pregnant cows PAG are detectable in the maternal circulation from days 24-25 after fertilization (Perenyi *et al*, 2002b). This period coincides with the time that the trophoblast attaches firmly to the uterine wall and when placentation begins (Roberts *et al*, 1996). PAG concentration increases gradually until Day 240 of gestation, and then more dramatically during the few days before delivery, reaching maximum levels around parturition (Zoli *et al*, 1992; Patel *et al*, 1997). Indeed, the mean PAG concentration between Days 20 and 10 prepartum approximately triples, but between days 10 prepartum and parturition, PAG values increase about threefold in singleton and fivefold in twin-bearing cows, with peak concentrations three days before parturition and on the day of parturition, respectively (Patel *et al*, 1997).

PAG are not expressed co-ordinately throughout pregnancy, some being expressed early, others only as pregnancy progresses (Green *et al*, 2000) (figure 2.3).

In particular, in the bovine species, two different patterns of expression were found: those PAG that are expressed predominantly in binucleate cells (PAG 1-subgroup) are invariably absent in term placenta, whereas those PAG expressed more uniformly throughout trophoblast (PAG 2-subgroup) are detectable at all stages of pregnancy (Green *et al*, 2000). For example, boPAG-9 is expressed predominantly in early pregnancy, being detectable at Day 25 and declining as pregnancy progresses until being

undetectable at term. On the contrary, boPAG-1 is not detectable at Day 25 but becomes prevalent at later stages, although it is absent in term placenta. Bovine PAG-2, -8, -10, and -11 are detectable throughout gestation and they are the only PAG present in term placenta (Green *et al*, 2000).

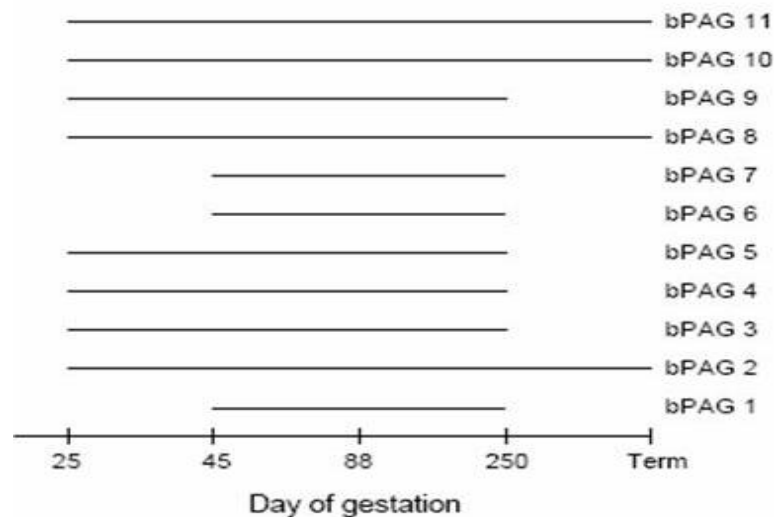


Figure 2.3 Diagrammatic representation of bovine PAG expression throughout pregnancy. The expression of specific bovine PAG are displayed as solid lines representing the stages of pregnancy at which a full-length protected probe can be detected by ribonuclease protection assay. Adapted by Green *et al*, 2000.

After parturition, PAG remain in the circulation for several weeks (Mialon *et al*, 1993; Haugejorden *et al*, 2006). Their concentrations decrease steadily in the postpartum period reaching undetectable levels only by Day 100 ± 20 post-partum (Zoli *et al*, 1992). The long time needed for PAG to be cleared from the maternal serum might be the result of either the very high concentration reached around parturition or the long half-life of the protein in blood (Zoli *et al*, 1992).

2.3.3 PAG function

Despite more than 20 years of study, the function of PAG in reproduction is still not completely known. In view of the magnitude of their production and their survival over millions of years of selective pressure, a lack of a role in pregnancy seems unlikely (Green *et al*, 1998).

While the enzymatic role of the aspartic proteinases, which PAG belong to, is well known, an enzymatic role of PAG seems unlikely, as at least some of the PAG have mutations likely to render them enzymatically inactive (Xie *et al*, 1991; Green *et al*, 1998; Perényi *et al*, 2002a). However, many of them can certainly bind peptide ligands. Therefore, it could be speculated that they are carrier molecules or their substrate-binding cleft may target them to specific receptors (Green *et al*, 1998).

In a recent immunoistochemical study, Wooding *et al*. (2005) suggested that the diverse localisation of the two PAG groups may imply different possible functions. The evolutionary more ancient group (PAG-2 subgroup) is mainly expressed at the foeto-maternal interface (microvillar junction) and PAG molecules are localised in the small apical vesicles in transit to the cell surface. This strategic orientation between the two epithelial surfaces could place the PAG in an ideal position to have a role in adhesion. Moreover, their relative absence in placental areas of less definitive contact, such as the cow interplacentomal regions, lends support to this hypothesis. The adhesion function could have been evolved from the PAG peptide-binding site and the N- and O-linked carbohydrate chains, which would allow PAG to act as bifunctional linking molecules, with the binding cleft engaging a protein (e.g. an integrin), and the carbohydrate a lectin (Wooding *et al*, 2005). One intriguing possibility argued by Wooding *et al*. (2005) is that if the pH of the microenvironment at the interface falls, e.g. perhaps at parturition, PAG proteolytic activity might be revived and participate in the release of the cotyledon from the caruncular crypts. A role of PAG in parturition has also been suggested by other authors by virtue of their peri-parturient peak (Green *et al*, 2005a), but also because altered PAG concentrations prior to calving have been observed in calving difficulty and stillbirth (Kindahl *et al*, 2002).

The more recently evolved PAG group (PAG-1 subgroup) is restricted to the binucleate cells located mainly in the maternal villi of the placentomes. This localisation suggests two main functions for this group. On one hand, since the binucleate cell system has presumably evolved to allow delivery of foetal products to the mother, PAG have been proposed to act as hormones targeted to the mother (Roberts *et al*, 1996), perhaps acting upon the corpus luteum (Del Vecchio *et al*, 1996; Weems *et al*, 1998). On the other hand, some of the PAG released into the maternal system become deposited in the stromal layer within the maternal caruncles, and such localisation is consistent with the formation of a physical or immunological barrier that protects the foetus (Wooding *et al*, 2005). PAG might either block lymphocyte or polymorphonuclear leukocyte migration and activation (Roberts *et al*, 1996), or compete with the major histocompatibility complex for peptides processed for antigen presentation, hence masking the trophoblast antigens from

maternal immune recognition (Zoli *et al*, 1992). Therefore, by acting as local immunosuppressive factors, PAG could be involved in the maintenance of the histoincompatible foeto-maternal unit.

2.3.4 Factors influencing PAG production

Factors affecting PAG production and secretion are still little known.

Peripheral concentrations of PAG are affected by the maternal environment (breed of the mother) (Zoli *et al*, 1992) and the sire of the foetus (Lopez-Gatius *et al*, 2007a). They are also directly correlated to the placental mass (Wallace *et al*, 1997) which, in turn, is related to the stage of pregnancy and the number of foetuses. Indeed, PAG concentrations have been reported to be controlled by number and sex of foetuses, being higher in cows carrying either twins (Patel *et al*, 1997; Lopez-Gatius *et al*, 2007a) or female foetuses (Zoli *et al*, 1992).

Also an influence of milk production on PAG production has been hypothesised. Milk production correlates negatively with plasma levels of PAG during the early foetal period in high producing cows (Lopez-Gatius *et al*, 2007a) although lactation number seems to exert no effect on PAG secretion (Ayad *et al*, 2007a; Lopez-Gatius *et al*, 2007a).

A role for several hormones in regulating PAG production has been proposed. Findings from Bridges *et al*. (1999) are consistent with the hypothesis that oestradiol-17 β regulates placental secretion of PSPB in sheep. Ayad *et al*. (2007a) observed higher PAG concentrations in cows with higher P4 concentrations in the early stages of pregnancy, suggesting a positive effect of P4 on PAG production.

2.3.5 The use of PAG for pregnancy diagnosis and monitoring embryo viability

Because of their expression by trophoblastic cells, the measurement of PAG in maternal blood has been used for both pregnancy diagnosis and follow-up of the trophoblastic function (Sasser *et al*, 1986; Zoli *et al*, 1992; Patel *et al*, 1997; Szenci *et al*, 1998; Perényi *et al*, 2002b; Lopez-Gatius *et al*, 2007b). The first aspect can be very useful in the management of reproduction, while the second one concerns more specifically clinicians and researchers in their investigation to establish differential diagnosis of pathologies affecting pregnancy (Sousa *et al*, 2006). For example, PAG measurement has been used

in several studies to monitor pregnancy failure during the late embryo and early foetal period (Mialon *et al*, 1993; Szenci *et al*, 2003; Lopez-Gatius *et al*, 2007b).

However, the effectiveness of the PAG test is compromised by two disadvantages. Firstly, positive pregnancy diagnosis in the first four weeks after insemination is rather uncertain because antigen concentrations in the maternal blood are low and somewhat variable (Sasser *et al*, 1986; Zoli *et al*, 1992; Mialon *et al*, 1993). Secondly, the presence of PAG in blood for nearly 100 days postpartum constitutes a problem for subsequent diagnosis of pregnancy if re-breeding occurs less than 80 days postpartum (Zoli *et al*, 1992).

A recent study aimed to solve the first problem, improving sensitivity and specificity of the PAG-RIA assay by raising antisera against different PAG molecules (Ayad *et al*, 2007b). Indeed, as previously described in this review, it has been demonstrated that various PAG are not expressed co-ordinately throughout gestation (Green *et al*, 2000). The current PAG assay (Sasser *et al*, 1986; Zoli *et al*, 1992) has been developed using antisera raised against antigens belonging to boPAG-1, which is expressed minimally, if at all, at day 25. It recognises also boPAG-4 which present the same pattern of expression as boPAG-1. This fact might explain the low sensitivity of the method when performed in early stages of pregnancy (Sousa *et al*, 2006). The study from Ayad *et al*. (2007b) clearly shows that the ability of PAG-RIA system to diagnose pregnancy correctly at day 30 after breeding can be improved by using a combination of antisera raised against different isoforms of PAG. A better candidate for a pregnancy test might be a protein such as boPAG-9 since it is expressed in early pregnancy and almost absent around parturition, hence not contributing to the high PAG levels after parturition detected by the current assay (Green *et al*, 2000). To avoid the interference given by high plasma levels of PAG in the post-partum, research has been focusing towards a new pregnancy test that measures PAG in milk, since the decrease of PAG concentration after parturition is reported to be faster and more accentuated in milk than in blood (Metelo *et al*, 2004). In dairy goats, for example, the milk PAG test seems to provide an accurate pregnancy diagnosis as soon as day 32 after breeding (Gonzalez *et al*, 2001). In dairy cows, however, the reliability of milk PAG test is still under investigation (Metelo *et al*, 2004).

2.4 Oxidative stress and embryo mortality

A vast literature exists on the role of oxidative stress (OS) in the development of many pathological conditions in humans as well as in farm animals. In particular, OS may be involved in the cause and progression of a number of pathological disorders related to

reproduction in mammals (Miller *et al*, 1993a). Therefore, the aim of the present review is to introduce the concept of OS and damage, and to provide an overview of the involvement of OS in reproductive disorders, EM in particular.

2.4.1 Oxidative status

2.4.1.1 Reactive Oxygen Metabolites (ROMs)

A free radical is a molecule with one or more unpaired electrons in its outer orbital. Such unpaired electrons make these species very unstable and therefore quite reactive, since the free radical tends to react with other molecules to pair this electron in order to generate a more stable species (Reilly *et al*, 1991). The most abundant free radicals in biological systems are the oxygen-centred free radicals and their metabolites, usually referred as “reactive oxygen metabolites” (ROMs) (Miller *et al*, 1993a).

All cells living under aerobic conditions are continuously exposed to large numbers of oxidants derived from various exogenous and endogenous sources. The exogenous sources of oxidants include ionising and non-ionising radiations, air pollution and natural toxic gases, as well as chemicals and toxins. The endogenous sources are several and the main one is the respiratory chain in the mitochondria that converts molecular oxygen to water. A few percent of oxygen molecules continuously leaks from the electron transport chain as reactive oxygen species (ROS) intermediates and this process results in a substantial basal level of oxidants (Lykkesfeldt and Svendsen, 2007).

ROMs are formed continuously as normal by-products of cellular metabolism and, in low concentrations, they are essential for several physiological processes, including protein phosphorylation, transcription factors activation, cell differentiation, apoptosis, oocyte maturation, corpus luteum functions, cell immunity and cellular defence against microorganisms (for review see Hoidal, 2001; Georgieva, 2005; Agarwal *et al*, 2005). An example of ROMs production during physiological processes is shown in figure 2.4

However, when produced in excess, ROMs can damage cell functionality as they can harm cellular lipid, proteins, and DNA (Sugino, 2006).

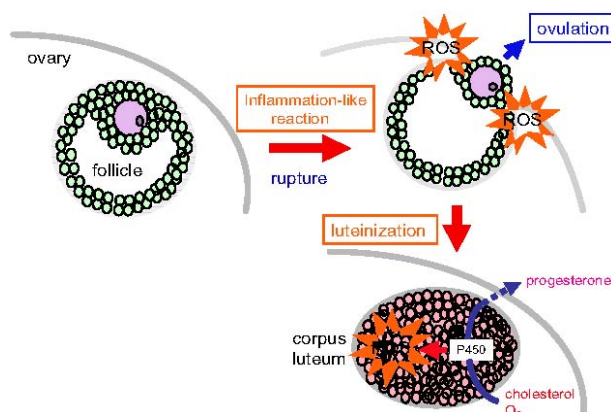


Figure 2.4 Generation of ROS during ovulation and steroidogenesis in corpus luteum. Ovulation appears to be an inflammation-like process. ROS is locally produced during follicular rupture and may be involved in the ovulation process. ROS is also generated by the corpus luteum via the monooxygenase reaction as a byproduct during steroid hormone. From Fujii *et al.* (2005).

2.4.1.2 Antioxidants

Aerobic organisms have evolved complex cellular defence strategies to prevent or limit damage caused by ROMs. The main defence against oxidants is the so-called antioxidants network. Antioxidants are a variegated group of compounds that are capable of donating electrons to oxidants, thus neutralising their reactivity and making them harmless to cellular macromolecules. The antioxidants thereby become radicals themselves, but these are far more stable compounds and are not capable of inducing cellular damage. The oxidised antioxidants are then reduced to their active state by several cellular processes so that they can be recycled into the antioxidant network (Lykkesfeldt and Svendsen, 2007).

The antioxidant network is represented by mechanisms that are either enzymatic, such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase, or non-enzymatic, such as vitamins A, C and E, urate, transferrin and even albumin (Reilly *et al.*, 1991; Agarwal *et al.*, 2005; Noyan *et al.*, 2006).

There are specific metalloenzymes to scavenge superoxide radicals: copper-zinc superoxide dismutase (Cu,Zn-SOD), located in the cytosol, and manganese SOD (Mn-SOD), located in the mitochondria. These antioxidant enzymes require some dietary essential trace elements, such as Mn^{2+} , Cu^{2+} , and Zn^{2+} for SOD, Se for glutathione peroxidase, and Fe for catalase (Miller *et al.*, 1993a). Catalase is found primarily within

peroxisome of most cells; it catalyses the conversion of hydrogen peroxide into water and oxygen. GSH-Px is a selenium containing-enzyme and catalyses the degradation of lipid peroxides as well as hydrogen peroxide leaking from the electron transport chain (Sugino, 2006).

There is also a second important level of defence against damages induced by ROMs. It is the ability to detect and repair or remove oxidised molecules. For instance, to deal with oxidised DNA a multitude of repair systems have evolved (i.e. base excision repair, ligation, and nucleotide excision repair) which are capable of repairing damaged DNA by detecting oxidised bases, cutting them out and inserting the correct undamaged base in the DNA (Loft *et al*, 2008). Other important mechanisms of defence include catabolism of non-functional or modified proteins and lipids by proteases and other enzymes (Ames *et al*, 1993). These scavenging processes are essential in order to avoid accumulation of these compounds which could lead to several diseases (e.g. Alzheimer's disease and atherosclerosis) as well as aging (Dean *et al*, 1997).

2.4.1.3 Oxidative stress

When free radicals are produced more and faster than they can be safely neutralised by antioxidant mechanisms, OS results (Bernabucci *et al*, 2002; Castillo *et al*, 2005).

OS leads to damage of biological macromolecules and disruption of normal metabolism and physiology (Trevisan *et al*, 2001). Excessive production of ROMs during OS has been reported to cause DNA damage, lipid peroxidation (which principally affects the membrane structure and function), and protein damage (Sugino, 2006).

Because free radicals are so highly reactive, they generally react with the first structure that they encounter, which is likely to be the lipid component of cell or organelle membranes (Reilly *et al*, 1991). In the past few decades, lipid peroxidation has been shown to be of great importance in mammalian physiology and physiopathology. Indeed, the mechanism of free radical-mediated lipid peroxidation is an example of a classic chain reaction. A single hydroxyl radical and molecular oxygen can react with polyunsaturated fatty acid to alter not only its structural and/or functional integrity, but also generate multiple fatty acid peroxy radicals, which subsequently react with other lipids, proteins, or nucleic acids. A cascade of electrons transfer is thereby propagated and consequent oxidation and destruction of these substrates occur. The cell injuries produced by lipid peroxidation of cell membranes can range from increased permeability to cell lysis (Reilly *et al*, 1991).

Toxic oxygen metabolites can also directly attack nucleic acids, causing base hydroxylation, cross-linking, or scission of DNA strands. This can result in cell death or mutation (Imlay and Linn, 1988; Takahashi *et al*, 2000). Oxidative modifications of DNA are also thought to play an important role in carcinogenesis (Loft *et al*, 2008).

Oxidation of proteins by free radicals results in modification of amino acid side-chain groups, degradation of peptide bonds, or formation of covalent bonds with oxidised products of lipids or carbohydrates (Davies, 1987). Protein oxidation involves several propagating radicals and its final products include several categories of reactive species as well as a range of stable products, which can be used as biomarkers (Dean *et al*, 1997).

Oxidised proteins are often functionally inactive and this makes proteins the most critical target of toxic damage. For example, the inactivation of a small portion of a ion transporter is likely to have much more drastic consequences than a similarly extensive alteration of sterol molecules (Dean *et al*, 1997).

Oxidized proteins often undergo unfolding or conformational change of their structure which affects their susceptibility to proteolysis. Proteases recognise proteins with a more open conformation (Moller and Kristensen, 2004). Moderately oxidised proteins are often more susceptible to proteolytic breakdown, while extensively damaged proteins appear sometimes to become less sensitive to proteinases (Moller and Kristensen, 2004). As a consequence, certain oxidised proteins are poorly handled by cells, resulting in accumulation and damaging actions observed in several pathological conditions.

Moreover, oxidised proteins may act as immunogenic neoepitopes, triggering the production of autoantibodies (Kurien *et al*, 2006) (see chapter 2.4.4.1, Inflammation, immune system activation and oxidative stress).

Furthermore, ROMs can affect cell function by controlling production or the activation of substances that have biological activities and lead to the modification of important physiological and metabolic functions, resulting in altered physiology and possibly pathology (Miller *et al*, 1993a; Sugino, 2006).

In humans, OS is related to aging (Ames *et al*, 1993) as well as to several diseases such as cancer (Loft *et al*, 2008), atherosclerosis (Gounopoulos *et al*, 2007; Matsuura *et al*, 2008), arthritis (Seven *et al*, 2008), renal diseases (Witko-Sarsat *et al*, 1998; Shi *et al*, 2008), diabetes (Kaluosava *et al*, 2002), Alzheimer's disease (Smith *et al*, 1992) and Parkinson's diseases (Jenner, 2003). It has also been hypothesised that OS is involved in the activation of resistance to chemotherapy (Toyokuni *et al*, 1995).

2.4.2 Oxidative stress biomarkers

As OS describes an imbalance between oxidants and antioxidants, it can be assessed either by measuring antioxidant or oxidant biomarkers and their products.

2.4.2.1 Oxidants

Plasma level of ROMs is considered an indicator of free radical production (Miller *et al*, 1993a). ROMs is a collective term that includes not only oxygen-centred free radicals such as superoxide anion ($^{\circ}\text{O}_2^-$) and hydroxyl radical (OH°), but also some non-radical derivatives of oxygen, such as hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) (Reilly *et al*, 1991).

2.4.2.2 Oxidation products

Advanced glycation end products (AGEs) are the products of non-enzymatic glycation/oxidation of proteins/lipids (Shi *et al*, 2008). Accumulation of AGEs has several toxic effects. AGEs can modify proteins, damage the structure and metabolism of extracellular matrix either directly or by their specific receptors (RAGE). In particular, the interaction between AGE and their receptors stimulates the transcription of genes for cytokines, growth factors and adhesive factors, as well as inducing the migration of macrophages (Kalousova *et al*, 2002). AGEs can also accumulate in tissues, thus taking part in the progression of several diseases, such as diabetes, chronic renal failure, atherosclerosis, and aging (Witko-Sarsat *et al*, 1998).

During OS new compounds and modified structures thus are formed, and one of them is advanced oxidation protein products (AOPP). AOPP are terminal products of proteins exposed to free radicals and arise from the reaction between plasma proteins and chlorinated oxidants mediated by a neutrophil enzyme myeloperoxidase (MPO) (Fialova *et al*, 2006; Noyan *et al*, 2006).

In human plasma, the spectrophotometric characterization of AOPP has shown two distinct peaks: one of high the other of low molecular weight corresponding to molecular mass of 600 and 80 kD, respectively. The high molecular weight fraction corresponds mainly to albumin, which appears to form aggregates resulting from disulphide bridges and/or dityrosine cross-linking. The low molecular weight fraction is mainly constituted by monomeric albumin (Witko-Sarsat *et al*, 1996).

Initially described in patient with chronic renal failure (Witko-Sarsat *et al*, 1998), over the last few years AOPP have been found to accumulate in several diseases. Examples are diabetes mellitus (Kaluosava *et al*, 2002), diabetic nephropathy (Shi *et al*, 2008), coronary artery diseases (Kaneda *et al*, 2002), and even obesity (Atabek *et al*, 2006).

In addition to being a biomarker of protein oxidation, AOPP have been suggested to exert also biological activity on their own (Drueke and Massy, 2002). Indeed, they can trigger a respiratory burst in neutrophils and monocytes and monocyte activation in patients with chronic renal insufficiency (Witko-Sarsat *et al*, 1998). Therefore, monocyte is apparently, at the same time, the elective cellular target of AOPP and a potential source of oxidants inducing AOPP. Accumulation of AOPP during the course of chronic renal failure is not only related to the decrease in kidney excretory function, but is also closely associated with several immunoinflammatory markers (Witko-Sarsat *et al*, 1998). Chronic accumulation of AOPP has been demonstrated to promote inflammatory processes in diabetic kidney too (Shi *et al*, 2008). These data suggest that AOPP might be potential inducers of cellular inflammation. A possible mechanism through which AOPP may exert their effect involves the interaction with RAGE. As a consequence of this interaction, the induction of proinflammatory activities and adhesive molecules and cytokines production are even more intensive than that caused by AGEs (Kalousova *et al*, 2005).

Being lipids the preferred substrate to free radicals damage via lipid peroxidation, they are considered the best biomarkers of OS (Georgieva, 2005). One of the lipid peroxidation end-products is malondialdehyde (MDA). MDA is one of the several low-molecular-weight end products formed during the radical induced decomposition of poly-unsaturated fatty acid (Janero, 1990). At low pH and elevated temperatures MDA reacts with a variety of nucleophiles giving rise to coloured adducts; in particular, MDA readily reacts with thiobarbituric acid producing a red pigment which can be easily measured by spectrophotometry in the form of thiobarbituric acid reactive substances (TBARS) (Janero, 1990). Lipid peroxidation can be measured also by oxidised low density lipoproteins (oxLDL). LDL is the major lipid carrier in plasma and consists of cholesterol ester, phospholipids, free cholesterol and triglycerides, and apolipoprotein B (Matsuura *et al*, 2008). OxLDL represent a variety of modification of both lipid and apolipoprotein B components by lipid peroxidation. They are cytotoxic, chemotactic and proinflammatory, and they play important roles in the development of atherosclerosis (Dean *et al*, 1997; Gounopoulos *et al*, 2007; Matsuura *et al*, 2008).

Finally, several established methods detect fragmented DNA damage that arises as a consequence of oxidative injury. These include HPLC fractionation of bases released as consequence of the damage, electrophoretic methods that detect directly fragmented DNA of lower molecular weight, and a single-cell gel electrophoresis assay (comet assay) for the detection of DNA damage in single cells (see Takahashi *et al*, 1999).

2.4.2.3 Antioxidants

In general, according to Bernabucci *et al*. (2005), endogenous antioxidants can be divided into three major groups. The first group comprises enzymatic antioxidants including SOD and GSH-Peroxidase, and represents the main form of intracellular antioxidant defence.

The second group includes non-enzymatic protein antioxidants which are primarily found in plasma. They are mainly represented by sulfhydryl groups of albumin and are considered a significant element of extracellular antioxidant defence system. A recent study (Castillo *et al*, 2005) has confirmed the role played by albumin as an important component with antioxidant capacity, particularly close to calving when animals usually do not receive any vitamin/mineral supplementation.

The third group, represented by the non-enzymatic low-molecular weight antioxidants, is found mainly in plasma but also in other extracellular and intracellular fluids. Examples are ascorbic acid, glutathione (GSH) and acid uric. In particular, GSH plays an important role in protecting cells against OS and toxic agents. It acts as substrate or co-substrate in enzymatic reactions and it also reacts directly with free radicals and lipid peroxides (Briviba and Sies, 1994).

Because of the difficulty in measuring each antioxidant component separately and their interaction in the plasma, several methods have been developed in the past few years to assess total antioxidant capacity. The measure of antioxidant capacity considers the cumulative action of all the antioxidants present in plasma and body fluids, thus providing an integrated parameter rather than the simple sum of measurable antioxidants (Ghiselli *et al*, 2000). Antioxidant capacity can be measured by means of several methods, such as Trolox Equivalent Antioxidant Capacity (TEAC) (Miller *et al*, 1993b), Total Radical-trapping Antioxidant Parameter (TRAP) (Ghiselli *et al*, 1995), Oxygen Radical Absorbance Capacity (ORAC) (Cao *et al*, 1993), or the Ferric Reducing Ability of Plasma (FRAP) (Benzie and Strain, 1996). Plasma antioxidant status is the result of the interaction of many different compounds and systemic metabolic interactions (Ghiselli *et al*, 2000). As a single measure, total antioxidant capacity provides relevant information that may

effectively describe the dynamic equilibrium between pro-oxidants and antioxidant in the plasma compartment (Cao and Prior, 1998; Ghiselli *et al*, 2000).

2.4.3 Oxidative stress in the cow

Dairy cows are exposed to various metabolic pressures of adaptation to high milk yield and environmental conditions and OS may develop. It has been proposed that the activation of antioxidant defences, is a preparative mechanism against OS caused by physiological stress situations (Hermes-Lima *et al*, 1998). It seems, indeed, that the entire antioxidant system is under homeostatic control. However, when metabolic reactions are overloaded, free radicals accumulate and the disturbance of the redox balance may occur, producing OS (Tanaka *et al*, 2007).

The possibility that cow metabolic activity may determine oxidant status has been suggested since various correlations between metabolic and oxidative biomarkers depending on the physiological condition of the cow have been detected (Castillo *et al*, 2005). During the transition period, OS is likely to occur as cows are activating different metabolic pathways and ROMs production is increased while the antioxidant system is decreased (Bernabucci *et al*, 2005). In particular, variation of plasma and erythrocytes oxidants and antioxidants during the transition period observed in the study of Bernabucci *et al*. (2002), revealed an alteration of the oxidative status of the cow after calving. In addition, in a subsequent study (Bernabucci *et al*, 2005), the same authors found that the peripartum period (particularly postpartum) was characterised by a depleted antioxidant status.

Birth means OS for calves, mainly due to the induction of pulmonary respiration. However, calves are able to counter effectively the oxidative stress inevitably present after the first inhalation of atmospheric oxygen thanks to their elevated plasma total antioxidant capacity (Gaál *et al*, 2006). Parturition has, instead, minor effects on the antioxidant system of the mother. In fact, Gaál *et al*. (2006) found changes in total antioxidant capacity only on the day of calving but not before or several days after calving.

It has been observed a progressive decline in antioxidant activity as lactation progresses, probably due to the depletion of fat-soluble antioxidants by milk (Castillo *et al*, 2005). Moreover, the metabolic demand imposed on cow by the onset of lactation may increase ROMs rate of production markedly, leading to an imbalance between oxidants and antioxidants (Castillo *et al*, 2005).

Also heat stress has been suggested to affect oxidative status. Evidence suggests that the effects of elevated temperatures on embryonic development involve changes in the metabolism of free radicals. A recent *in vitro* study (Sakatani *et al.*, 2008) demonstrated that heat shock increased intracellular ROMs in cultured bovine embryos, which in turn delayed or blocked embryo development. Moreover, exposure of cultured mouse embryos to elevated temperatures causes a reduction in GSH (Aréchiga *et al.*, 1995).

The situation *in vivo* is still somehow controversial. Bernabucci *et al.* (2002) found higher erythrocyte oxidant and antioxidant biomarkers in cows calving in summer than in cows calving in spring, which would indicate some effects of hot weather on the oxidative status of transition cows. Also Di Trana *et al.* (2006) found higher plasma ROMs concentrations in lactating dairy goats in summer compare to spring. Different result has been found by Trout *et al.* (1998). In their study heat stress did not increase lipid peroxidation or lipid-soluble antioxidant concentrations in blood of lactating cows. However, the animals objective of these studies were in different physiological status, which might be responsible for the discrepancies of the results.

Taking all together, these results may suggest that the effects of heat stress on oxidative status are probably related to the physiological phase of the animals, and also that these effects might be different at local (embryonic development within the uterus) or systemic level (plasmatic OS biomarkers).

2.4.4 Oxidative stress and impaired fertility

In spite of the great importance that has been ascribed to OS in a broad variety of pathologies, studies on the role of OS in cow's reproduction are still lacking.

In dairy cows, OS has been associated with several pathological conditions, such as retained placenta, udder oedema, mastitis, which in turn may impair reproductive performances. For instance, deficiency of antioxidants may contribute to impaired muscle tone and thus uterine contractibility. This can result in decreased transport of sperm to ova or retained placenta (for review see Miller *et al.*, 1993a).

On the contrary, in human medicine, there is growing literature on the effects of OS in the female reproduction (for review see Agarwal *et al.*, 2005). ROMs can affect a variety of physiological functions in the reproductive tract, and excessive levels can result in precipitous pathologies affecting female reproduction.

For example, pre-eclampsia is a systemic maternal disease, potentially fatal to both mother and foetus, characterized by defective placentation and reduced placental

perfusion in the early stages of pregnancy. Endothelial dysfunction is generally observed in affected women and thought to cause the systemic disease (Ness, 2004). It has been reported that OS and inflammatory mechanisms are involved in endothelial cell activation and dysfunction in pre-eclampsia. In particular, Redman *et al.* (1999) have argued that excessive inflammation, revealed by elevated titer of granulocytes and monocytes, characteristic of acute sepsis, as well as proinflammatory cytokines, provides the oxidative insult to the endothelium observed in this disease.

A role for OS also in the pathogenesis of endometriosis, a chronic disease characterized by the presence and growth of functional endometrial glands and stroma at extrauterine (ectopic) sites, in women has been proposed (Agarwal *et al.*, 2005).

Finally, the excess of ROMs production that occurs in OS has been reported to impair embryo development leading often to embryo death (Guerin *et al.*, 2001).

2.4.4.1 Mechanisms of action of oxidative stress on reproductive physiology

Luteolysis and oxidative stress

ROMs are produced in the steroidogenic cells and mononuclear phagocytes in the corpus luteum and the increase in ROMs in the CL seems to be involved in luteolysis (Kato *et al.*, 1997; Sugino, 2006). In addition, ROMs have been reported to inhibit P4 synthesis by the inhibition of cytochrome P450 scc, intracellular transport of cholesterol to mitochondria, and by impairing LH receptors (for review see Sugino, 2006). Since ROMs are generated and SOD is expressed in CL, there is the possibility that ROMs and SOD work as local regulators of the CL function, and an imbalance between oxidants and antioxidants, as in OS, could impair the regulation of CL activity leading to premature luteolysis (Sugino, 2006). Indeed, Wu *et al.* (1993) reported that ROMs cause several changes that disrupt the plasma membrane of luteal cells, which are often seen in the regression of the CL. One of the causes for the increase in ROMs in CL during the regression phase could be the decrease in Cu,Zn-SOD expression observed, for example, in CL of pseudopregnant rats exposed to PGF_{2α} administration (Shimamura *et al.*, 1995). Also PGF_{2α} may contribute to ROMs increase since it induces lipid peroxidation (Shimamura *et al.*, 1995) and stimulates superoxide radical production (Tanaka *et al.*, 2000). Also macrophages have been hypothesised to be involved, at least in part, in luteolysis. Macrophages produce ROMs and damage cells when they are activated. They increase in number in the regressing CL and produce cytokines that inhibit P4 production by luteal cells (Sugino, 2006).

In addition, a decrease in blood flow occurs in the ovary during the CL regression (Pang and Behrman, 1979), and this may cause tissue damage via reactive oxygen species generation by the mechanism of ischemia-reperfusion injury (Sugino, 2006).

Embryo development and oxidative stress

ROMs are generated during normal embryo metabolism (Guerin *et al*, 2001). The prevention against ROMs formation as well as their destruction seem to be linked both to internal embryo protection (e.g. SOD and GSH-Px) and external protection through the oviductal environment (e.g. transferrin, ascorbic acid) (Guerin *et al*, 2001). However, due to continuous and abundant ROMs production during embryo development, OS may occur. OS is involved in the aetiology of defective embryo development, being responsible for numerous types of embryo damages (Guerin *et al*, 2001). ROMs can damage preimplantation embryos by increasing lipid peroxidation (Guerin *et al*, 2001) and DNA fragmentation (Takahashi *et al*, 2000), by altering either enzymes or mitochondrial structures and functions, and leading often to embryonic death (Guerin *et al*, 2001; Agarwal *et al*, 2005).

OS might also be considered one of the mechanisms that link inflammation with embryo mortality. For example, observations from cows experiencing mastitis suggest that activation of inflammatory or immune responses can lead to embryonic mortality (Hansen *et al*, 2004). These embryonic losses appear to follow activation of multiple pathways that disrupt the reproductive axis at several points including the hypothalamic-pituitary axis, ovary, oocyte and the embryo (see figure 2.5).

On one hand, activation of inflammatory and immune responses leads to an increase in cytokines production, which in turn can increase secretion of other molecules detrimental for embryo survival and development, such as PGF_{2α} or nitric oxide (NO) (Hansen *et al*, 2004). On the other hand, elimination of bacterial infections through phagocytosis involves activation of neutrophils which, once activated, undergo the respiratory burst releasing ROMs intended to target foreign pathogens, but the lack of specificity in these reactions may result in tissue damage to the host (Lykkesfeldt and Svendsen, 2007).

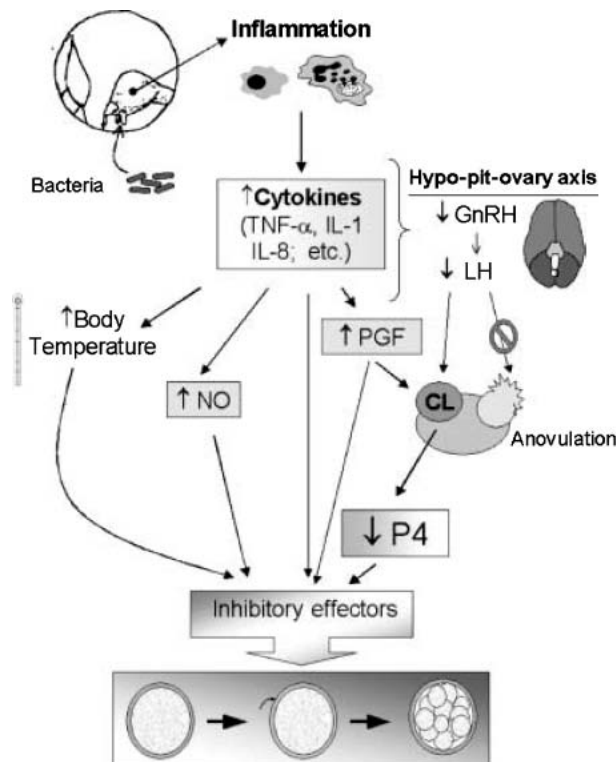


Figure 2.5. Potential pathways by which infection in the mammary gland can reduce embryonic survival. According to this model, the central phenomenon leading to embryonic mortality associated with mastitis is increased secretion of cytokines (either produced in the mammary and draining lymph nodes and secreted or produced in other tissues in response to mammary-derived signals) that in turn modulate reproductive function at several levels. From Hansen *et al*, 2004.

Inflammation, immune system activation and oxidative stress

Inflammation, immune system activation and oxidative stress have been hypothesised to be part of the pathogenetic mechanism of several diseases. For example, it has been suggested a role for OS in the development of mastitis, since marked decreases in ascorbate concentration have been found in cows with either clinical or subclinical mastitis (see Lykkesfeldt and Svendsen, 2007).

Pregnancy itself involves increase in activated monocyte and macrophages, as well as markers of OS, consistent with a pro-inflammatory state (Ness, 2004). Also Fialová *et al.* (2006) found a positive correlation between AOPP and C-reactive protein in pregnant women suggesting an association between inflammatory mechanisms and OS in pregnancy.

Moreover, it is well known that immune cells generate large amount of ROMs when stimulated (Santanam *et al*, 2002; Noyan *et al*, 2006; Lykkesfeldt and Svendsen, 2007; Spears and Weiss, 2008).

At the moment, the reciprocal role of each actor in respect to the others is far from being elucidated. Several pathways might be activated but a central role of oxidation products seems likely.

Oxidation products can:

a) accumulate in body tissues, since, for example, oxidised proteins are less susceptible to proteolysis and scavenging systems (Moller and Kristensen, 2004). This is what happens with ageing (Smith *et al*, 1992; Ames *et al*, 1993) and in pathologies like atherosclerosis (Matsuura *et al*, 2008) or Alzheimer's diseases (Smith *et al*, 1992).

b) act as neoepitopes inducing production of autoantibodies, which in turn enhance activation of mediators of inflammation. A typical example of this mechanism is the lipoperoxidation that occurs in atherosclerosis, a pathological condition in which arteries undergo gradual intima thickness, causing decreasing elasticity, narrowing and reduced blood supply (Matsuura *et al*, 2008). Oxidation of lipoproteins is intimately involved in all stages of atherosclerosis and oxidative by-products are involved in inflammatory cells activation. When LDL is oxidised, it becomes a ligand for scavenger receptors on macrophages leading to generation of foam cells, which accumulate on vessel wall causing its thickening (Dean *et al*, 1997). Moreover, oxLDL is not only pro-inflammatory and pro-atherogenic, but several of the neoepitopes generated during its oxidation are highly immunogenic and result in the generation of autoantibodies (Palinski and Witztum, 2000; Gounopoulos *et al*, 2007; Matsuura *et al*, 2008).

There is significant evidence suggesting critical roles of an inflammatory-type immune response also in the pathogenesis of endometriosis (Shanti *et al*, 1999; Bholer *et al*, 2007). Inflammatory cytokines, chemokines, T cells, macrophages and oxLDL have been observed in women affected by this pathology (Santanam *et al*, 2002). Activated macrophages react with LDL leading to their oxidation. Oxidation products of LDL are cytotoxic, chemotactic, and proinflammatory (Gounopoulos *et al*, 2007). Moreover, there is evidence that autoantibodies against oxLDL are produced in endometriosis (Shanti *et al*, 1999). The antigenicity is attributed to specific modified epitopes and not to the protein backbone. As a consequence, an inflammatory reaction through the recruitment of monocyte/macrophages and activation of inflammatory genes is generated.

In light of what discussed in the present literature review, an involvement of OS in different aspects of the pathophysiology of reproduction in mammals seems likely. In spite of this,

very few studies have been carried out so far to elucidate the role that OS might play in the development of reproductive diseases, particularly in farm animals. Therefore, it was considered worthwhile to investigate the relationship between OS and EM in the cow.

Laboratory Methods

3.1 Plasma and whey progesterone

3.1.1 General aspects of the solid-phase progesterone RIA

Radioimmunoassay (RIA) was used to measure plasma and whey progesterone (P4). This assay is based on the ability of nonlabeled hormone (Ag) to compete with a fixed amount of isotopically labeled hormone (Ag*) for the binding sites on a fixed and lacking amount of specific antibody (Ab). The nonlabeled hormone reduces the availability of free binding sites on the Ab to the labeled hormone. As the degree of the inhibition of binding of Ag* to the Ab is a function of the concentration of Ag present in the solution, the reaction is quantified by the determination of the amount of the Ab-bound labeled hormone. The solid-phase method used in this study allows a easier separation between free and bound hormone. A 96-wells plate is firstly coated with antirabbit IgG antibody, incubated and thus rabbit anti-progesterone antibody is added. After an overnight incubation period Ag and Ag* are added to start the reaction. At equilibrium, the free hormone (both Ag and Ag*) is easily separated from the Ab-bound hormone by aspiration of the supernatant and the radioactivity of Ab-bound Ag* can be counted in a β counter.

3.1.2 Blood and whey samples

Blood samples were collected in 10 ml tubes containing lithium or sodium heparin as anticoagulant and centrifuged at 2500 x g for 15 minutes. Plasma was stored at -20°C until assayed.

Plasma volumes of 100 μ l of samples and quality controls were extracted with 8 ml of petroleum ether in 10 ml glass tubes by shaking for 15 minutes at room temperature. After centrifugation at 1500 x g for 5 minutes at +5°C, the tubes were frozen for at least 1 hour. The supernatant was thus easily transferred into other glass tubes. The solvent was removed by evaporation under nitrogen and the residue resuspended in 1 ml of RIA buffer (PBS 0,1% BSA, pH 7.4). In order to increase assay sensibility, in the samples

taken at the day of artificial insemination (when P4 is supposed to be in low concentration), the steroid was concentrated extracting 200µl of plasma, then redissolved in 500 µl of RIA buffer.

Milk samples were collected at the beginning of milking into a polyethylene tube and immediately frozen (-20°C). Whey samples were prepared according to the method described by Prandi *et al.* (1994) and Comin *et al.* (2005). Whey was obtained by incubating 5 ml milk with 0.25 mg (approximately 5 Units) calf chymosin (Sigma Chemical Co., St, Louis, MO) at 40°C for 45 min to allow clotting. Whey was separated from fat and curd phases following centrifugation at 4500 × *g* for 15 min at 4°C and used for analysis.

3.1.3 Antiserum and radiolabeled hormone (tracer)

Antiserum anti-P4-7-CMO-BSA raised in rabbit was used (working dilution 1:7000). Crossreaction with progesterone was 100%, with 11-α-hydro-progesterone 77%, with 11-β-hydro-progesterone 65%, with 17-α-hydro-progesterone 2,9%, with 20-α-hydro-progesterone 0,01%, with 20-β-hydro-progesterone <0,001%.

The radiolabeled hormone was NEN 1,2,5,6-³H Progesterone dissolved in RIA buffer with a final dilution of 30pg/well/10µl (tot cpm).

3.1.4 Standard curve

As a basis for the quantification of hormone present in each sample, a standard curve ranging from 320 to 5 pg/200µl/well and from 320 to 2,5 pg/200µl/well for whey and plasma P4, respectively, was developed (Standard progesterone, Sigma Chemical Co., St Louis, MO). To minimize non-specific interference due to whey components, a progesterone free whey ("stripped whey") as a matrix to give the standard curve was used. The "stripped whey" was obtained adding a solution of dextran-activated charcoal dissolved in RIA-BSA buffer (1%) to whey belonging to cows on oestrus or 4-5 days after calving, when progesterone is supposed to be very low. After an overnight incubation whey was centrifuged until a clear solution was obtained.

3.1.5 Assay procedures

Plasma and whey P4 was measured by the specific microtitre radioimmunoassay described by Battocchio *et al.* (1999).

Briefly, RIA buffer, stripped whey, standard, samples and tracer were added to duplicate wells of 96well plates (Perkin Helmer, optiplate 96 well) coated with anti-progesterone antibody, as shown in tables 3.1 (plasma assay) and table 3.2 (whey assay).

Well	RIA Buffer	Standard	Sample	Tracer
Tot activity				10
Blank	200			10
Control	200			10
Standard	150	50		10
Samples	100		100	10
CTRL	100		100	10

Table 3.1 P4 plasma assay. The amount of RIA buffer, standard, samples and tracer are expressed in $\mu\text{l}/\text{well}$.

Well	Stripped whey	RIA Buffer	Standard	Sample	Tracer
Tot activity					10
Blank	100	100			10
Control	100	100			10
Standard			200		10
Samples		100		100	10
CTRL		100		100	10

Table 3.2 P4 whey assay. The amount of stripped whey, RIA buffer, standard, samples and tracer are expressed in $\mu\text{l}/\text{well}$.

Plates were shaken, incubated overnight at 4°C and washed 4 times with RIA buffer to remove any unbounded hormone. Two-hundred μl of a scintillation fluid (MICROCSINT-20, Perkin Helmer) were added to each well and the bound radioactivity was read by a β

counter (Top-Count, Packard Instrument Company, USA). Concentration of P4 were calculated by computer program (Riastar, Packard) mounted on β counter. The intra- and inter-assay coefficients of variation (CV) were 7.0% and 5.1% respectively.

3.2 Plasma and milk PAG

3.2.1 General aspect of liquid-phase PAG RIA

PAG concentrations in plasma and whey samples were determined using a liquid-phase RIA. The basic principle is once again a competitive reaction between labeled (Ag^*) and non-labeled (Ag) antigen with a fixed amount of a specific antibody ($Ab1$), but it differs from the solid-phase RIA as the separation between free and bound hormone is achieved by means of a second antibody ($Ab2$) raised against the first antibody. In this way, a macromolecular complex ($Ab2-Ab1-Ag^*$) is formed that can be precipitated by centrifugation. The free Ag^* is then eliminated by decanting the supernatant, while the $Ab2-Ab1-Ag^*$ complex stays on the bottom of the tube so that the radioactivity can be read by a γ counter. A better efficiency of $Ab2-Ab1-Ag^*$ complex precipitation can be achieved by adding the $Ab2$ in a buffer containing polyethylene glycol (PEG).

3.2.2 Blood and milk samples

Blood samples were collected in 10 ml tubes containing sodium heparin as anticoagulant and centrifuged at $3500 \times g$ for 15 minutes. Plasma was stored at $-20^\circ C$ until assayed. Milk samples were collected in 50mL tubes and a tablet of sodium azide was added to prevent enzymatic coagulation. Samples were stored at $-20^\circ C$ until processed. PAG assay was performed on whey samples obtained by ultracentrifugation at 20000 rpm for 30 minutes at $+4^\circ C$ (Beckman, SW 70 Ti rotor). The supernatant was then stored at $-20^\circ C$ until assayed.

3.2.3 Antigen and Antiserum

Pure boPAG_{67 kDa} preparation was obtained according to the protocol of Zoli *et al.* (1991) and used as standard and tracer for PAG assay. Rabbit polyclonal antiserum R#497 was

raised against boPAG_{67 kDa} (accession number A61232; Zoli *et al*, 1991) and prepared according to the Vaitukaitis *et al.* (1971) method.

3.2.4 Iodination

The iodination (Na-I¹²⁵, Amersham Biosciences, Uppsala, Sweden) was carried out according to the chloramines T method previously described by Greenwood *et al.* (1963). Briefly, the antigen (boPAG_{67 kDa}; 1.0 mg/mL) was dissolved in phosphate buffer 0.5 M (pH 7.5) while the chloramine T hydrate (5 mg/mL; Sigma-Aldrich Co., St. Louis, MO, USA) and sodium metabisulfite (30 mg/mL; Sigma-Aldrich Co.) were dissolved in distilled water. The radio-iodination mixture contained 10 µg of boPAG_{67kDa}, 10µl of Na¹²⁵I (1 mCi, approximately 3.7×10^7 disintegrations/s) and 10 µl of chloramine T solution. After 1 min of stirring, 10 µl of metabisulfite solution was added. Separation of free and ¹²⁵I labelled boPAG_{67 kDa} was performed on a 1 x 30 cm G-75 column (Amersham Biosciences) equilibrated and eluted with the buffer containing Tris-HCl (0.025 M, pH 7.5) and bovine serum albumin (BSA; 1.0 g/L; Fraction V; Sigma, St Louis, MO, USA). Eluted aliquots of 1.0 mL were collected, submitted on a test with anti-PAG sera, and selected according to the non-specific binding (NSB) value and binding/total (B₀/Tc) ratio. The best fractions were stored at +4°C until RIA procedure.

3.2.5 Second antibody

The second antibody precipitation system consisted of a mixture of sheep anti-rabbit immunoglobulin (0.83% vol/vol), normal rabbit serum (0.17% vol/vol), polyethylene glycol 6000 (20 mg/mL; Fluka Biochemika, Buchs Switzerland), microcrystalline cellulose (0.05 mg/mL; Merck, Darmstadt, Germany) and BSA (2 mg/mL; Fraction V; Sigma, St Louis, MO, USA) diluted in Tris-HCl assay buffer (25 mM MgCl₂, and 0.02% wt/vol NaN₃, pH 7.5).

3.2.6 Standard curve

As a basis for the quantification of proteins present in each sample, a standard curve ranging from 100 to 0,8 ng/ml and from 25 ng/ml to 0,2 ng/ml for plasma and milk PAG,

respectively, was developed. To minimize non-specific interference due to serum proteins, virgin heifer serum (PAG-free serum; 0.1 mL) was added to each tube of the standard curve. After the addition of an appropriate dilution of antisera (0.1 mL), the serum samples and the standard tubes were incubated overnight at room temperature (20 to 22 °C). The following day, 0.1 mL of I125-PAG ($\approx$ 25,000 cpm) was added and the tubes were incubated for 4 hours at room temperature. The total assay volume was 0.6 mL.

In order to find out the most accurate test for milk PAG, the assay was developed both in TRIS-BSA buffer (pH 7.5) and in nonpregnant cow de-fatted milk (PAG-free milk) as assay buffer and matrix for the standard curve.

TRIS-BSA buffer was rejected as matrix for the assay since it gave rise to much more non specific interferences than PAG-free milk, detecting PAG molecules even in samples PAG-free.

3.2.7 Assay procedure

For plasma PAG assay, TRIS buffer, standard, quality control, PAG-free serum, antibody (Ab1) and unknown samples were added to each tube, as shown in table 3.3, and incubated overnight at room temperature.

Tube	TRIS	Serum free	Standard	Sample	CTRL	Ab1
Blank	300	100				
Control	200	100				100
Standard	100	100	100			100
Samples	200			100		100
CTRL	200				100	100

Table 3.3 PAG plasma assay. The amount of TRIS buffer, serum free, standard, samples, quality control (CTRL) and antibody (Ab1) are expressed in μ l/tube.

Then, 100 μ l of tracer (^{125} I-PAG, approximately 25,000 cpm/tube) were added, and the reaction mixture was incubated for 4 hours at room temperature. Finally, the PEG-Ab2 solution (1 ml) was added to each tube except TC tube, the tubes were carefully mixed

and incubated for 30 min. The reaction was interrupted by the addition of 2 ml Tris-BSA buffer, and tubes were centrifuged at 3500 rpm for 20 minutes at 4°C. The supernatant was discarded and radioactivity of the precipitate was counted in a γ counter (DPC gamma-C12). The intra- and inter-assay coefficients of variation (CV) were 3.08% and 10.25%, respectively.

Milk assay procedure was the same as for plasma and the amount of reagents added to each tube is shown in the table 3.4.

	PAG-free milk	Standard	Samples	CTRL	Ab1
Blank	600				
Control	500				100
Standard	400	100			100
Sample			500		100
CTRL				500	100

Table 3.4 PAG milk assay. The amount of PAG-free milk, standard, samples, quality control (CTRL) and antibody (Ab1) are expressed in μ l/tube.

3.3 Malondyaldehyde (MDA)

Plasma concentration of malondyaldehyde was estimated according to Yoshida *et al.* (2005). Briefly, the reaction was carried out by mixing 0,2 ml sodium dodecyl sulphate solution (8,1%, w/v – Sigma-Aldrich 71729), 1,5 ml acetic acid buffer (20%, v/v, pH 3,5 – Sigma-Aldrich 45732), 1,5 ml thiobarbituric acid (1%, v/v – Sigma-Aldrich T5500), 0,7 ml water, and 0,1 ml plasma. The reaction mixture was incubated at 100°C for 60 minutes and then cooled in ice. One ml of water and 5 ml of n-butyl alcohol and pyridine (Fluka 82704; MW 79,10) (15/1, by volume) were added. The mixture was centrifuged (2500rpm at 0°C) for 10 minutes and the supernatant was measured spectrophotometrically at 535 nm. MDA (Tetramethoxypropane – Sigma-Aldrich 87670) was used to perform a standard curve ranging from 0,154 to 5 μ M.

3.4 Advanced Oxidation Protein Products (AOPP)

Plasma concentration of AOPP was estimated according to Witko-Sarsat *et al.* (1998). Briefly, AOPP were measured by spectrophotometry on a microplate reader (Spectracount; Packard) at 340nm. Two-hundred μ l of plasma diluted 1/5 in PBS (5 mM; pH7,2) was placed into wells of a 96-well plate (PS microtiter plate with 96 flat bottom wells; PerkinElmer) and 20 μ l of glacial acetic acid (Fluka 45732; MW 60.05) was added. A chloramine-T solution (Sigma-Aldrich; MW 281.69) was used to perform a standard curve ranging from 0 to 200 μ mol/liter. In standard wells, 10 μ l of 1,16 M of potassium iodide (Sigma-Aldrich ReagentPlus®; MW 166.00) was added to 200 μ l of the chloramines-T solution followed by 20 μ l of acetic acid. The absorbance of the reaction mixture is read in 5 minutes against a blank containing 200 μ l of PBS, 10 μ l of potassium iodide and 20 μ l of acetic acid. AOPP concentration were expressed as micromoles per litre of chloramine-T equivalents.

3.5 Total plasma glutathione (GSH)

Total plasma glutathione (GSH) concentrations were determined according to the method described by Tietze (1969) with some modifications to adapt the assay to a microplate reader (Baker *et al.*, 1990).

Briefly, GSH present in plasma samples and standards is oxidised by adding ditiobis-2-nitrobenzoic acid (DTNB). Samples and standards are incubated at 37°C for 10 minutes until the redox reaction reaches equilibrium. A first read is performed to be used as blank. Then, the enzyme glutathione reductase is added to reduce total GSH present in each sample with NADPH as source of reducing power. As a result of this redox reaction, NADP⁺ is generated. Its concentration is proportional to total GSH present in each sample and it can be quantified thanks to its chromogenic properties. Reads will be performed every minute for 10 minutes until the signal is constant, indicating that the redox reaction has reached equilibrium. Blank is then subtracted from the last read and the OD values obtained are used to calculate total plasma GSH using a standard curve.

The following reagents (Sigma-Aldrich) were used to develop the assay: reduced glutathione; glutathione reductase; NADPH; NaH₂PO₄; TRIS buffer; NaEDTA; 5,5'- DTNB. The following stock solutions were prepared

- EDTA 0.5M (4.65g/25mL; pH 7)

- TF-E: NaH_2PO_4 0.1M + EDTA 0.5M (pH 7)
- Standard GSH solution: 325.42 μM
- GSH reductase solution (1.7 U/ml TF-E pH 7.4)
- NADPH 10mM (8.3 mg/mL H_2O)
- TRIS buffer 0.1M
- DTNB 10mM (3.96 mg/ ml in TRIS buffer; pH 7.4 – 8)
- Reaction Solution (RS): 12mL TF-E + 180mL DTNB + 300 mL NADPH

Standard curve was generate using 0.339 to 21.69 $\mu\text{mol/L}$ GSH diluted in TF-E. Two-hundred μL of reaction solution and 30 μL of standards or samples (diluted 1:60) were added to wells of 96well plate (PS microtiter plate with 96 flat bottom wells; PerkinElmer) in duplicate. After a 10 min incubation the absorbance was measured spectrophotometrically at 410 nm. Twenty-five μL of reductase solution were added to each well. Reads were performed every minute for 10 minutes. GSH concentrations were calculated as previously described.

3.6 The assessment of oxidant and antioxidant status: ROMs and BAP

To assess oxidant and antioxidant status, the FRAS (Free Radical Analytical System) was used. This system has been developed by “DIACRON International S.a.S Grosseto -Italy-” in order to assess the pro-oxidant component (by the d-ROMs test) and the antioxidant barrier (by the BAP test) in humans.

3.6.1 The d-ROMs test

3.6.1.1 General aspect of the d-ROMs test

The d-ROMs test is a photometric test which measures hydroperoxides blood concentration (ROOH), a class of chemical oxidant species belonging to the wide family of Reactive Oxygen Metabolites (ROMs). Mixing blood with an acidic buffered solution, transferrin present in the sample releases iron ions which can catalyze the hydroperoxides breakdown with generation of free radicals such as alkoxyl ($\text{RO}^\bullet$) and hydroperoxyl radicals ($\text{ROO}^\bullet$). When an aromatic amine (N,N-diethylpraphenylendiamine) is added to this solution, the free radicals put out an electron from the aromatic amine which becomes

a coloured radical cation and can be detected and measured photometrically. As the radical cation concentration is proportional to the colour intensity, it is possible to evaluate the free radical concentration and then the hydroperoxides concentration of the samples. The results of the d-ROMs test are expressed as CARR U, i.e. Carratelli Units, by the initial of the chemist who first developed this test. One CARR U is equivalent to 0.08 mg/dl of a hydrogen peroxide water solution.

As this test has been developed to assess ROMs concentration in human blood, the values were corrected with cow hematocrit, according to the equation $ROOH (UCarr) * 58 / (100 - \text{cow PVC})$.

3.6.1.2 Assay procedure

Heparinized blood samples only were used because the chelating agents such as ethylene diamine tetraacetate (EDTA) or citrate may interfere with the chemical reactions of the test leading to an underestimation of the results.

Twenty μL of fresh blood were diluted in an acidic buffered solution (pH 4,8) (reagent 2) and a chromogen mixture (reagent 1) was added. After centrifugation for 60 seconds at 37°C , the absorbance of the solution was measured by spectrophotometry at 505 nm.

3.6.2 The BAP test

3.6.2.1 General aspect of the BAP test

The BAP test is a photometric test which evaluates blood Biological Antioxidant Potential (BAP) by measuring some antioxidant species, including uric acid, ascorbic acid, proteins, α -tocopherol and bilirubin. This assay is based on the ability of an antioxidant solution, such as plasma, to reduce iron from its ferric (Fe^{3+}) to its ferrous (Fe^{2+}) form. First, a coloured solution is prepared immediately before performing the test by mixing a thiocyanate derived (the chromogen) and a ferric chloride solution. A first read at 505 nm is performed. Blood sample is added and a second read is performed. The intensity of the decolouration obtained will be directly proportional to the antioxidant agents concentration of the sample. The results of BAP test are expressed as micromoles of reduced iron per litre of biological sample.

3.6.2.2 Assay procedure

As for the d-ROMs test, heparinized blood samples only were used because chelating agents, such as ethylene diamine tetraacetate (EDTA) or citrate, may interfere with the chemical reactions of the test leading to an underestimation of the results.

The absorbance of a coloured solution prepared immediately before performing the test by mixing a thiocyanate derived (reagent 1) and a ferric salt (reagent 2) was measured at 505nm. Then 10 μ L of plasma were added and, after mixing gently, the absorbance was read again at 505 nm.

3.7 Metabolic indicators

Plasma samples collected during experiment 2 were analysed by means of Automatic biochemical analyzer Roche BM Hitachi 911 at the “Istituto Zooprofilattico delle Venezie” in Legnaro, Italy. They were analyzed for: albumin, Kit *Roche* ALB plus, cod.1970569 ; total proteins, kit *Roche* TP, cod. 1553836 ; cholesterol, Kit *Roche* CHOL, cod. 1489232; glucose, Kit *Roche* GLU, cod. 1447513; urea, Kit *Roche* UREA/BUN cod. 1489364; triglycerides, Kit *Roche* TG, cod. 1488872; β -hydroxybutyrate, Kit *Randox*; NEFA, kit *Randox*, cod. 9420.

Plasma samples collected during experiment 3 were analysed for: glucose by the Cayman's Glucose Assay kit; albumin by QuantichromTM BCG Albumin Assay kit (DIAG-250), Bioassay Systems; proteins by QuantichromTM Protein Assay kit (QCPR-500) Bioassay Systems; urea by QuantichromTM Urea Assay kit (DIUR-500) Bioassay Systems; cholesterol by Randox; NEFA by Randox; Triglycerides by Randox, haptoglobin by PHASETM RANGE Haptoglobin Assay, Tridelata Development.

Experiment 1: Factors affecting the release of pregnancy-associated glycoprotein and its use for diagnosing embryo mortality in the dairy cow.

4.1 Introduction

Embryo mortality (EM) represents one of the major sources of economic wastage for the modern dairy industry. Most of the pregnancy losses occur during the first days after fertilization and during the process of implantation (Silke *et al*, 2002; McNeill *et al*, 2006).

Despite the important role that EM has on infertility in the dairy cow, causes and incidence of this phenomenon are still poorly investigated. The main reason is that it is difficult to discriminate non-pregnant and pregnant animals with an early diagnosis. Indeed, a diagnosis of EM can be made when, after a first positive pregnancy diagnosis, the cow is diagnosed not pregnant at subsequent examinations. Therefore, timing and accuracy of pregnancy diagnosis are to be considered essential requirements to investigate EM.

In this study artificial insemination (AI) outcome during the first 90 days post AI in dairy cows was monitored. A new method for bovine pregnancy diagnosis was used, never before applied in Italy. It incorporates the measurement of pregnancy-associated glycoproteins (PAG) in maternal blood. These proteins are produced by the binucleated cells of the trophoctoderm and can be detected in the maternal blood from 22-24 days after breeding until 80-100 days after delivering (Zoli *et al*, 1992). Once PAG are released in the maternal circulation, they can be used for pregnancy diagnosis, pregnancy follow-up, and for the monitoring of the trophoblastic function (Perényi *et al*, 2002b). PAG can also cross the mammary barrier, thus being detectable in milk throughout the gestation and up until parturition. Indeed, PAG molecules belonging to the previous gestation are not transferred from plasma to milk at the beginning of the new lactation.

PAG are considered an indicator of the presence of a viable embryo (see Lopez-Gatius *et al*, 2007a), and therefore plasma PAG assay could be a very useful method to diagnose embryo deaths. However, the use of milk to determine levels of PAG has attracted much attention lately, since the PAG belonging to the previous gestation decrease faster and more intensively in milk than in plasma and therefore they are no longer detectable in the early post-partum period. This would avoid false-positive diagnoses when AIs are performed before 80-100 days post-partum. Moreover, milk sampling avoids stressful

effects of venipuncture, does not require special expertise and is easier to collect and store than blood.

Little information is available in literature about PAG functions and PAG production regulation. PAG have been hypothesised to act as local immunosuppressive factors (Roberts *et al*, 1996) or as carrier molecules (Green *et al*, 1998), or else to have a role in parturition (Green *et al*, 2005a) or an endocrine function, perhaps acting upon the corpus luteum (CL) (Green *et al*, 1998). In particular, the latter aspect is of great interest because, CL being essential for pregnancy, it might help to better understand the mechanisms of the establishment and maintenance of pregnancy. The knowledge in regards to this is still controversial: some works have suggested (Weems *et al*, 1998; Del Vecchio *et al*, 1996) or not (Del Vecchio *et al*, 1995) an influence of PAG on P4 production by CL *in vitro*, whereas a more recent work *in vivo* reported an effect of P4 concentration on PAG production by the trophoblast (Ayad *et al*, 2007a).

Therefore, the objectives of this study were:

- (a) To verify whether the plasma PAG assay is a reliable method to perform pregnancy diagnosis in commercial dairy farms.
- (b) To develop a reliable milk PAG assay as a non-invasive method for measuring PAG.
- (c) To verify whether the PAG assay allows differentiation between an embryo mortality and inseminations that do not result in pregnancy.
- (d) To verify whether a relationship between PAG secretion and CL function exists and, in particular, whether P4 produced by CL at early pregnancy could affect the secretion of PAG.

4.2 Materials and methods

4.2.1 Experimental design

Sixty-one lactating cows and 18 heifers were used in this study which was conducted in a mixed breed herd comprised of Holstein-Friesian, Bruna Italiana, and Pezzata Rossa cows. The herd was located in north-eastern Italy (Vicenza) (45° 44' N; 11° 39' E) and it was ideal to test the PAG assay as a method to diagnose EM because of the high number of returns to oestrus after a positive pregnancy diagnosis reported by the farmer.

The reproductive status of the herd at the beginning of the observations was evaluated. Some productive and reproductive features of the herd are shown in table 4.1.

The study was conducted between December 2005 and August 2006 and involved all the cows and the heifers undergoing artificial insemination (AI). Blood samples were collected on Days 0 (AI) and, whether cows did not return to oestrus, on Day 21, 28, 30, 45, 60 and 90 after insemination. Samples were collected in tubes containing lithium heparin as an anticoagulant and analysed weekly for plasma concentrations of P4 and PAG as described in chapter 3.

Total number of cows	110 head
Total number of heifers	70 head
Breed	60%Pezzata rossa; 20%Holstein Friesian; 20%Bruna
Milk yield (average)	36 L/cow.day
Calving – 1 st AI interval	50 days
Days open	110 days
N° AI/conception	3 AI
1 st AI – conception interval	60-70 days

Table 4.1 Productive and reproductive features of the herd at the beginning of the observations.

Since PAG are not detectable in maternal serum during the first three weeks of pregnancy, PAG concentration was not measured on day 0. Milk samples were collected on the same days as the blood samples and analysed for PAG concentration as described in chapter 3.

Pregnancy diagnosis was carried out by means of PAG assay only. Pregnancy confirmation was performed by trans-rectal palpation after Day 90.

4.2.2 Data analysis

AI outcomes were classified in positive (AI+), negative (AI-), and embryo mortality (EM) according to the pregnancy diagnosis outcome.

Pregnancy diagnosis was performed by means of PAG concentrations at day 28 and 30 (according to Szenci *et al*, 1998 and Lopez-Gatius *et al*, 2007a):

- pregnant when $PAG \geq 0.8$ ng/ml

- not pregnant when PAG<0.8 ng/ml

P4 profile was also considered from Day 0 to Day 28 and 30. Luteal function was considered consistent with pregnancy when:

- at Day 0: P4<1 ng/ml
- from Day 21 on: P4>4 ng/ml

EM was diagnosed by considering the whole PAG profile, rather than just one threshold concentration of PAG, in association with P4 profile. Als presenting at least one value of PAG typical of early pregnancy (i.e. above the threshold concentration of 0.8ng/ml) followed by a steady decrease in PAG concentrations were classified as EM. At the same time, P4 profile had to show a functional CL at least until Day 21 followed by a decrease in P4 concentration.

In order to investigate a possible relationship between CL function and PAG secretion, P4 concentrations were classified into two groups depending on the level of P4 on Day 21: P4 concentrations higher than the mean were allotted to “high-P4 group”, whereas P4 concentrations lower than the mean were allotted to “low-P4 group”, as proposed by Ayad *et al.* (2007a).

P4 and PAG concentrations were analysed by the MIXED model of SPSS. The term of the model were the groups (High P4 vs Low P4), the cow within group, the days from AI, and the interaction group by day.

4.3 Results and discussion

4.3.1 Fertility, general observations

The reproductive situation observed in this farm far exceeded expectations. Almost half of the cows (49%) got pregnant at the first AI (figure 4.1), the days-open interval was 105 days, and the average number of AIs per conception was 2.15. These data show a clear improvement compared to the reproductive situation observed at the beginning of the trial (see table 4.1).

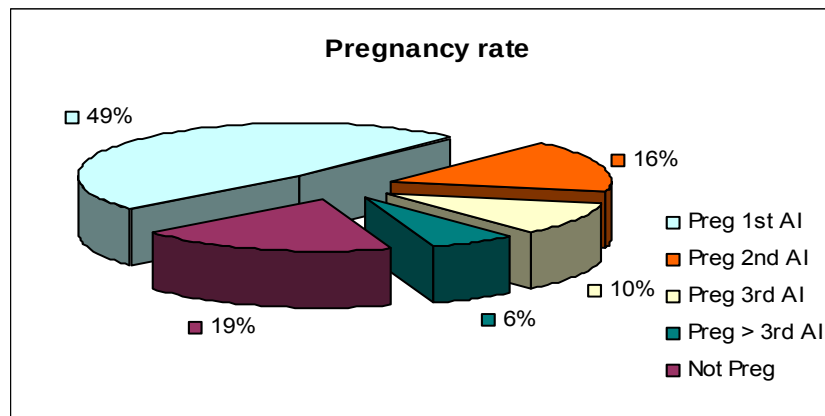


Figure 4.1 Distribution (%) of the pregnant cows per progressive AI.

An incidence of 9% EM was observed (figure 4.2). It was possible to identify those embryo deaths occurring after day 28 only, which according to the classification proposed by Humblot (2001), have to be considered LEM. Therefore, a 9% incidence of late embryo losses is in line with other studies reported in literature (for review see Santos *et al*, 2004).

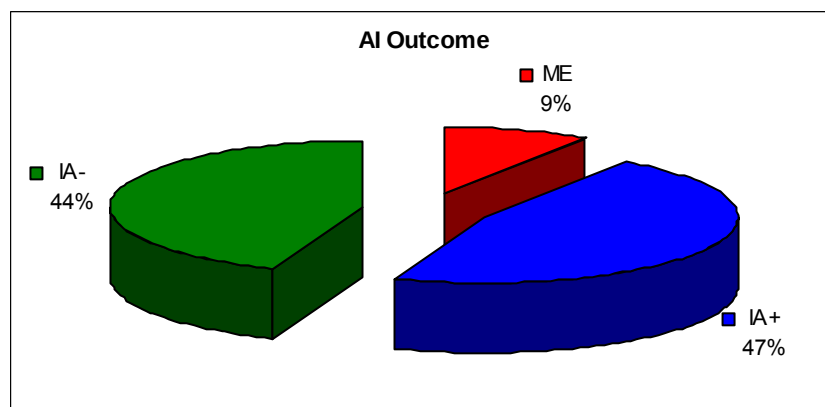


Figure 4.2 Distribution (%) of the AI outcome.

4.3.2 Positive AI

As previously described, AI outcome was assessed by means of PAG concentrations at days 28 and 30, in association with P4 profile. Considering the average profile of total positive AIs, the appearance of PAG molecules in detectable concentrations was observed from Day 28. In fact, at Day 21 PAG were not detectable in maternal circulation despite pregnancy being already established (figure 4.3). To some extent, this confirms the warning of Zoli *et al.* (1992) about the results of any PAG assay performed before Day 28 after AI in cows bred after 80 days post-partum. Indeed, due to individual variation in

PAG production, PAG are not always detectable in all pregnant cows before Day 28-30 post-AI. Therefore, PAG concentrations on Day 21 should be interpreted cautiously.

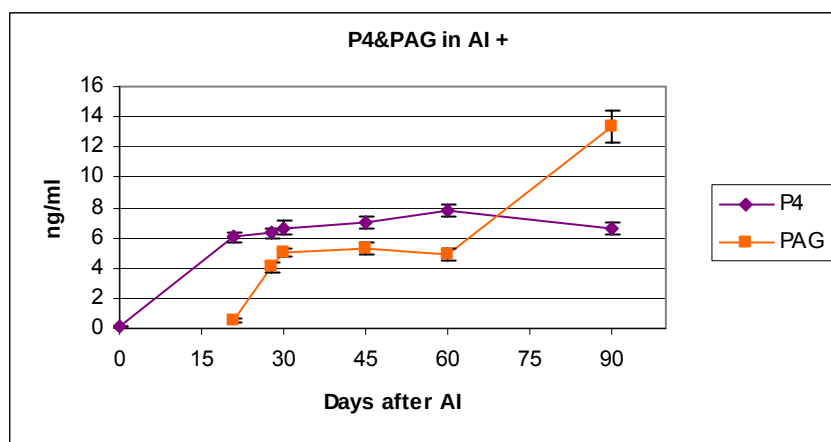


Figure 4.3 Mean P4 and PAG plasma concentrations in positive AI.

After the first appearance of PAG at Day 28, a plateau phase until Day 60 was observed. Similar trend was found by Sasser *et al.* (1986) who observed relative stable levels (3 ± 0.6 ng/ml) of pregnancy specific protein B (PSPB) between Days 42 and 70.

Throughout the whole sampling period there was a considerable amount of individual variation in the concentration of PAG (Table 4.2), as it is well documented in literature (Zoli *et al.*, 1992; Patel *et al.*, 1997; Green *et al.*, 2005a; Lopez-Gatius *et al.*, 2007a). In particular, several pregnant cows showed undetectable PAG level until Day 45. A similar pattern was reported also by Lopez-Gatius *et al.* (2007a) between Days 35 and 56.

	Mean± S.D. (ng/ml) (minimum-maximum)	
	Progesterone	PAG
Day 0	0.14 ± 0.10 (0.06-0.39)	
Day 21	6.02 ± 2.25 (0.75-12.14)	0.56 ± 1.08 (0.00-5.04)
Day 28	6.28 ± 3.12 (1.15-17.84)	4.07 ± 2.54 (0.00-15.06)
Day 30	6.65 ± 3.79 (1.00-21.59)	5.06 ± 2.24 (0.00-10.50)
Day 45	7.00 ± 2.81 (1.47-16.21)	5.28 ± 3.08 (0.00-16.23)
Day 60	7.80 ± 3.42 (1.57-15.36)	4.89 ± 2.85 (1.18-13.71)
Day 90	6.64 ± 3.12 (1.21-15.07)	13.37 ± 7.16 (3.85-34.20)

Table 4.2 P4 and PAG mean, minimum and maximum concentrations throughout the sampling period.

Despite a large range of individual concentrations observed in almost all the sampling days (Table 4.2), mean progesterone plasma concentrations clearly showed a fully functional corpus luteum. P4 concentrations increased from Day 0 (0.14 ± 0.10 ng/ml) to Day 21 (6.02 ± 2.25 ng/ml) remaining then relatively constant until Day 90.

There was no difference in PAG concentrations during the observation period between pregnant cows and heifers, therefore data collected from both cows and heifers were analysed together.

4.3.2.1 PAG and P4 profiles interpretation in positive AIs

Here some examples of PAG and P4 profile in positive AIs are shown.

All the following AIs were confirmed as positive and cows delivered healthy calves at the end of the gestation period.

Cow 342 showed typical P4 and PAG profiles of positive AIs (figure 4.4).

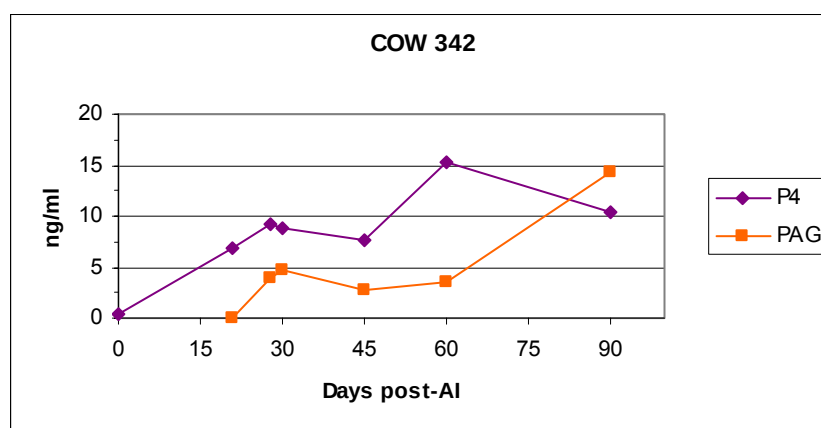


Figure 4.4 P4 and PAG plasma concentrations in the positive AI of cow 342.

A slight decrease of PAG concentration between Day 30 and 45 was observed. For this period of pregnancy, similar decrease has been found by other authors (Mialon *et al*, 1993; Perenyi *et al*, 2002b) suggesting that this kind of profile could be considered physiological.

However, not all profiles were so straightforward to interpret.

a) Normal P4 profile but very low PAG concentrations (cow 101; figure 4.5).

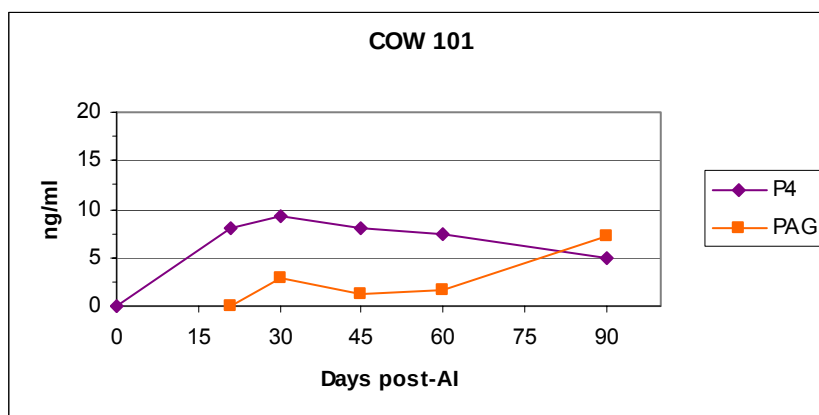


Figure 4.5 P4 and PAG plasma concentrations in the positive AI of cow 101.

PAG concentrations, particularly on days 45 and 60 (1.30ng/ml and 1.61ng/ml, respectively) were below the average concentrations reported in previous studies (Ayad *et al*, 2007a). They returned to normal pregnancy level only at day 90 (7.22 ng/ml).

b) Both PAG and P4 concentrations low (cow 350; figure 4.6).

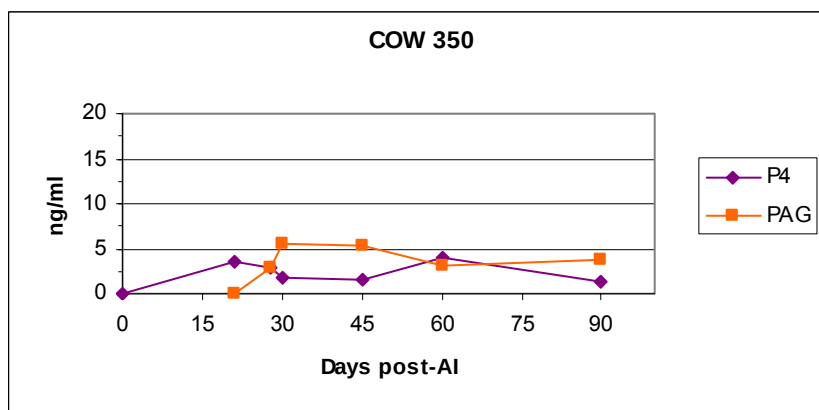


Figure 4.6 P4 and PAG plasma concentrations in the positive AI of cow 350.

P4 profile of cow 350 was not typical of pregnancy, showing concentrations <2ng/ml at days 30, 45 and 90 of pregnancy. This could be explained by the fact that, as P4 is transferred locally from the ovary to the uterus (Weems *et al*, 1989), peripheral concentrations of P4 may be less relevant for embryo survival than local (uterine tissue and lumen) concentrations (Lucy, 2001; Chagas e Silvas and Lopes da Costa, 2005).

Also PAG concentrations were slightly below the average concentrations reported in literature for this stage of pregnancy, particularly at day 60 (3.07ng/ml) and day 90 (3.85ng/ml). This could suggest a relationship between plasmatic P4 concentrations and PAG secretion in early stages of pregnancy.

4.3.3 Negative AI

Negative AIs were classified on the basis of the possible cause of failure of pregnancy establishment, by means of P4 and PAG profiles and the farmer's observations of the single cows (i.e. return to oestrus) (figure 4.9).

One of the aims of this trial was to differentiate between EM and AIs that do not result in pregnancy. Hence, negative AIs were divided into two main subpopulations: embryo mortality (EM) and all the rest (AI-). The next two graphs (figure 4.7 and figure 4.8) show quite clearly the difference in PAG and P4 profiles in cows that experienced EM and AI-.

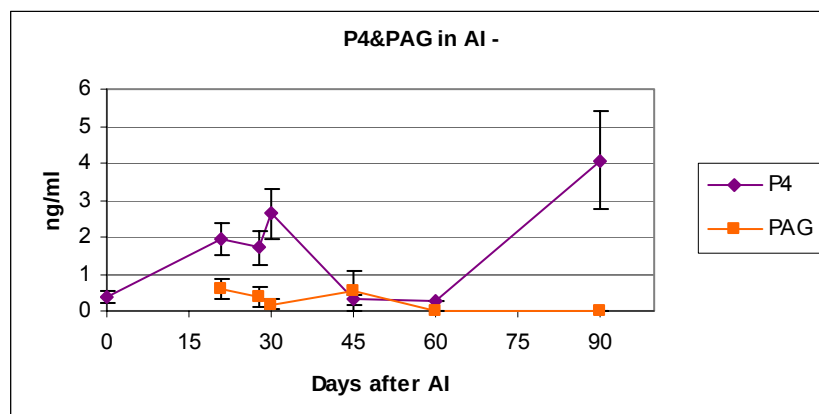


Figure 4.7 Mean P4 and PAG plasma concentrations in negative AIs.

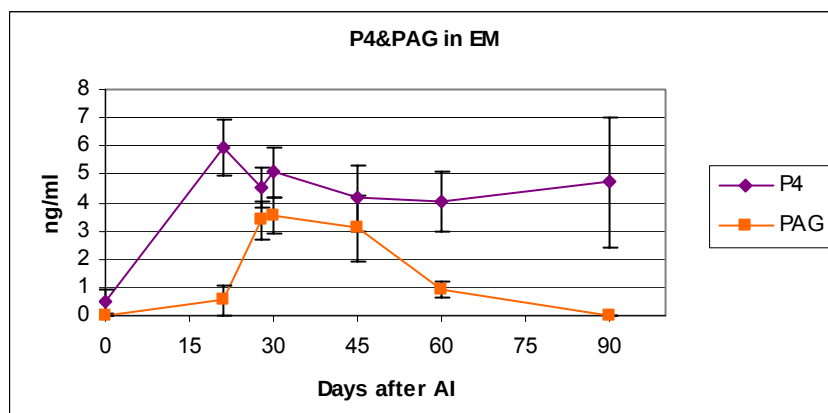


Figure 4.8 Mean P4 and PAG plasma concentrations in EM.

The little amount ($<0.8\text{ng/ml}$) of PAG observed in AI- (figure 4.8) could probably be due to the post-partum period when the samples were taken. Indeed, before 80 days post-partum, PAG belonging to the previous gestation are still detectable in the maternal serum. However, there could also be another explanation for this. Since PAG or a PAG-like protein has been found in bulls (Zoli *et al*, 1990 and 1992) and unbred heifers (Zoli *et al*, 1990 and 1992; Green *et al*, 2005a), it has been suggested that possible extraplacental sources of PAG, such as gonads, may be responsible for the detection of low concentrations of PAG in absence of pregnancy. These results mean that the detection of very low levels of PAG may not necessarily indicate pregnancy.

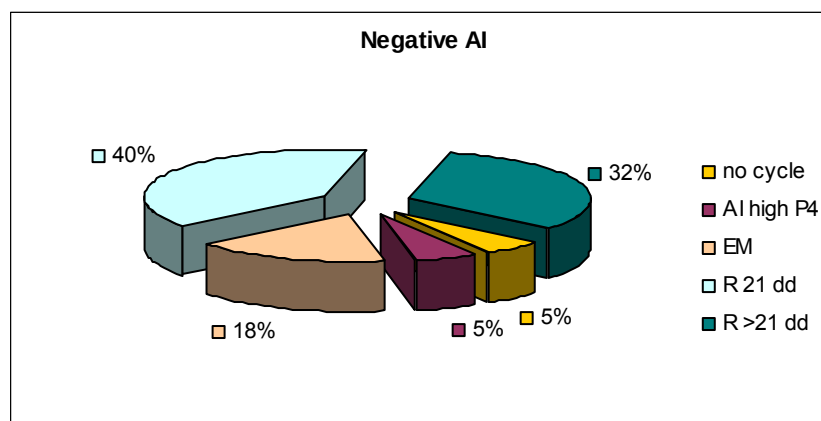


Figure 4.9 Distribution (%) of negative AI. Lack of ovarian activity (no cycle; $n=4$); AI performed with high P4 concentrations (AI high P4; $n=4$); EM ($n=13$); Return to oestrus at 21 days (R 21 dd; $n=29$); Return to oestrus after 21 days (R > 21 dd; $n=24$).

The following examples briefly explain some cases of negative AI.

1) “AI high P4”

In four cases (5.5% of total AI) cows were not inseminated during the oestrus phase of the cycle. Indeed, P4 concentration at Day 0 was $>1\text{ng/ml}$ which, according to Battocchio *et al*. (1999), represents an evolving CL.

2) “no cycle”

Cow 347 (figure 4.10) showed the typical P4 profile in the case of lack of resumption of ovarian activity after calving (Shrestha *et al*, 2004b; Kawashima *et al*, 2006).

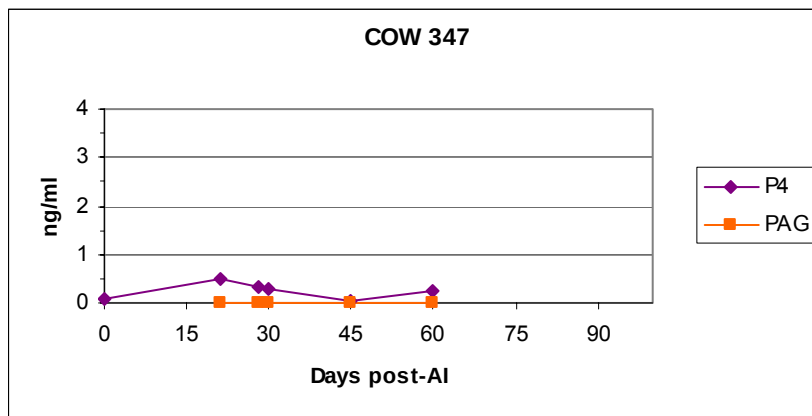


Figure 4.10 P4 and PAG plasma concentrations in the negative AI of cow 347 – an example of lack of ovarian activity.

3) “return to oestrus beyond Day 21”

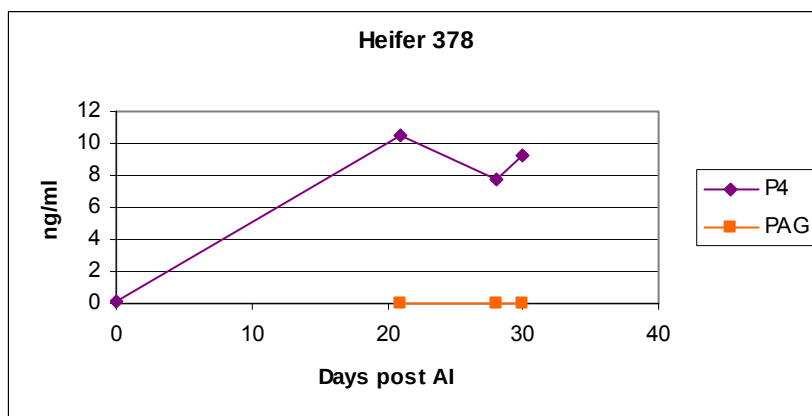


Figure 4.11 P4 and PAG plasma concentrations in the negative AI of cow 378 – an example of return to oestrus beyond Day 21.

In heifer 378 (figure 4.11), P4 levels increased from Day 0 to 21, remaining then around 8-10ng/ml until Day 30. P4 profile considered separately from PAG profile could be misleading by suggesting an embryo mortality case. Nevertheless, since there was no PAG production, this AI was classified as negative.

With a return to oestrus beyond 21 days post-AI, the presence of ovarian cysts, of which heifer 378 could be an example, or an embryo death occurring before PAG production begins could be hypothesised.

Luteal cysts are believed to be follicular cysts in later stages (Garverick, 1997). Cystic ovarian follicles develop as a consequence of a dysfunction of the hypothalamic-pituitary-ovarian axis that alters LH release. Due to their steroidogenic activity, cystic ovarian

follicles clearly delay/interfere with the physiological ovarian cycle, leading to an increased interval between calving and conception, number of services per conception and decreased pregnancy rate at the first insemination (Vanholder *et al*, 2006).

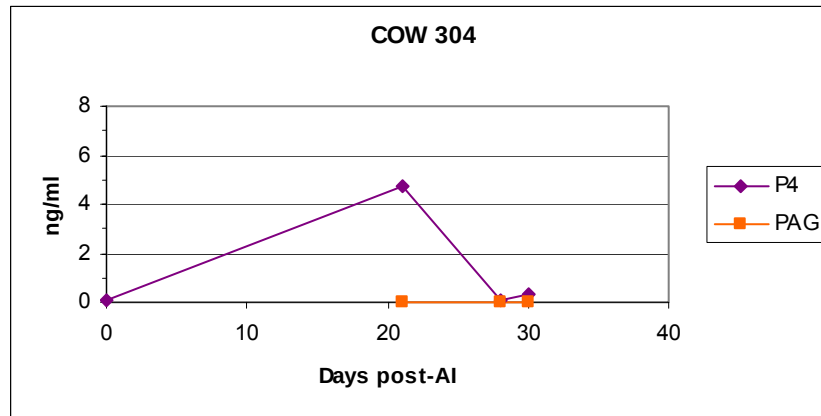


Figure 4.12 P4 and PAG plasma concentrations in the negative AI of cow 304 – an example of return to oestrus beyond Day 21.

Returns to oestrus beyond 21 days may also occur as a consequence of embryo death (Humblot, 2001). For example, in the case of cow 304 (figure 4.12), decrease in P4 concentration occurred between Day 21 and 28, indicating a prolonged lifespan of CL. It could be contested that, since there was no PAG production, the presence of an embryo should “not be supposed”. Actually, an embryo could die either before PAG production begins, or while PAG concentration is still too low to be detected (Humblot, 2001). In such situations, luteolysis is likely to be delayed and this may explain the high P4 level at Day 21. It can be hypothesised that the embryo starts to signal its presence to the mother which, consequently, prevents luteolysis. However, the embryo, perhaps not vital enough to fuel the maternal recognition of pregnancy process, eventually dies.

4) “return to oestrus at Day 21”

In most of these cases cows were inseminated again on the day of new oestrus, starting another cycle of sampling. One possible cause of return to oestrus is the repeated breeding syndrome. Repeated breeders are likely to present hormonal asynchrony around oestrus which leads to a series of dysfunctions such as prolonged standing oestrus and delayed ovulation (Båge *et al*, 2000). Particularly, with a delayed ovulation, the chances of performing AI at the right timing are impaired, resulting in fertilization failure or in asynchronous uterine environment and, consequently, early embryonic death.

4.3.4 Embryo Mortality

As described in the “Material and Method” of this chapter, to perform a EM diagnosis it is necessary to observe first a rise in both PAG and P4 concentrations (which indicates the presence of a viable embryo), followed then by a steady decrease.

In four cases of pregnancy failures the decrease first occurred in the P4 profile (e.g. cow 174, figure 4.13), in five cases it first occurred in PAG profile (e.g. cow 325, figure 4.14), in two cases P4 and PAG concentrations decreased at the same time (e.g. cow 224 3rd AI, figure 4.15), and in two cases it was not possible to see any decrease in the hormonal profiles because the cows returned to oestrus before the following sample was collected, starting a new cycle of sampling.

It could be speculated that pregnancy losses where “abnormality” first occurred in P4 profile were probably originating from a failure in the mother reproductive function.

The embryo loss experienced by Cow 174 (figure 4.13) might be an example of this.

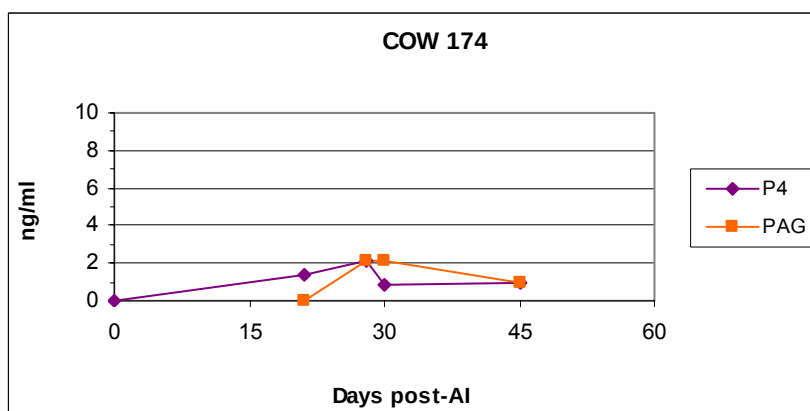


Figure 4.13 P4 and PAG plasma concentrations in the EM experienced by cow 304.

A deficiency of luteal function, characterized by the falling P4 levels at Day 30, could be observed prior to the embryo death, which in turn is indicated by a decrease in PAG secretion between Day 30 and 45. Probably, as a consequence of low P4 production, pregnancy recognition signals were not strong enough to assure adequate uterine functions, such as endometrial gland secretions and inhibition of myometrium contractivity, leading to the loss of the conceptus. This hypothetical pattern would be in line with previous studies reported in literature (Kerbler *et al*, 1997; Mann and Lamming, 2001; Whates *et al*, 2003).

The hormonal profile of cow 325 (graph 4.14) shows the opposite situation: “abnormality” first occurred in PAG concentration, as it can be observed from its sharp decline after Day 30, while the luteal function, according to the P4 profile, could be considered physiological until the day of embryo death.

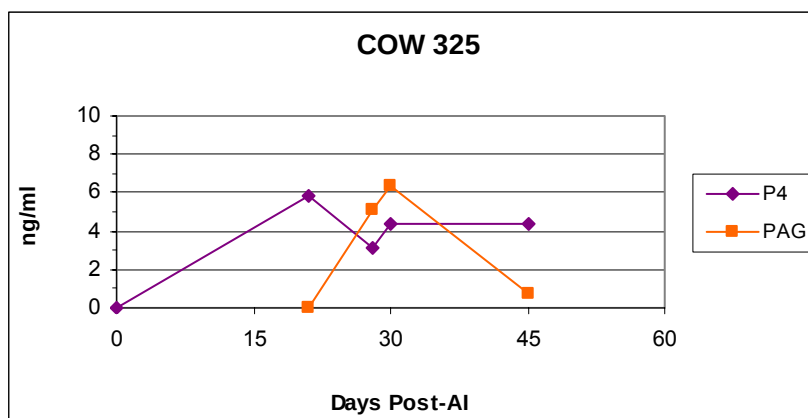


Figure 4.14 P4 and PAG plasma concentrations in the EM experienced by cow 325.

In this particular example, the decrease in P4 concentration can not be observed because the cow returned to oestrus before the sampling on Day 60 and was immediately inseminated. Overall five cases like this one were observed. The reason for the embryonic loss was probably the embryo. Genetic factors, such as chromosomal abnormalities, can impair the embryo’s ability to proceed through development (Hansen, 2002). Also placental abnormalities can cause embryo loss: underdeveloped placenta has less and smaller foetomaternal exchange areas than normal, which in turn would cause starvation and death of the embryos because of the insufficient supply of nutrients (Hill *et al*, 2000).

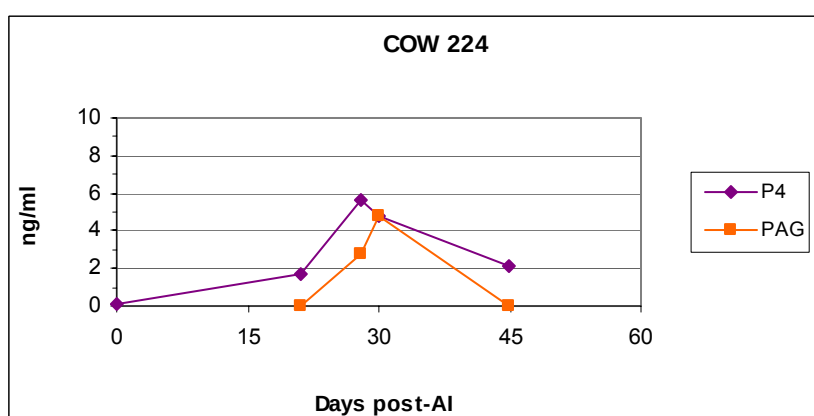


Figure 4.15 P4 and PAG plasma concentrations in the EM experienced by cow 224.

In two cases the decline in PAG and P4 concentrations occurred at the same time (cow 224; figure 4.15). In these cases the origin of the embryonic death could be in the mother (decreased, abnormal luteal function) or/and in the embryo (decreased viability of the trophoblastic cells).

In light of these hormonal profiles, it can be concluded that PAG profile combined with P4 profile could be a very efficient way to diagnose embryo mortality.

However, if considered separately, PAG and P4 concentrations may mislead sometimes. In particular, P4 profile is independent from embryo death if the corpus luteum persists some time after embryonic death. Szenci *et al.* (2003) found that, when embryo death occurs after implantation, complete luteolysis occurs within five to nine days after induction of embryo mortality. Therefore P4 production could be maintained for several days after embryo death.

On the other hand, in several studies (Humblot *et al.*, 1988; Szenci *et al.*, 2000; Serrano *et al.*, 2008) PAG have also been detected in the maternal circulation after embryonic death occurred, leading to the conclusion that embryonic membranes function with a certain independence of the embryo, continuing some of their functions following embryo death (Serrano *et al.*, 2008).

This suggests that PAG may be a better indicator of trophoblast function rather than embryo viability.

4.3.5 PAG and days post-partum

PAG concentrations decrease steadily in the post-partum period, and can still be detected in the maternal circulation 80-100 days after calving (Zoli *et al.*, 1992; Mialon *et al.*, 1993). PAG concentrations were analysed in relation to the post-partum period to verify whether the PAG molecules belonging to the previous gestation were still detectable in the maternal serum in the early post-partum. For this reason AIs performed in heifers were excluded from this analysis.

Plasma PAG concentration were significantly higher ($P < 0.01$) in samples collected before 80 days post-partum (figure 4.16).

The presence of PAG belonging to previous gestation in maternal serum before 80 days post-partum was even more evident when we considered negative AIs. Negative AIs were divided into two groups, according to the time of sample collection at D21 before or after 80 days post-partum. Mean PAG concentration of samples collected before 80 days post-

partum (n=6) was 3.72 ± 1.14 ng/ml, whereas in samples collected after 80 days post-partum (n=29) it was 0.48 ± 0.04 ng/ml. Among the 29 samples collected after 80 days postpartum, 27 samples had not detectable PAG concentration, while 2 samples, collected at Day 86 and Day 89, showed 4.19 ng/ml and 0.65 ng/ml of PAG, respectively. According to Zoli *et al.* (1992), undetectable concentrations (<0.2 ng/ml) are reached by Day 100 ± 20 post-partum. Likely, the long time needed for PAG to be cleared from maternal serum might be the result of either the very high concentrations reached at parturition or the long half-life of the protein in the blood (Zoli *et al.*, 1992).

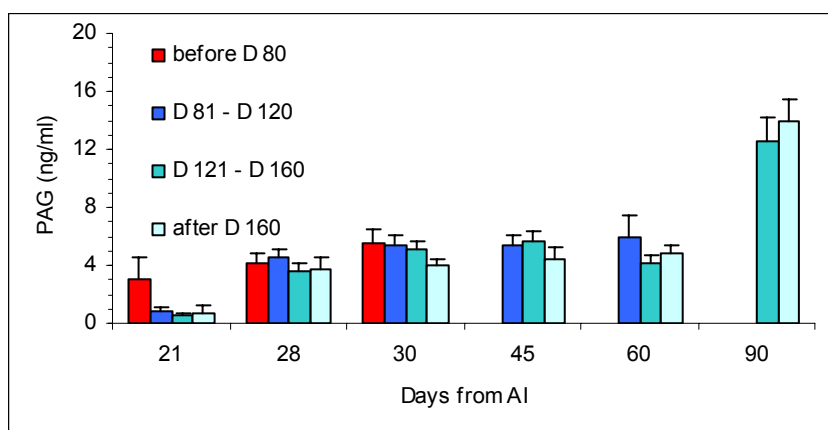


Figure 4.16 PAG concentrations in relation to days from AI and post-partum period in positive AIs.

The presence of PAG in maternal blood for nearly 100 days after calving can compromise pregnancy diagnosis in cows bred within this early post-partum period. For instance, 44.9% of false positives found by Szenci *et al.* (1998) were due to AIs that occurred within 70 days after calving.

Six cases of false-positive due to early post partum period were observed.

Therefore, plasma PAG can not be used as a pregnancy diagnosis method before 80 days post partum in dairy cows.

4.3.6 Relationship between P4 and PAG concentrations

In order to investigate a possible relationship between PAG secretion and CL function in early pregnancy, PAG and P4 profiles were analysed in positive AIs of 44 of the cows studied in this experiment. The animals used for this investigation were selected on the basis of three criteria: (1) only cows; heifers have been reported to produce more P4 than lactating cows (Sartori *et al.*, 2004; Wolfenson *et al.*, 2004), so, in order to obtain an

homogeneous group of animals, they were excluded; (2) positive pregnancy diagnosis and pregnancy carried to term; (3) AIs performed after 80 days post-partum to avoid interference with the PAG belonging to the previous gestation.

A luteotropic function of PAG has been suggested (Del Vecchio *et al*, 1996; Weems *et al*, 1998) but the results obtained by these *in vitro* studies can hardly reflect what actually might happen *in vivo*. In fact, in these studies, 5mg or 4µg/ml of PSPB, respectively, were added to mid-phase CL (12-14 days of the oestrus cycle) which are not the physiological concentrations of PSPB for this stage of pregnancy and CL development. Indeed, Humblot (2001) reported PSPB concentration to rise from Day 15 to 35 reaching 2 to 3 ng/ml at this stage in the cow.

The hypothesis that P4 produced by CL at early pregnancy (Day 21) affects the secretion of PAG was tested instead. This hypothesis has been investigated only recently (Ayad *et al*, 2007a) and it seems to confirm the pivotal role of P4 in regulating embryo development and function, as revealed by the positive correlation between P4 concentrations and PAG production.

Concentrations of P4 were classified into following two groups depending on the level of P4 on Day 21: P4 concentrations higher than the mean of $6,09 \pm 0,39$ ng/ml were allotted to “High P4 group”, whereas P4 concentrations lower than the mean were allotted to “Low P4 group” (figure 4.17).

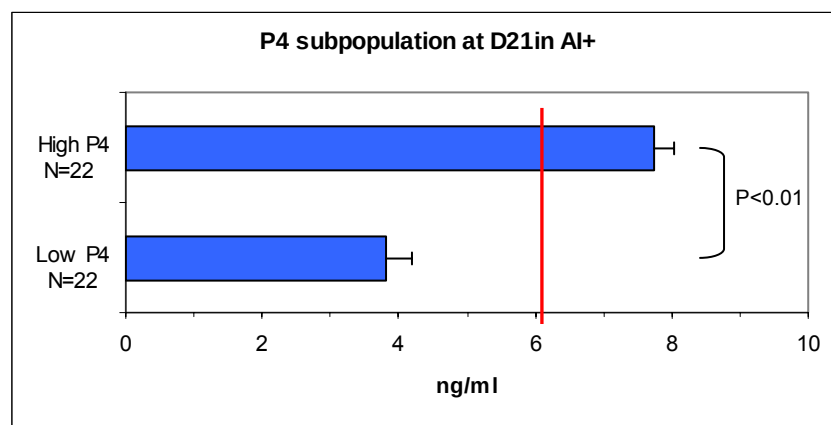


Figure 4.17 P4 mean concentrations in the two P4 subpopulations at Day 21 in positive AIs. The red line indicates the mean P4 concentration at Day 21 (6,1ng/ml).

Twenty-two animals were allocated to the Low-P4 group and twenty-two animals to the High-P4 group. Mean plasma concentrations of the two groups were significantly ($P < 0.01$)

different one another (Low P4 group: 4.77 ± 0.18 ng/ml; range 1.09-6.08 ng/ml; High-P4 group: 7.43 ± 0.17 ng/ml; range 6.13-12.28 ng/ml).

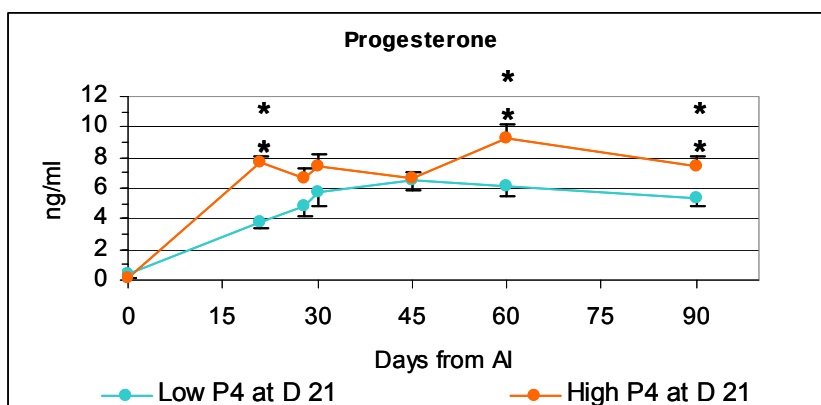


Figure 4.18 P4 profiles in the two P4 subpopulations at Day 21: “Low P4 group” and “High P4 group” (**, $P < 0.01$).

Progesterone profiles of the two groups of cows remained very different during the whole sampling period, particularly on Days 21, 60 and 90 (figure 4.18).

The high P4 group showed significantly higher overall PAG concentration between days 30 and 60 (figure 4.19 and figure 4.20).

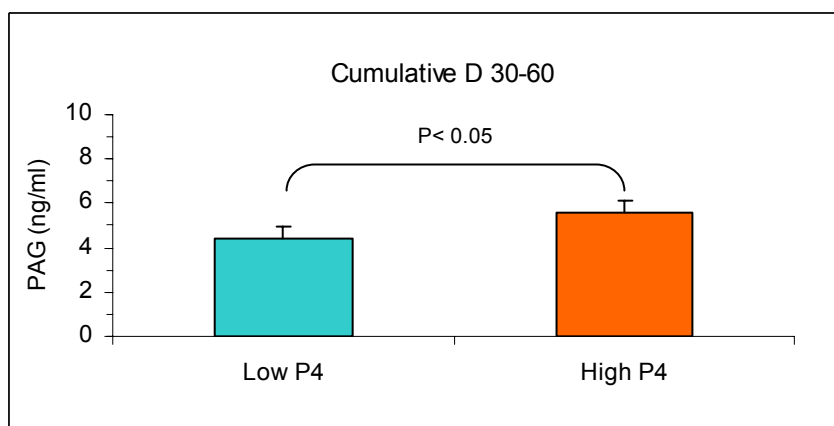


Figure 4.19 PAG concentrations in “low P4 group” and “high P4 group” between Day 30 and 60.

In particular, a significant difference was observed on day 60 ($P < 0.05$). The lack of significant difference in PAG concentration at Day 30 and 45 was possibly due to the high individual variation in PAG concentrations: at Day 30 mean PAG was 5.08 ± 0.34 ng/ml (range 0-10.50 ng/ml), and at Day 45 mean PAG was 5.03 ± 0.43 ng/ml (range 1.23-13.07 ng/ml).

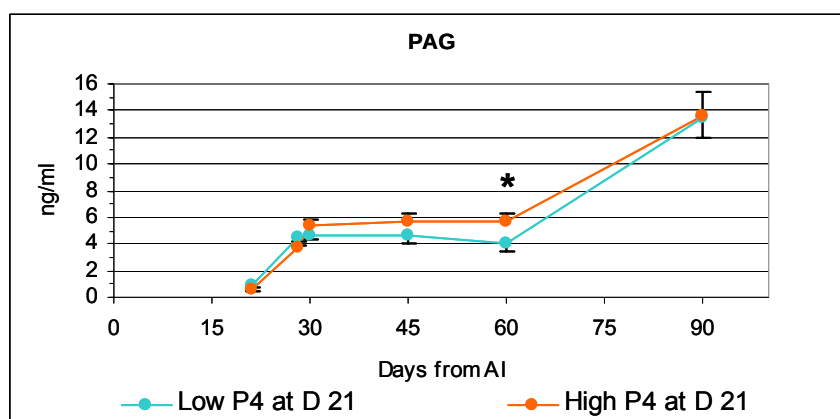


Figure 4.20 PAG profiles in the two P4 subpopulations at Day 21: “Low P4 group” and “High P4 group” (*, $P < 0.05$).

A similar result has also been found by Ayad *et al.* (2007a). In their study PAG concentrations at Day 80 of gestation were higher in high-P4 group at Day 21 than in low-P4 group at Day 21.

This result suggests that P4 concentration around the time of embryo implantation can affect the subsequent secretion of PAG by trophoblastic cells.

A similar effect of P4 has also been found on interferon-tau ($IFN\tau$) secretion by the mononucleate cells of the trophoblast: higher maternal P4 resulted in higher production of $IFN\tau$ (Kerbler *et al.*, 1997; Mann and Lamming, 2001). Since both $IFN\tau$ and PAG are produced and secreted by mono- and binucleate cells of the trophoblast, it can be concluded that high P4 concentrations during early pregnancy can positively affect trophoblastic functions. However, this result seems to be in contrast with previous findings of Lopez-Gatius *et al.* (2007a). They found no correlation between mean P4 concentrations on late embryonic period (Day 35-42) and PAG levels.

Considering all these results together, it may be suggested that P4 influence on PAG production probably occurs in the early embryonic period. A possible explanation might be that high P4 in early luteal phase provides a more suitable uterine environment for the developing embryo. This results in a conceptus that is larger and production of more trophoblast proteins, such as PAG and $IFN\tau$, that reach high concentrations in a shorter time.

On day 90 the difference in PAG concentration between Low and High P4 group was no longer evident. This result can not be compared with other results in literature because there are no other studies investigating the influence of P4 on PAG production later than day 80 of gestation in dairy cows.

4.3.7 PAG in milk samples: preliminary results

The PAG milk test is considered a better method for pregnancy diagnosis than the plasma one since milk sampling is easier to collect and store and, most importantly, the decrease of PAG concentration after parturition is faster and more accentuated in milk than in plasma (Metelo *et al*, 2004).

It was therefore considered relevant to test the reliability of the PAG milk test compared to the PAG plasma test.

Milk samples of 5 pregnant cows were analysed and then compared with plasma profiles of the same cows (figure 4.21).

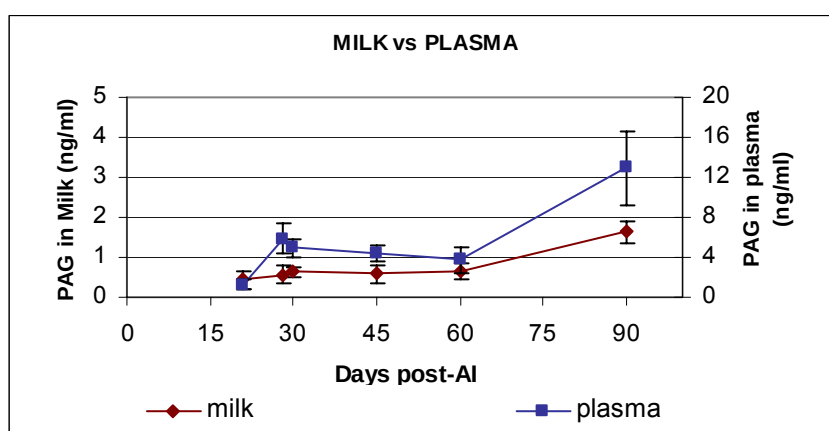


Figure 4.21 PAG profiles in milk and plasma of five pregnant cows.

During the first 60 days of pregnancy, PAG concentrations in milk were less than 1ng/ml. A significant increase in PAG concentration occurred only between Day 60 and Day 90. The low PAG concentrations observed in the early stages of pregnancy might pose a problem when using the milk PAG assay to perform pregnancy diagnosis in dairy cows. On the contrary, in dairy goats, milk PAG assay is considered an accurate method to perform early pregnancy diagnosis (PAG is detectable already from Day 32 after breeding; Gonzalez *et al*, 2001). However, from these preliminary results, it appears to be not the same for dairy cows, or at least not before 80-90 days after breeding. Therefore, as suggested by Metelo *et al.* (2004), the PAG milk test could be used as a later pregnancy diagnosis method in dairy cattle or for pregnancy confirmations.

Milk PAG profiles were compared with plasma profiles of the same cows. PAG concentrations in milk were much lower than in plasma (figure 4.21). The same pattern has been described in dairy goats, in which PAG concentrations in milk were about 10-

fold lower than in plasma (Gonzalez *et al*, 2001). This might be due to a high selectivity of the milk barrier towards PAG molecules. However, the kinetics of PAG in the maternal blood stream and its transport to other body compartments is still unknown.

It can be concluded that PAG concentrations in milk are lower than in plasma in the dairy cow.

4.4 Take-home message

The use of PAG provides complete monitoring of the pregnancy development and has a great prognostic value. However, the reliability of the PAG test and its use depends on its application.

PAG test application on commercial dairy farms as the only pregnancy diagnosis method needs to be evaluated carefully. In this study, sometimes AI outcomes were ambiguous (e.g. PAG concentrations too close to the threshold concentration for pregnancy, or abnormal fluctuations in PAG profiles) requiring a double check by either ultrasound or transrectal palpation. It would be appropriate to compare pregnancy diagnosis outcomes obtained by means of a PAG test with outcomes obtained by means of other methods (e.g. ultrasonography, transrectal palpation, or milk P4 profile), in order to better evaluate the PAG test as a routine pregnancy diagnosis method to use in commercial dairy farms.

However, the PAG test turned out to be very useful for research purposes not only because it performs a pregnancy diagnosis but also because it detects the presence of a viable embryo and allows one to follow either its development or death. In this way, investigations could aim to establish differential diagnosis of pathologies affecting pregnancy and the pathogenetic mechanisms involved in embryo losses. Moreover, the possibility to discriminate between EM and AIs that do not result in pregnancy is of great help. Indeed, the presence of an embryo in the uterus makes a big difference in terms of nutrients requirement for the mother and, most importantly, changes in her own physiology. Therefore, the factors involved in the negative outcome of AIs are likely to be different depending on whether the negative outcome is due to, for example, a fertilization failure or an embryo death.

P4 concentration in maternal blood around the time of embryo implantation seems to affect PAG production in early pregnancy. However it would be interesting to see whether P4 concentration in earlier or later stages of pregnancy has the same effect on PAG production.

Experiment 2: Relationship between embryonic mortality and oxidative stress in intensive dairy production system

5.1 Introduction

In the previous experiment the reliability of PAG assay as pregnancy test in commercial dairy farms was tested with satisfactory results. Although it allowed to perform pregnancy diagnosis in most of the cases, in some cases a check by ultrasounds was needed, particularly in case of embryo mortality (EM). Therefore, in the present study, in order to diagnose EM with greater precision, PAG test was combined with transrectal ultrasonography and P4 measurements in both plasma and whey. A refined method to diagnose pregnancy losses is an essential requirement to investigate the causes of EM.

Embryonic losses represent a major limitation to fertility in the modern dairy system but, despite the extensive investigation, the causes of this phenomenon are still poorly understood. Several factors have been suggested as implicated in EM: poor oocyte quality (Perry *et al*, 2002), an inadequate uterine environment (Gray *et al*, 2001a), asynchrony between mother endocrinology and embryo development (Roberts *et al*, 1996; Goff, 2002; Johnson *et al*, 2003) and maternal failure to respond to embryonic signals (Hansen, 2002; Green *et al*, 2005b). These events are strictly related to poor P4 production. Indeed, adequate corpus luteum (CL) activity is essential for the establishment and maintenance of pregnancy. CL activity, in turn, seems to be related to the resumption of ovarian activity (ROA) in the post-partum (Staples *et al*, 1990, Kawashima *et al*, 2006). Therefore, in this study, ROA and some features of ovarian cycle were investigated in order to establish whether they could be related to embryo survival and/or affect artificial insemination (AI) outcome.

Several papers indicate reactive oxygen species (ROS) and oxidative stress (OS) having a role in different aspects of the pathophysiology of reproduction in mammals, such as fertilization and early embryo development (for review see Agarwal *et al*, 2005; Fujii *et al*, 2005).

Dairy cows are exposed to various metabolic pressures to face high milk yield and environmental conditions. In such conditions, metabolic reactions can be overloaded and free radicals can accumulate. If free radicals generation exceeds body's antioxidant

production capacity, disturbance of the redox balance may occur and OS develops (Castillo *et al*, 2005; Tanaka *et al*, 2007).

In dairy cows, OS has been associated with several pathological conditions, such as retained placenta, udder oedema, and mastitis, which in turn may impair reproductive performances. For example, antioxidants deficiency may contribute to impaired muscle tone and thus uterine contractibility, resulting in decreased transport of sperm to ova or retained placenta (Miller *et al*, 1993a).

Excessive production of ROMs during OS has been reported to cause DNA damage, lipid peroxidation, cell membranes alteration, and protein damage (Sugino, 2006). In particular, radical-mediated protein oxidation is a complex process that may generate multiple products from aminoacid residues and the peptide backbone. Oxidized proteins are often functionally inactive and they can be more or, on the opposite, less susceptible to protease breakdown leading to accumulation (Moller and Kristensen, 2004). Moreover, they may activate the immune reaction and be the target of autoantibodies (Shanti *et al*, 1999; Palinski and Witztum, 2000; Kurien *et al*, 2006).

Advanced oxidation protein products (AOPP) are proteins damaged by oxidative stress and they arise from the reaction between plasma proteins and chlorinated oxidants mediated by a neutrophil enzyme myeloperoxidase (MPO) (Fialova *et al*, 2006; Noyan *et al*, 2006). In humans, they are responsible of induction of proinflammatory activities and cytokines, and increase in the circulation in some pathological conditions. Moreover, they are referred to as markers of OS and neutrophil activation (Witko-Sarsat *et al*, 1998; Shi *et al*, 2008).

Therefore, the hypothesis of a relationship between the AI outcome and OS biomarkers was tested. In particular, AOPP, as indicators of protein oxidation in dairy cows, were used in order to investigate the implication of protein oxidation in EM.

Finally, in the previous experiment P4 produced on Day 21 was found to affect PAG production (see chapter 4.3.6). It would be interesting to investigate whether an effect on PAG production is exert also by P4 produced in earlier stages of CL development (mid-luteal phase) since a positive effect of P4 levels around the time of implantation on trophoblastic function has already been proved by several authors (Kerbler *et al*, 1997; Mann and Lamming, 2001) by observing greater IFN- τ production associated with high P4 levels.

5.2 Materials and methods

5.2.1 Experimental design

This study was conducted in two high-yielding dairy herds located in the North of Italy (Mantova and Brescia, Herd A and Herd B, respectively) and selected because of their high incidence of embryonic losses, as referred by the farmers. Fifty Holstein-Friesian and 19 Jersey were used for this study, which was conducted over a ten months period from February to November 2006.

Blood samples were collected on Days 0 (AI) and, whether cows didn't return to oestrus, on Day 15, 28, 35, 45, and 60 after AI. Heparinized tubes were used to collect the samples. Plasma concentrations of P4, PAG, malondialdehyde (MDA), glutathione (GSH), AOPP, total proteins (TP), albumins (ALB), globulins (GLOB), urea, glucose (GLUC), cholesterol (CHOL), triglycerides (TG), NEFA, and β OH-butyrate were measured as described in chapter 3. Since PAG can not be detected in maternal blood before day 24-25 after breeding, PAG concentration was not measured on day 0 and 15. In order to assess whey progesterone, milk samples were taken every 3-4 days from day 15 post-partum to day 45 after each positive AI confirmed by pregnancy diagnosis carried out by transrectal ultrasonography between day 25 and 35 after AI. Confirmation of pregnancy was performed after day 45, once the attachment period was completed and the placenta well developed.

5.2.2 Data analysis

5.2.2.1 Criteria used to establish AI outcome

Pregnancy diagnosis were performed by transrectal ultrasonography between days 25 and 35 after AI. According to the criteria used also by Szenci *et al.* (1998) and Romano *et al.* (2006), a positive pregnancy diagnosis was made when a non-echodense fluid in the uterine lumen, a corpus luteum on the ipsilateral ovary, the allantochorion and an embryo were seen. If an embryo was seen its heart beat was evaluated.

AI outcome was classified as EM when the presence of an embryo without a heart beat was detected at the time of the ultrasonography diagnosis, or when a positive ultrasound test was followed by a negative one (according to Szenci *et al.*, 1998).

Pregnancy diagnosis was also performed by means of PAG concentrations at day 28 and 35 in association with plasma P4 profile, as described in the chapter 4.2.2.

AI outcomes were then classified as positive (AI+:AI resulted in pregnancy), negative (AI-: AI not resulted in pregnancy), or embryonic mortality (EM: AI resulted in embryonic loss).

The degree of agreement between the clinical diagnosis performed between day 25 and day 35 after AI using transrectal ultrasonography and biochemical diagnosis based on PAG and P4 profiles in the maternal blood between day 28 and day 45 was evaluated by the calculation of the Cohen's kappa. The kappa value ranges between 0 and 1. A value of 1 indicates perfect agreement, while a value of 0 indicates that agreement is no better than a chance (SPSS).

The distributions of the two related variables (clinical and biochemical diagnosis) was studied for each clinical outcome using the marginal homogeneity test for categorical variables, and each clinical outcome was compared with the related distribution of the biochemical diagnosis (SPSS).

5.2.2.2 Criteria used to classify features of ovarian activity

Whey P4 profiles were used to classify some features of ovarian activity, such as resumption of ovarian activity after calving (ROA), and length of follicular and luteal phases, some of which are shown in figure 5.1.

Resumption of ovarian activity: at least 1 sample with $P4 > 200 \text{ pg/ml}$

- early: if before 42 days post-partum
- late: if after 42 days post-partum

Length of follicular phase: on the basis of the number of samples with $P4 \leq 100 \text{ pg/ml}$

- short: less than 3 days (1 sample)
- physiological: 4-6 days (2 samples)
- long: more than 7 days (3 or more samples)
- absent: when AI occurred at the first heat, with no previous ovarian activity

Length of luteal phase: on the basis of the number of samples with $P4 \geq 200 \text{ pg/ml}$

- short: less than 10 days (1-3 samples)
- physiological: 11-18 days (4-6 samples)
- long: more than 18 days (7 or more samples)

- absent: for luteal phases preceding AIs performed at the first heat, with no previous ovarian activity

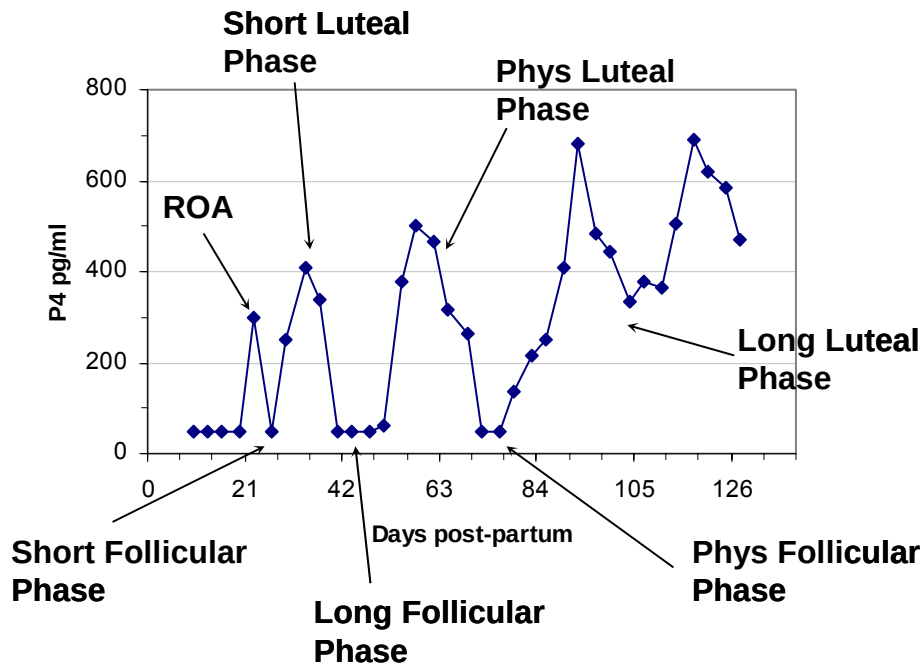


Figure 5.1 Whey P4 profile.

The relationships between the AI outcome and the ROA, and between the AI outcome and the length of oestrous cycle phases were evaluated by the calculation of the contingency coefficient. This is a measure of the degree of association between two nominal variables and varies between 0 and 1. A value close to 0 indicates poor association, while a value near 1 indicates a high degree of association (SPSS).

5.3 Results and discussion

5.3.1 Fertility, general observations

The number of cows that became pregnant at each progressive AI is shown in figure 5.2. It is interesting to note that only 20 animals (29%) became pregnant at the first AI, and, in both herds, several other attempts had to be performed in order to reach a pregnancy rate of 88.2% and 77.1% for herd A and herd B respectively, at the end of the

experimental period. Indeed, the number of AIs per conception was 2.9 and 2.3, respectively.

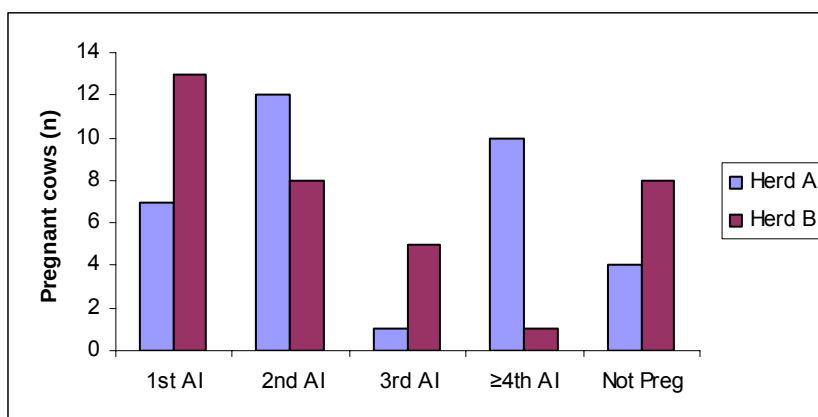


Figure 5.2 Distribution (N) of pregnant cows per progressive AI in Herd A and Herd B.

Fifty-nine AI+, 89 AI- and 9 EM were observed (figure 5.3 and table 5.1).

It is possible to identify late embryo deaths only (i.e. those occurring after days 25-35), therefore compared to the incidence of late embryonic losses reported by Santos *et al.* (2004) (12.8% after day 27), the 6% observed in this study has to be considered below the average. In fact, it is interesting to note that the farmers had a wrong perception of the incidence of embryonic losses in their farms, since they considered their herds as highly subject to pregnancy losses. This is particularly evident in Herd B, in which only 2 cases of EM were reported (see table 5.1).

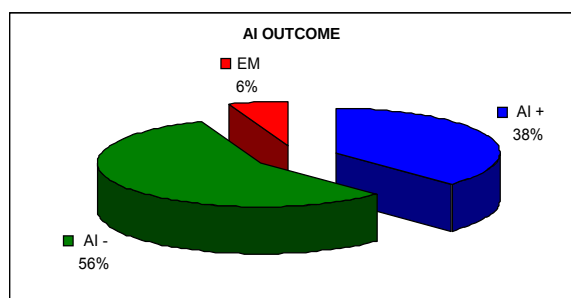


Figure 5.3 Distribution (%) of the AI outcome.

	Herd A	Herd B
AI +	32	27
AI -	54	35
EM	7	2
tot AI	93	64

Table 5.1 Distribution (N) of the AI outcome in the two herds

5.3.2 Comparison between clinical and biochemical pregnancy diagnosis

The outcome of pregnancy diagnosis performed by transrectal ultrasonography (clinical diagnosis) was compared with the outcome obtained by the simultaneous examination of PAG in plasma and P4 in whey and plasma (biochemical diagnosis) (table 5.2).

			Clinical Diagnosis (N)		
			EM	AI-	AI+
			8	89	59
Biochemical Diagnosis (N)	EM	7	5	1	1
	AI-	83	0	83	0
	AI+	66	3	5	58
Sig.			0.050	0.102	0.317

Table 5.2. Agreement between clinical diagnosis performed between day 25 and day 35 after AI using transrectal ultrasonography and biochemical diagnosis based on PAG and P4 profiles in the maternal blood between day 28 and day 45. Each clinical outcome was compared with the related distribution of the biochemical diagnosis using the marginal homogeneity test (SPSS). Criteria for PAG and P4 profiles interpretation are described in Material and Methods (chapter 4.2.2).

Legend: EM: Embryonic Mortality; AI-: artificial insemination not resulted in pregnancy; AI+: artificial insemination resulted in pregnancy.

One AI was omitted by the statistical analysis because the clinical diagnosis did not fully respect the criteria established by the experimental design. It is reported below since it represents an interesting case that may occur in commercial dairy farms.

Clinical diagnosis = EM, biochemical diagnosis = AI- (cow 712, Herd A; figure 5.4).

In this case, the veterinarian saw the uterus and CL typical of pregnancy but not the vesicle with the embryo. He hypothesized the presence of an embryo which had died and the vesicle reabsorbed before the ultrasonographic diagnosis was performed. Therefore, the clinical diagnosis was of EM. P4 profiles showed a long luteal phase after the AI (more than 28 days) which could aid to make a positive pregnancy diagnosis. However,

no maternal plasma PAG increase was observed, suggesting no signs of trophoblastic function.

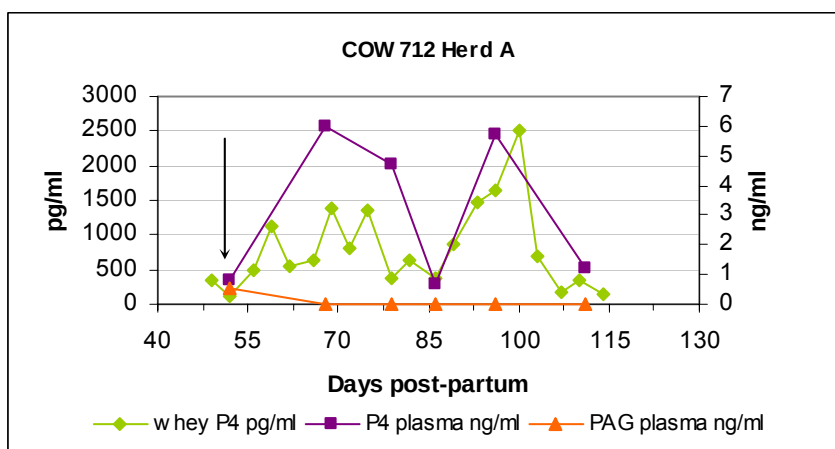


Figure 5.4 P4 and PAG profiles of cow 712, herd A. Black arrow indicates AI. Clinical diagnosis = EM, biochemical diagnosis = AI-.

The two methods of diagnosis showed an overall high level of agreement ($K=0.869$; $T=12.45$; $P<0.001$). Indeed, total agreement between the two methods was observed in 144 AI outcomes out of 156 (92.3%), being the three hormonal profiles (plasma PAG, plasma and whey P4) in agreement with clinical diagnosis and within each other. An example of this is shown in figure 5.5 (cow 268 herd B, case of EM occurred after Day 35, according to both clinical and biochemical diagnosis).

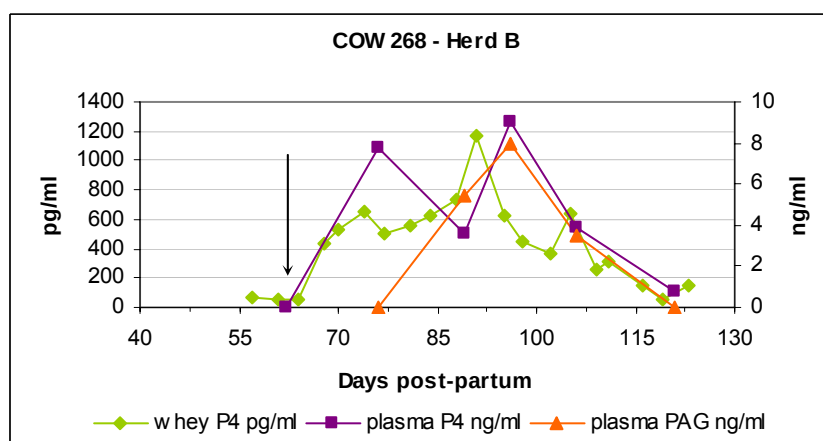


Figure 5.5 P4 and PAG profiles of cow 268, Herd B. Black arrow indicates AI. Clinical diagnosis = EM, biochemical diagnosis = EM

However, some cases of disagreement between the two diagnosis were found. In particular, the cases of EM as diagnosed by the evaluation of P4 and PAG profiles were

significantly less than those diagnosed by transrectal ultrasonography (respectively: 5 vs 8; $P < 0.05$), and three cases of false positive AI were observed.

Below, some cases of disagreement between the two diagnosis are reported as examples.

a) Clinical diagnosis = EM; biochemical diagnosis = AI+ (cow 292, Herd A, both AIs; figure 5.6).

In both the AIs performed in the cow 292, the veterinarian saw the vesicle with the embryo but the conceptus did not show the typical signs of vitality (e.g. heart beat) so $\text{PGF}_{2\alpha}$ was prescribed in order to induce vesicle expulsion and return to oestrus. The sharp decrease in P4 concentration typical of $\text{PGF}_{2\alpha}$ treatments can be clearly observed after day 35 in both whey and plasma profiles. When the clinical diagnosis was performed, PAG concentrations were still high giving rise to false positive (see Day 35 in both AIs).

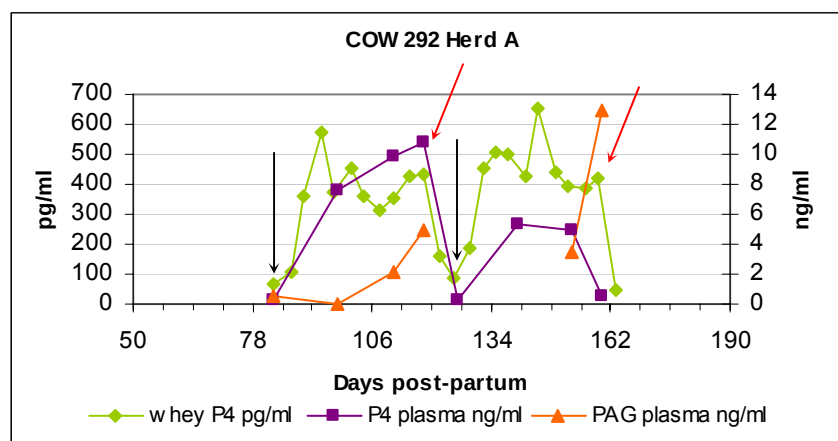


Figure 5.6 P4 and PAG profiles of cow 292, Herd A. Black arrows indicate AI; red arrows indicate $\text{PGF}_{2\alpha}$ treatments. Clinical diagnosis = EM, biochemical diagnosis = AI+

Considering this example, it could be hypothesised that the trophoblastic cells keep producing PAG for several days after embryo death. That could explain a diagnosis of EM (vesicle with embryo but no heart beat) while PAG concentration was still high. If this were true, one should ask if PAG analysis is a reliable method to monitor embryo viability or rather if it is a method to evaluate the trophoblastic cells function. Other authors had to face this question, since pregnancy losses have been observed in cows with high PAG concentrations (Lopez-Gatius *et al*, 2007b; Serrano *et al*, 2008). These authors came to the conclusion that, in the cow, foetal membranes are capable of continuing some of their

activities following embryo death and, hence, low PAG values can be indicators of placental disturbances more than foetal defects.

This observation would also confirm the results found in the previous experiment of this thesis (chapter 4.3.4), bracing the hypothesis that PAG may be a better indicator of trophoblastic function rather than embryo viability.

b) Clinical diagnosis = AI+, biochemical diagnosis = EM (cow 52 Herd B; figure 5.7).

Sometimes the disagreement between clinical and biochemical diagnosis may be due to the different time when the two pregnancy diagnosis are performed. In this study, two inseminations presented this situation and one of them is shown in figure 5.7.

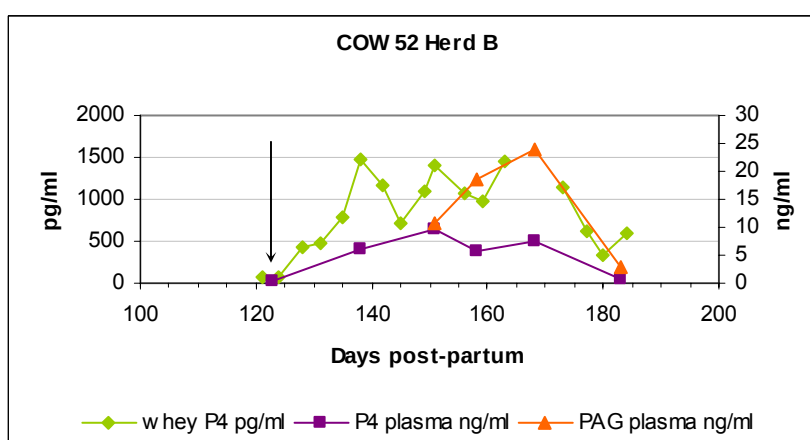


Figure 5.7 P4 and PAG profiles of cow 52, Herd B. Black arrow indicates AI. Clinical diagnosis = AI+, biochemical diagnosis = EM

The clinical diagnosis was performed between day 25 and 35 showing a viable embryo, whereas embryo death occurred after day 45, as clearly indicated by the decrease of both PAG and plasma and whey P4 concentrations.

c) Clinical diagnosis = AI-, biochemical diagnosis = AI+ (cow 25 Herd B; figure 5.8).

This is a good example that highlights that there is not a perfect method to perform a pregnancy diagnosis in dairy cows. In this case, after observing carefully the three hormonal profiles, as well as considering when the subsequent AI was performed, the final diagnosis could be of EM. Indeed, the AI did not result in pregnancy as the cow returned to oestrus and was inseminated again, but at the same time there was production of PAG and, likely, the sample at day 60 (if done) could have shown the decrease in PAG concentration characteristic of embryo death.

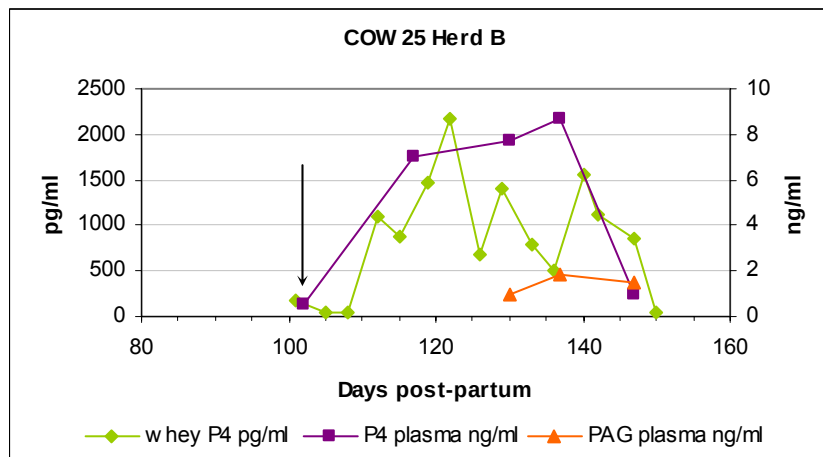


Figure 5.8 P4 and PAG profiles of cow 25, Herd B. Black arrow indicates AI. Clinical diagnosis = AI-, biochemical diagnosis = AI+

d) Clinical diagnosis = AI-, biochemical diagnosis = AI+ (cow 483, Herd A; figure 5.9). The case of cow 483 is even more interesting.

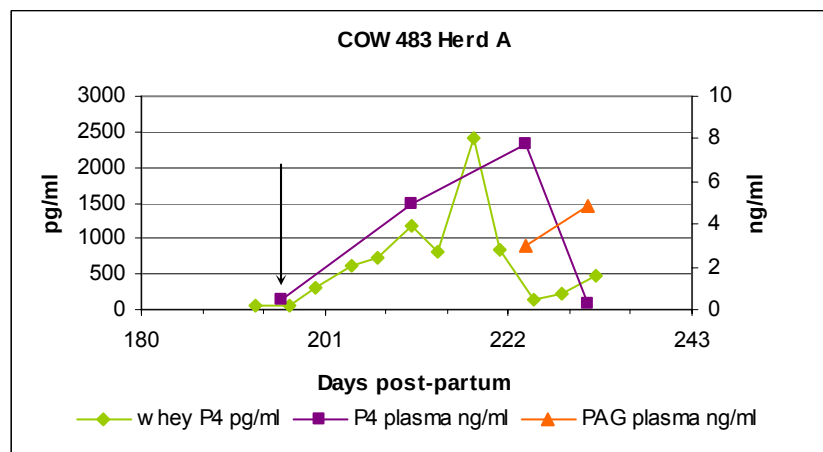


Figure 5.9 P4 and PAG profiles of cow 483, Herd A. Black arrow indicates AI. Clinical diagnosis = AI-, biochemical diagnosis = AI+

Probably the embryo was so small and poorly developed that it was not detectable by ultrasounds. Therefore the clinical diagnosis was negative. On the contrary, PAG concentrations on days 28 and 35 were typical of pregnancy (2.96 ng/ml and 4.83 ng/ml, respectively) and also plasma P4 at Day 28 seemed to support this diagnosis. Nevertheless, the pregnancy was not maintained and the final AI outcome was negative.

Overall, some limitations of PAG as pregnancy diagnosis test were found in this study, in particular in case of EM. An example is the cow 292 herd A (figure 5.6), where, despite

the embryo being diagnosed as not vital, PAG levels were still high. Other authors found a similar situation. According to Szenci *et al.* (1998 and 2000), a limitation to the accuracy of pregnancy diagnosis by PAG assay is that, after EM (accurately diagnosed by ultrasonography), protein levels remain above the threshold level for several days, giving rise to false positive results. This might be due to the long half life of PAG (Szenci *et al.*, 2000) but also to the fact that, apparently, the trophoblast keeps delivering PAG into the maternal blood stream also after embryo death (Serrano *et al.*, 2008). Also in another study (Semambo *et al.*, 1992) EM was most conveniently estimated by the ultrasound investigation rather than by PSPB or P4 assays: P4 was maintained at concentrations similar to those achieved during a normal pregnancy for a period of at least 20 days after the induction of abortion, whereas PSPB showed a plasma half-life of 7 days after that. Therefore, it could be concluded that the assay of PAG is very useful for diagnosing embryo mortality, using serial plasma samples from the same cow.

However, as previously described in this thesis (Chapter 2.2.3), the ideal pregnancy diagnosis method should be able to determine pregnancy status at the time the test is performed, not by means of two subsequent tests or comparisons between two or more hormonal profiles. Some of the examples reported above do question reliability of one single measurement of PAG as pregnancy diagnosis method. This disagrees with the findings of Lopez-Gatius *et al.* (2007b) who argue that PAG measurements from one single sample taken at day 35 of gestation provide more useful information than a series of values obtained from day 35 to 63 of gestation, and they can be indicators of subsequent foetal loss.

5.3.3 Relationship between P4 and PAG concentrations

On the basis of the results obtained in the previous experiment (chapter 4.3.6), where P4 produced on Day 21 was related to PAG concentrations, the relationship between P4 produced in earlier stages of CL development (mid-luteal phase) and PAG was here investigated.

Positive AIs were classified into two groups depending on the level of P4 on Day 15: AIs with P4 concentrations at Day 15 higher than the mean (6.26 ng/ml) were allotted to “High P4 group”, whereas AIs with P4 concentrations at Day 15 lower than the mean were allotted to “Low P4 group”. P4 and PAG concentrations were then analysed by the MIXED model of SPSS, as described also in chapter 4.2.2.

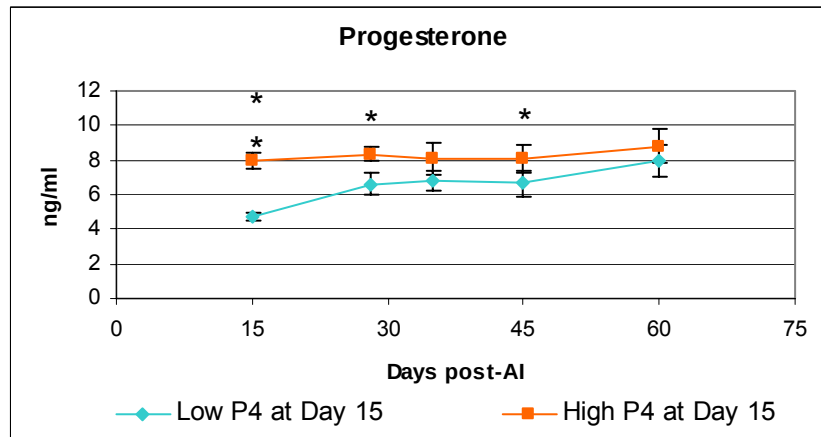


Figure 5.10 Progesterone profiles in the two P4 subpopulations at Day 15: “Low P4 group” and “High P4 group” (**, $P < 0.01$; *, $P < 0.05$)

Thirty-two AIs were allocated to the low-P4 group and twenty-three AIs to the high-P4 group. Mean plasma concentrations of the two groups were significantly different one another (Low P4 group: 4.92 ± 0.18 ng/ml; range 1.93-6.20 ng/ml; High P4 group: 8.12 ± 0.35 ng/ml; range 6.33-12.92 ng/ml; $P < 0.01$). P4 profiles of the two groups remained very different during the whole sampling period (figure 5.10).

Although a positive effect of P4 levels around the time of implantation (Day 14-16) on trophoblastic function has been proved by observing greater IFN τ production associated with high P4 levels (Kerbler *et al*, 1997; Mann and Lamming, 2001), in this study no difference in PAG concentration between Low P4 group and High P4 group was observed (figure 5.11).

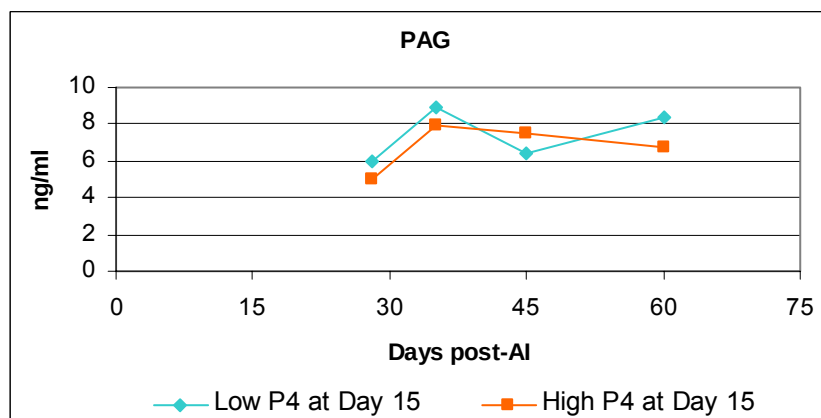


Figure 5.11 PAG profiles in the two P4 subpopulations at Day 15: “Low P4 group” and “High P4 group”.

5.3.4 Resumption of ovarian activity (ROA)

Dairy cows have classically been described as ovulating between 14 and 21 days after calving (Lucy *et al*, 2001). However, in modern dairy cows, the average interval to first ovulation has become longer (29.7 days, deVries and Veerkamp, 2000). Even longer interval from calving to first ovulation has been described by Shrestha *et al*. (2004a): in 63% of cows ROA did not occur until >45 days post partum.

Early resumption of normal ovarian function is directly related to higher fertility since intervals from calving to first ovulation, first service, and conception are extended when ROA is delayed (Staples *et al*, 1990; Royal *et al*, 2000; Westwood *et al*, 2002). For instance, Kawashima *et al*. (2006) found that a larger number of cows that had ROA >21 days after calving experienced abnormal subsequent cycles (short or prolonged luteal phases) than cows with ROA within 3 weeks. Early ROA allows cows to have two or more oestrus cycles prior to breeding, which improves luteal function from first to subsequent oestrus cycles after calving (Staples *et al*, 1990, Kawashima *et al*, 2006). Adequate luteal activity, in turn, is essential for the establishment and maintenance of pregnancy (Goff, 2002; Spencer *et al*, 2004). Therefore, factors affecting luteal activity in the post partum may have effects on the AI outcome.

In this study 57% and 43% cows with early (<42days after calving) and late (>42days after calving) ROA, respectively, were found (figure 5.12).

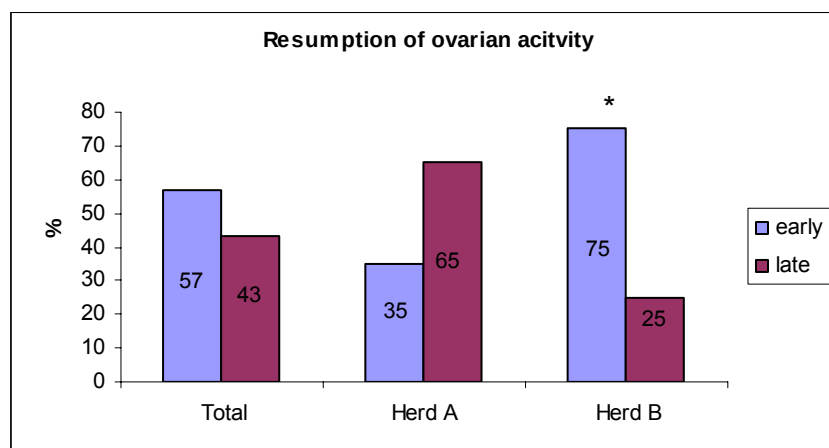


Figure 5.12 Distribution (%) of cows showing early or late ROA in Herd A, Herd B, and in total (*, $P < 0.05$)

Since there was a significant difference within Herd B, which was the herd with two different breeds (Holstein and Jersey), a breed effect on ROA was hypothesized. Indeed,

as reported by Campbell *et al* (1999), Jerseys are more likely to restart ovarian activity earlier than Holsteins. In spite of this, there was no difference between the two breeds since 75% of the Holsteins ($P=0,046$) and 79% of the Jerseys ($P=0,025$) had an early ROA.

The AI outcome was not associated with the ROA (table 5.3), and the contingency coefficient was 0.146.

		AI outcome (N)		
		EM	AI-	AI+
ROA	Before day 42	2	41	32
	After day 42	7	47	27

Table 5.3 Relationship between the AI outcome and the beginning of oestrous activity.

Although early ROA cows should be more likely to receive the first AI sooner than late ROA ones, there was no difference in term of days post-partum when the 1st AI was performed. A significant difference was observed in coincidence with the 2nd and the 3rd AIs instead. Late ROA cows had 2nd and 3rd AI later on the post partum (129 ± 11.7 days post-partum and 156 ± 12.2 days post-partum, respectively) compared to early ROA cows (2nd AI at 94 ± 7.9 days post-partum and 3rd AI at 132 ± 13.5 days post-partum; $P=0,044$) (figure 5.13).

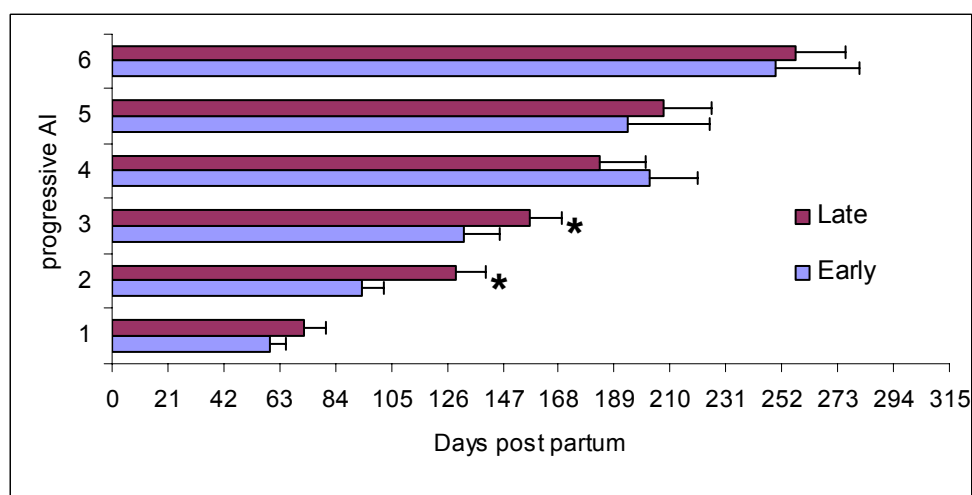


Figure 5.13 Relationship between days post-partum and progressive AI in Early ROA and Late ROA cows (*, $P<0.05$).

In particular, between the first and the second AI of late ROA group, a further oestrus cycle may have occurred (the interval was 43 days). If so, a poor oestrous detection may be responsible for the delay in performing the second AI, suggesting then a management cause. However, no herd effect was found in support of a management problem.

There could be a physiological reason responsible for this, and may relate to the length of the luteal phases following the AIs that did not result in pregnancy (i.e. AI- and EM) (see table 5.4).

	Luteal phase after AI	Early ROA	Late ROA
1st AI	Short	35% (n=7)	13.6% (n=3)
	Physiological	50% (n=10)	63.6% (n=14)
	Long	15% (n=3)	22.7% (n=5)
2nd AI	Short	50% (n=5)	27.3% (n=3)
	Physiological	40% (n=4)	45.5% (n=5)
	Long	10% (n=1)	27.3% (n=3)
3rd AI	Short	/	/
	Physiological	50% (n=2)	66.7% (n=6)
	Long	50% (n=2)	33.3% (n=3)

Table 5.4 Relationship between length of the luteal phases following AIs that did not result in pregnancy (i.e. AI- and EM) and progressive AI in Early ROA and Late ROA cows.

Early ROA cows had a greater number of short luteal phases after AI (both 1st and 2nd) compared to late ROA cows, and this would allow to perform the following AI sooner. For the late ROA cows, there was a greater incidence of long luteal phases after both the 1st and the 2nd AI compared to early ROA cows, thus delaying the interval to the following AI. This feature of the luteal phases was no longer evident after the 3rd AI and, in fact, there was no difference between early and late ROA cows in coincidence with the 4th AI (see figure 5.11).

5.3.5 Length of ovarian cycle phases in relation to AI outcome

There is a wealth of literature that investigates the effects of abnormal ovarian cycles during the pre-service post-partum period on subsequent reproductive performance of

dairy cows (Smith and Wallace, 1998; Shrestha *et al*, 2004b; Kawashima *et al*, 2006), whereas there are no studies specifically aimed to investigate the features of ovarian cycle phases related to AI and the AI outcome. Therefore, the following results can hardly be compared with others.

Abnormal ovarian activity (such as prolonged luteal phase or delayed ovulation) during pre-service period seems to have negative effects on days open, number of services per conception, and conception rate (Lamming and Darwash, 1998).

The length of the luteal phase appears to be the primary determinant of number of follicular waves occurring during an oestrous cycle which, in turn, may affect AI outcome (Inskeep, 2004). Growth of vesicular follicles in cattle occurs in a wavelike pattern. A follicular wave is characterized by continued development of a single follicle and regression of several subordinates. Since a follicular wave occurs about every 7 to 10 days, a cow may have two or three waves during an oestrous cycle (Roche *et al*, 1999). The ovulatory follicle in cows with two follicular waves is older and larger than the ovulatory follicle in cows with three waves, and this may impair oocyte quality and embryo survival (Ahmad *et al*, 1995; Revah and Butler, 1996).

Also the length of the follicular phase when AI is performed may affect AI outcome. It has been reported that prolonged lifespan of the preovulatory follicle is often associated with delayed ovulation and return to oestrus (Båge *et al*, 2002).

Unfortunately, the effects of abnormal ovarian cycles on reproductive performance have not been studied exhaustively, and the mechanisms involved are not well understood.

In this study, only the AIs with at least two ovarian phases clearly classified were analysed. For some AIs, in fact, there was a lack of milk sampling so it was not possible to determine the length of each phase.

The AI outcome was associated with neither the luteal phase length of the cycle before the AI nor the follicular phase length at the time of AI (Tables 5.5 and 5.6), and the contingency coefficients were 0.165 and 0.210, respectively.

However, it is interesting to note that the great majority of short (68%) and long (75%) luteal phases pre-AI was followed by AIs that did not result in pregnancy. This points out the important role that an adequate CL activity has for the establishment and maintenance of pregnancy.

		Luteal phase length (N)			
		Not Present	Short	Physiological	Long
AI outcome	EM	1	2	4	1
	AI-	5	32	28	8
	AI+	1	16	38	3

Table 5.5 Relationship between the AI outcome and the luteal phase length of the cycle before the AI.

		Follicular phase length (N)			
		Not Present	Short	Physiological	Long
AI outcome	EM	1	3	2	2
	AI-	5	15	26	36
	AI+	1	6	15	27

Table 5.6 Relationship between the AI outcome and the follicular phase length of the cycle of AI.

The positive AIs were removed from the evaluation of the relationship between the AI outcome and the length of the luteal phase following the AI, and the results are shown in Table 5.7.

		Luteal phase length (N)			
		Not Present	Short	Physiological	Long
AI outcome	EM	0	0	0	9
	AI-	0	22	48	9

Table 5.7 Relationship between the AI outcome and the luteal phase length of the cycle after AI.

The two variables were significantly associated (contingency coefficient=0.554; $P<0.001$). All EM were characterised by a long luteal phase, while luteal phases following a

negative AI were either short or physiological, although few cases with a long luteal phase were observed. With a long luteal phase after a negative AI, the presence of either ovarian cysts or an embryo death occurring before PAG production begins could be hypothesised (see chapter 4.3.3). Indeed, an embryo could die either before PAG production begins, or while PAG concentrations are still too low to be detected in the maternal serum (Humboldt, 2001). In such situations, luteolysis is likely to be delayed, thus determining a prolonged luteal phase.

5.3.6 Blood indicators

Blood indicators were analysed in relation to the AI outcome, ROA, physiological phase, sample, and animal (Table 5.8).

	Outcome (O)	ROA	Physiol Phase (PP)	Sample (S)	Animal (A)	O*S	O*ROA	O*PP
MDA	NS	NS	**	NS	*	NS	*	**
GSH	NS	NS	§	NS	***	NS	NS	NS
AOPP	***	NS	*	NS	***	NS	NS	NS
TP	NS	*	***	NS	***	NS	NS	NS
ALB	NS	***	***	NS	***	NS	NS	NS
GLOB	NS	NS	***	NS	***	NS	NS	NS
UREA	**	NS	**	NS	***	NS	NS	§
GLUC	NS	NS	NS	NS	NS	NS	NS	§
CHOL	NS	NS	***	NS	***	NS	*	NS
TG	NS	***	NS	NS	***	NS	NS	NS
NEFA	NS	NS	*	§	§	NS	NS	NS
3OHB	NS	**	§	NS	***	NS	NS	NS

Table 5.8 Relationship between blood indicators and AI outcome, ROA and Physiological Phase, Sample, and Animal

NS not significant; § P<0.1; * P<0.05; ** P<0.01; *** P<0.001

O: AI outcome (AI+, AI-, EM); ROA: resumption of ovarian activity (before D 42 vs after D 42 post calving); PP: Physiological Phase (D 30-60; D 61-90; D 91-120; D 121-180; after D180 post calving)

Almost all blood indicators showed a great variability among individual cows (P<0.001).

Plasma TP, ALB and GLOB were not affected by the AI outcome, but they were significantly affected by the physiological phase ($P<0.01$), increasing during the post partum (figure 5.14).

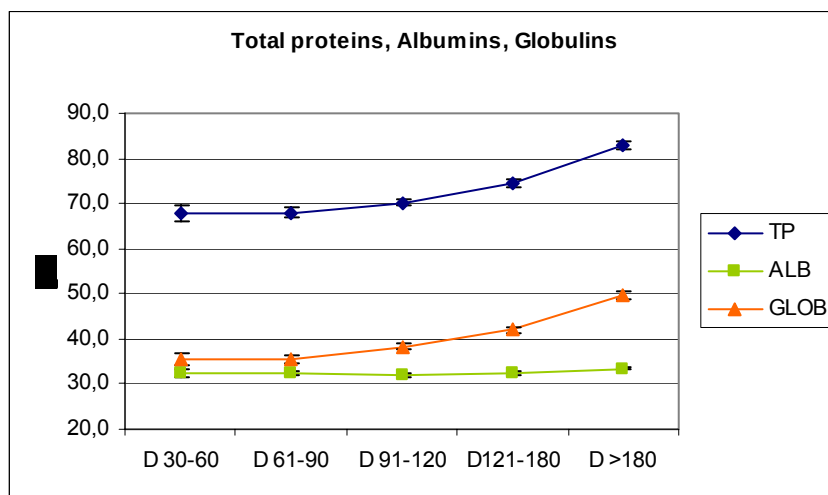


Figure 5.14 Total proteins (TP), albumins (ALB) and globulins (GLOB) concentrations in relation to the physiological phase.

TP and ALB were significantly higher in early ROA cows ($P<0.05$ and $P<0.001$, respectively).

Plasma urea increased steadily throughout the post partum (physiological phase, $P<0.05$) and it was affected by the AI outcome ($P<0.01$), being significantly lower in EM. This result was unexpected and difficult to explain by the present experimental design. However, several hypothesis could be made:

- a) slower protein turn over
- b) higher urea excretion rate
- c) higher urea metabolism

In any case, the plasma urea concentrations were within the physiological range (Kaneko, 1997) in all AI outcomes, and the small variations observed need further investigations with appropriate experimental protocols.

5.3.7 Relationship between OS biomarkers and AI outcome

The AI outcome affected AOPP plasma concentrations that were significantly higher in EM ($P<0.01$; figure 5.15).

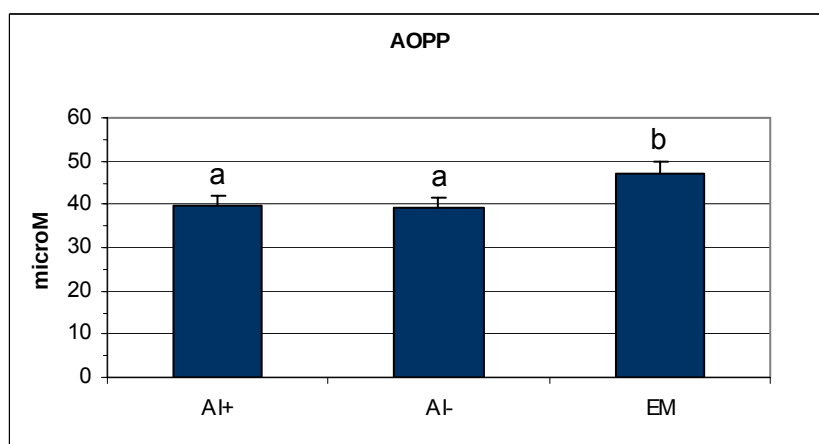


Figure 5.15 Relationship between AOPP concentrations and AI outcome. Different letters indicate significantly different means; ($P < 0.01$).

On the contrary, plasma MDA and GSH were not affected by the AI outcome. However, AOPP showed weak but significant correlations with both MDA ($r^2 = 0.323$; $P < 0.001$) and GSH ($r^2 = 0.096$; $P < 0.05$), suggesting that AOPP may be used as a marker of OS not only in humans (Witko-Sarsat *et al*, 1998), but in the cow as well.

Although GSH is the most abundant non-protein thiol in mammalian cells and can be considered a good indicator of the blood oxidative scavenging capacity, it is important to remember that GSH is also an important source of storage and transport of cysteine (Wu *et al*, 2004). Thus, total plasma GSH is possibly more related to metabolic adaptations, such as sulphur amino acid spearing, rather than to oxidative stress (Gabai *et al*, 2004), and for this reason the relationship between AOPP and GSH was not very strong in this experiment.

In this study, for the first time the variations of AOPP in parallel with those of circulating proteins in the cow plasma were reported. Plasma AOPP variations were independent from those of TP, A and G, suggesting a greater degree of protein oxidation in case of EM.

Besides being markers of OS, AOPP are also considered responsible of induction of proinflammatory activities (Kalousova *et al*, 2005; Shi *et al*, 2008). Hence, it is possible that the higher levels of AOPP observed in EM may reflect subclinical inflammatory events at uterine levels that can compromise the correct embryonic development. Several authors agree that activation of inflammatory or immune responses can lead to EM (Zavy, 1994; Soto *et al*, 2003; Hansen *et al*, 2004). Many hypothesis about the mechanisms involved in embryo death have been made. Some molecules that are produced during inflammation, such as $\text{PGF}_{2\alpha}$ or nitric oxide (NO), may affect embryonic development by acting either on the oocyte or on the developing embryo (Soto *et al*, 2003). Moreover,

PGF_{2α} is transferred locally from the uterus to the ovary and acts on the CL causing its destruction and a decline in P4 secretion (McCracken *et al*, 1999). Cytokines produced during inflammation have been suggested to affect embryonic survival by disrupting function of the hypothalamus, pituitary, ovary, and uterus (Hansen *et al*, 2004).

The lack of significant modifications of MDA and GSH in case of EM seems to support the hypothesis of an involvement of an inflammatory process. Witko-Sarsat *et al*. (1998) found a similar pattern. In their study, AOPP accumulated while plasma concentration of MDA remained stable. These authors suggested that AOPP are more accurate markers of oxidative stress than lipid peroxidation products. Indeed, the MDA assay has often been criticised for low specificity and artefact formation, since only a fraction of the MDA measured is generated *in vivo* while several MDA species derive from the assay procedure itself (Lykkesfeldt and Svendsen, 2007).

A close relationship between AOPP and inflammation is suggested by the mechanism that leads to AOPP formation, in particular the crucial importance of chlorinated oxidants in the formation of AOPP. Studies have demonstrated that AOPP arise from the reaction between plasma proteins and chlorinated oxidants (Witko-Sarsat *et al*, 1999; Fialova *et al*, 2006; Noyan *et al*, 2006). The generation of chlorinated oxidants is a feature of phagocytic cells which possess myeloperoxidase (MPO) (e.g. neutrophils), the only enzyme able to generate chlorinated oxidants. Neutrophils are the predominant cells present in the early stages of acute inflammation, being responsible for ingestion and destruction of invading pathogens. Phagocytosis of bacteria is accompanied by the activation of an NADPH oxidase complex in the cell membrane, resulting in generation of superoxide (O^{2-}) and the release of cytoplasmic granule proteins into the phagosome. One of the major granule proteins is the enzyme MPO. The combination of O^{2-} production and MPO release gives neutrophils a unique oxidative potential: hydrogen peroxide (H_2O_2), generated by dismutation of O^{2-} , is utilised by MPO to oxidise chloride to generate hypochlorous (HOCl), which is the major end-product of the neutrophil respiratory burst (Noyan *et al*, 2006). In light of these mechanisms, it could be stated that AOPP might constitute a new molecular basis for the deleterious activity of oxidants and hence be considered as true mediators of proinflammatory effects of OS.

5.4 Take-home message

In this study ultrasound examination was apparently more precise than PAG analysis in

performing pregnancy diagnosis, particularly in case of EM, where some limitations of the PAG test were found. In three cases ultrasounds allowed to perform an EM diagnosis whereas, according to the PAG profile, those outcomes were classified as positive. This disagreement was probably due to the insufficient number of samples collected after the embryo death: likely, with few additional samples, it would be possible to detect a decrease in PAG concentrations, thus indicating the embryo death. It is hence a matter of number of samples rather than the ability of PAG test to diagnose an EM. It could be concluded that PAG assay is very useful for diagnosing EM using serial plasma samples from the same cow.

Although only 9 cases of EM were observed at the end of the experiment, a relationship between protein oxidation and EM could be hypothesised. It would be interesting to further investigate the role of OS in EM.

More information about the role of protein oxidation in EM could be obtained by the comparison of AOPP with other indicators of protein oxidation, such as advanced glycation end products (AGE). In particular, it would be extremely important to establish whether the possible inflammatory event is still present at the time of EM, or it has already been resolved, and EM could be considered as a carry-over effect.

The inflammatory events generated by either AOPP or AGE seem to be activated by the interaction with the same receptors (RAGE) (Kalousová *et al*, 2002). However, while a correlation between AGE and inflammatory parameters is usually not found or is only weak, the induction of proinflammatory activities caused by AOPP seems to be more intense (Witko-Sarsat *et al*, 1999; Kalousová *et al*, 2005). This suggests that OS has a closer relationship to inflammation and acute phase reactions than advanced glycation process and its end products. AOPP could thus better describe acute inflammation, while AGE might serve more as a marker of chronic long-lasting damage (Kalousová *et al*, 2005).

Experiment 3

Relationship between AI outcome and oxidative stress in dairy cows in pasture-based system

6.1 Introduction

The results reported in Chapter 5, based on cows reared on an intensive system, showed a relationship between some oxidative stress (OS) biomarkers (i.e. AOPP) and embryo mortality (EM). OS develops when reactive oxygen metabolites (ROMs) are produced in excessive quantities, or faster than they can be safely removed by antioxidant mechanisms (Bernabucci *et al*, 2002; Castillo *et al*, 2005). The antioxidant network is represented either by enzymes such as glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD), or vitamins like vitamin A, E and C (Reilly *et al*, 1991; Agarwal *et al*, 2005; Noyan *et al*, 2006). It is important to consider that the activity of these compounds is influenced by nutrition (Andrés *et al*, 1997; Gatellier *et al*, 2004). For example, fresh pasture is particularly rich in natural antioxidants such as vitamins from group A, C, and especially E, and so it offers a great protection against lipids and proteins oxidation (Gatellier *et al*, 2004). Antioxidants content and properties of pasture are in turn affected by environmental effects such as air temperature, altitudinal location of pasture, light intensity and nutrient availability in the soil (Tamura and Nishibe, 2002; Al-Mamun *et al*, 2007).

Many studies have investigated the effects of pasture-based diets on meat (Gatellier *et al*, 2004; Realini *et al*, 2004; Descalzo *et al*, 2005 and 2007) and milk quality (White *et al*, 2001; Kay *et al*, 2005; Calderon *et al*, 2007), whereas very little attention has been paid so far to a possible role of pasture feeding in relation to OS and fertility in dairy cows.

Therefore, it would be interesting to investigate oxidative status of dairy cows pasture fed and, then, to verify whether the oxidative status of animals at pasture could affect AI outcome, including EM.

6.2 Materials and methods

6.2.1 Experimental design

Forty pasture fed Holstein-Friesian cows were selected from the dairy facility of the University of Sydney (Corstorphine Dairy, Camden, NSW Australia) and studied for a 4 months period from May to August 2007.

Camden region (150°39'E, 34°3'S) has a temperate climate with warm summers and mild winters, and rainfall spread throughout the year. During the four months of observations, minimum and maximum temperatures were in the mid-range with the average temperatures of the last 34 years, while the rainfall occurring in June was extraordinary high (see figure 6.1 and 6.2).

Cows grazed on a short rotation ryegrass pasture (*Lolium Multiflorum* L.) (11kg/head/day dry matter intake). As the energy content of the ryegrass is deficient when the plant is in a vegetative state (between May and August) (Fulkerson *et al*, 1998), the cows were supplemented with maize silage (7kg/head/day dry matter intake) fed out on a feed pad, and concentrates (4kg/head/day dry matter intake) fed individually at each milking.

The cows object of this study calved between February and March 2007, and at the beginning of the observations they were all in early lactation (DIM 70±2). Starting on May cows were checked twice a day for oestrus signs in coincidence with the morning and afternoon milking. Oestrus was detected also by placing KaMaR Heatmount Detectors (Kamar Inc., Steamboat Springs, CO) on the tailhead of the cows. Cows detected on oestrus were artificially inseminated by the same trained inseminator.

On the day of the AI (Day 0), and on Days 30 and 42 after AI, blood samples were collected. The choice of Day 30 and 42 as sampling days was made to detect most of the embryo losses. On one hand, Day 30 is the first day that allows detecting PAG in concentrations higher than 0.8ng/ml in all cows (Sousa *et al*, 2006; Ayad *et al*, 2007b). Although PAG can be detected in some pregnant females at earlier gestation periods (see chapter 4), accuracy of pregnancy diagnosis before Day 30 after conception can be compromised by individual differences in patterns of PAG secretion (Perényi *et al*, 2002b). On the other hand, since most of pregnancy losses are likely to occur within 42 days after breeding (Santos *et al*, 2004), in case of EM, the sampling on Day 42 would show the decrease of PAG concentration occurring after embryo death.

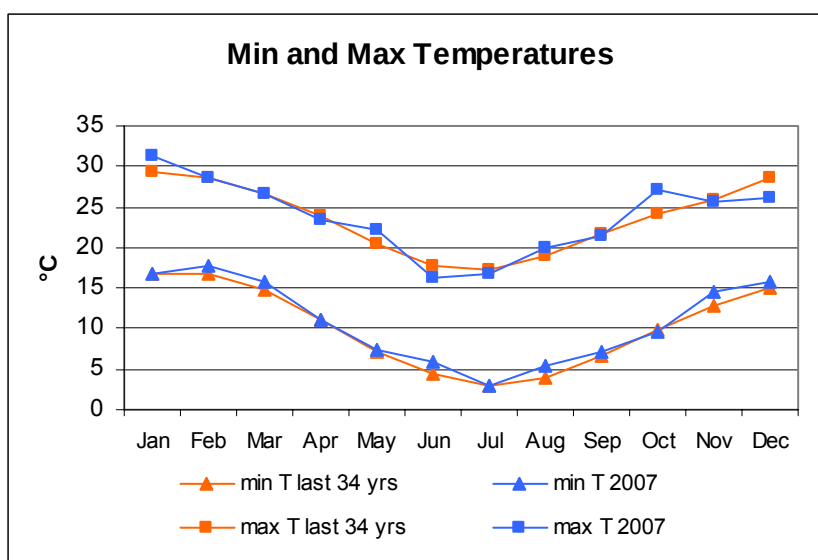


Figure 6.1 Long term (34 years) average and actual for 2007, monthly minimum and maximum temperatures (°C) for Camden (NSW); data from Bureau of Meteorology.

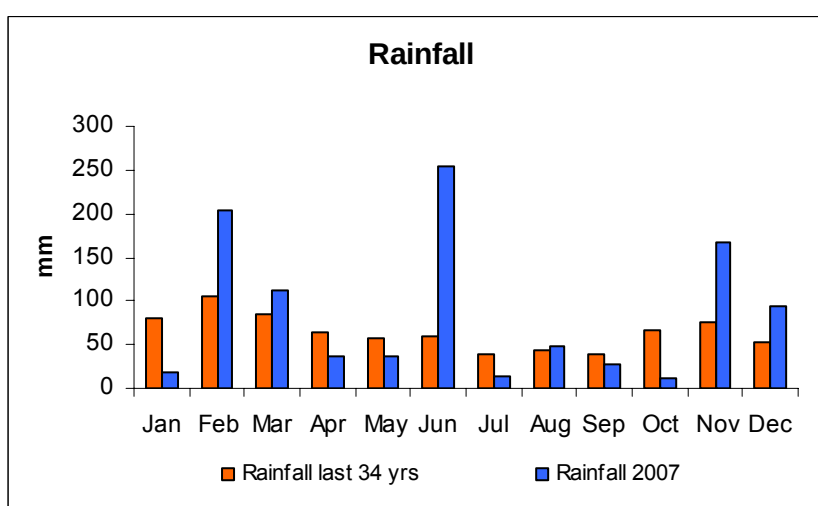


Figure 6.2 Long term (34 years) average and actual for 2007, monthly rainfall (mm) for Camden (NSW); data from Bureau of Meteorology.

Reactive oxygen metabolites (ROMs) concentrations and biological antioxidant potential (BAP) were determined by a commercial kit (Diacron, Grosseto, Italy) on fresh blood by FRAS4 (H&D limited, Parma, Italy). Blood was centrifuged immediately and plasma was stored at -20°C until assayed for its concentrations of P4, pregnancy associated glycoprotein (PAG), advanced oxidation protein products (AOPP), glutathione, glucose, albumin, total protein, urea, haptoglobin, cholesterol, NEFA, and triglycerides as described in chapter 3.

All cows had their body condition score (BCS) assessed on a scale of 1-5 in coincidence with the blood sampling.

Pregnancy diagnosis was carried out by means of PAG and P4 assay, as described in the chapter 6.2.2. Pregnancy confirmation was performed by trans-rectal palpation after Day 60.

6.2.2 Data analysis

AI outcomes were classified in positive (AI+), negative (AI-), and embryo mortality (EM) according to PAG and P4 concentrations at Days 30 and 42. AI outcomes were considered positive when $\text{PAG} \geq 0.8$ ng/ml, while when $\text{PAG} < 0.8$ ng/ml, AIs were classified as negative (according to Szenci *et al*, 1998 and Lopez-Gatius *et al*, 2007a).

P4 profile was also considered from Day 0 to Day 30. Luteal function was considered consistent with pregnancy when at Day 0 $\text{P4} < 1$ ng/ml and at Day 30 $\text{P4} > 4$ ng/ml (Battocchio *et al*, 1999).

EM was diagnosed by considering the whole PAG profile, rather than just one threshold concentration of PAG, in association with P4 profile. AIs presenting PAG concentration at Day 30 typical of early pregnancy (i.e. above the threshold concentration of 0.8ng/ml) followed by a steady decrease in PAG concentrations were classified as EM. At the same time, P4 profile had to show a functional CL at least at Day 30 followed by a decrease in P4 concentration. Also the outcome of the diagnosis carried out by transrectal palpation at around Day 60 was used to detect embryo losses occurring beyond Day 42.

Samples were divided according to the post-partum period when they had been collected: Physiological phase 1 = <80 days post-partum; Physiological phase 2 = 81-120 days post-partum; Physiological phase 3 = >121 days post-partum.

Data were analysed by linear regression (GenStat). Analysis included between-subjects main effect of AI outcome, within-subjects main effect of postpartum period, and their interactions.

6.3 Results and discussion

6.3.1 Fertility, general observations

One cow left the trial due to acute mastitis and was omitted from data analysis.

For the other 39 cows, some parameters of reproductive performance were evaluated, taking into account the different Australian mating systems proposed by the Australian *InCalf* project. The *InCalf* project is a learning program funded by Australian dairy farmers that aims to achieve measured improvement in herd reproductive performance and also gives useful guidelines to assess overall herd reproductive performance.

According to *InCalf*, the best overall measures of reproductive performance in split calving herds are the 6-weeks in-calf rate, the not-in-calf rate, and the 3-weeks submission rate. The 6-weeks in-calf rate represents the percentage of cows that become pregnant in the first 6 weeks of the mating period. A high percentage (optimum is 71%) means that a large number of cows will calve early in the following calving period making the most of pasture availability.

The not-in-calf rate describes the percentage of cows that remain not pregnant at the end of the breeding season. These cows are more likely to be sold due to low production following a prolonged lactation. For a mating period of 15 weeks it should be not more than 17%.

The 3-weeks submission rate represents the percentage of cows detected in oestrus and inseminated within three weeks of commencement of the mating period. A high percentage (more than 75%) possibly allows good 6-weeks in-calf rates.

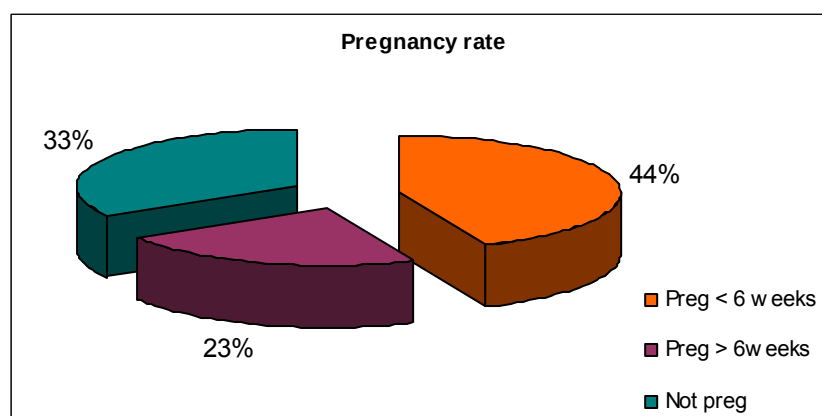


Figure 6.3 Pregnancy rate (%). Cows pregnant < 6 weeks ($n=17$), cows pregnant > 6 weeks ($n=9$), and cows not pregnant ($n=13$) by the end of the breeding season.

In the present study, the 6-weeks in-calf rate was 44%, the not in-calf rate was 33% (figure 6.3), and the 3-weeks submission rate was 79.5%.

Despite the good 3-weeks submission rate, the reproductive performance of the herd was lower than the optimum reference values reported by *InCalf* for the Australian split calving system.

Looking at the AI outcomes (figure 6.4), it appears clear that the management of modern high genetic merit Holstein dairy cows presents a major challenge in pasture-based production. A great percentage (62%) of the AIs performed during the mating period did not result in pregnancy and 5% resulted in embryo loss. Moreover, very few AIs were performed before 80 days post-partum (n=10) and only two of them were positive (figure 6.5). In pasture based system, genetic selection for increased milk production, which is generally accompanied with increased proportion of North American Holstein Friesian genes, is associated with greater milk production but also with reduced fertility (Dillon *et al*, 2006).

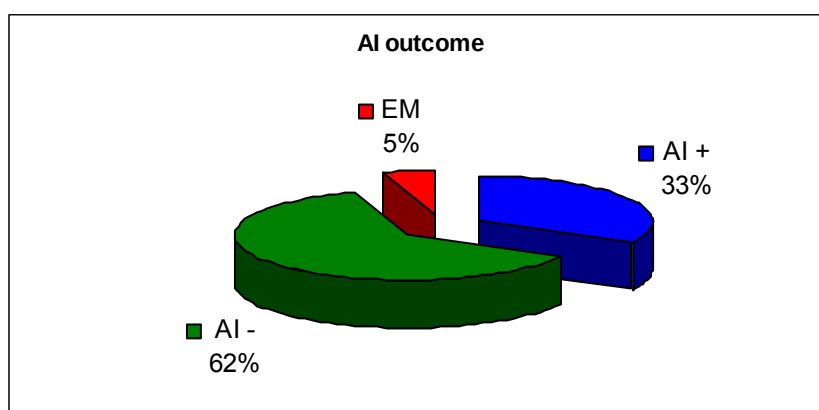


Figure 6.4 Distribution (%) of AI outcome.

Moreover, the increased selection for milk production has resulted in cows that are not able to achieve desired grass intake from the pasture (for review see Dillon *et al*, 2006). This could possibly address the negative energy balance (NEB) after calving and affect the ability of the cow to recover soon from this condition. Greater and extended NEB in early lactation is reported to have negative consequences on health and fertility of the cow (Veerkamp *et al*, 2003). In particular, NEB reduces post-partum LH pulsatility and, therefore, delays the resumption of ovarian activity (Beam and Butler, 1999; Butler, 2000). As a consequence, interval from calving to first ovulation, first service, conception, and calving are extended when resumption of ovarian activity is delayed (Westwood *et al*, 2002).

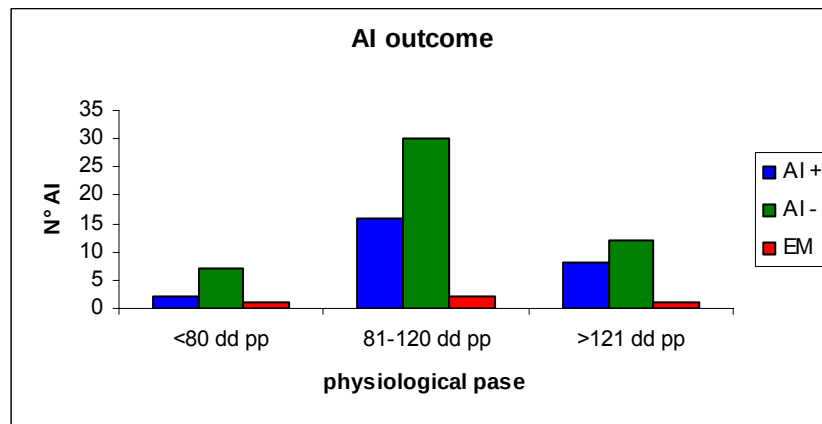


Figure 6.5 Distribution (N) of AI outcome in relation to the physiological phase.

An incidence of 5% (n=4) EM was observed (figure 6.4). In one case pregnancy loss occurred after Day 42 and was detected by the negative outcome of the clinical diagnosis at Day 60. It is difficult to compare this result with others reported in literature because of the lack of studies on EM in dairy cows managed on pasture-based system. In one study (Silke *et al*, 2002) the extent of embryo/foetal losses diagnosed by ultrasounds from days 28 to 84 of gestation was 7.2% with 3.2% of losses occurring between days 28 and 42 of gestation. Similarly, another study (Horan *et al*, 2004) reported an overall embryo/foetal loss incidence of 7.5% between days 30 and 67 of gestation.

Cows that experienced embryo loss had lower BCS (2.5 ± 0.1) at Day 0 compared with cows that had positive or negative AIs (2.8 ± 0.06 and 2.9 ± 0.05 , respectively; $P < 0.05$). Similar result was found also by Moreira *et al*. (2000) who suggested that EM may occur at a greater rate in low BCS cows in the early stages of pregnancy (between Day 20 and 27). Silke *et al*. (2002) and Lopez-Gatius *et al*. (2002) observed that cows with lower BCS in early post-partum had a higher embryonic loss rate.

However, as extensively reviewed by Lopez-Gatius *et al*. (2003), discrepancies regarding the effects of BCS on establishment and maintenance of pregnancy in dairy cows are common in literature. In fact, Starbuck *et al*. (2004) observed that cows in greater body condition (≥ 3.50) maintained fewer pregnancies than those with 2.75-3.25 BCS, whereas an *in vitro* experiment demonstrated that embryonic development to the blastocyst stage was not affected by body condition score of the oocyte donor at all (Fazio *et al*, 1999).

In conclusion, although it is widely accepted that nutritional status can affect embryo survival and development (see Boland and Lonergan, 2003), the mechanisms by which dietary intake and diet impair early embryonic development remain to be established.

6.3.2 Blood indicators

Total proteins, albumin, urea, and haptoglobin concentrations were within the normal ranges (Kaneko, 1997). Haptoglobin is one of the major acute phase proteins in cattle, and increasing concentrations are attributable to inflammatory reactions (Hachenberg *et al*, 2007). Therefore, the haptoglobin concentrations observed in this study suggest that the cows did not experience any clinical or subclinical inflammatory process.

Triglycerides and cholesterol levels were constant during the post-partum and within the optimum range (Kaneko, 1997). NEFA and glucose showed the typical pattern of the final stages of recovery from negative energy balance: NEFA levels gradually decreased from physiological phase 1 to physiological phase 3, whereas glucose steadily increased during the post-partum ($P < 0.05$ and $P < 0.03$, respectively).

NEFA reflect the magnitude of fat mobilization from adipose tissue in response to negative energy balance during post-partum. In cows at pasture, post-partum NEFA levels are reported to be higher during a longer period than reported for cows reared in intensive system (Cavestany *et al*, 2005). This result suggests that in pasture-based system, cows are likely to experience a longer period of negative energy balance which would probably take longer to be recovered.

It is widely accepted that the increase of glucose levels during the post-partum may reflect the recovery feed intake and improving energy status of the cow (Vazquez-Anon *et al*, 1994).

In addition, a recover from the negative energy balance is suggested also by an increase of BCS during the post partum ($P < 0.05$).

6.3.3 Oxidative status in dairy cows in pasture-based system

6.3.3.1 ROMs and BAP profiles

In this study, for the first time, ROMs and BAP profiles were studied in dairy cows reared in a pasture-based system. The mean concentrations of both ROMs and BAP relatively to the postpartum period are shown in the table 6.1.

Unfortunately, there are no reference values for BAP or ROMs in dairy cows at pasture, thus it is not possible to establish whether or not the concentrations observed in this study were within physiological range.

	< 80 dd pp	81-120 dd pp	> 121 dd pp
ROMs (mgH ₂ O ₂ /dL)	9.73±0.67 ^a	11.12±0.35 ^{ab}	12.36±0.37 ^b
BAP (μEq/L)	2775.8±94.67 ^{ab}	2871.8±38.47 ^b	2733.31±29.58 ^a

Table 6.1 ROMs and BAP mean concentrations in relation to the postpartum period (Different letters indicate significantly different means; Duncan test, P<0.05).

The only studies to report ROMs concentrations in dairy cows are the ones by Bernabucci *et al.* (2002 and 2005). However, comparisons can hardly be done since those studies involved cows in different physiological phases (i.e. transition period) and reared in an intensive system. In particular, the rearing system may determine important differences in terms of oxidative status of animals. Indeed, being at pasture may affect oxidative status of cows. Pasture-based system could improve oxidative status of animals in two ways: the elevated antioxidants content of green grass (Gatellier *et al.*, 2004; Realini *et al.*, 2004; Descalzo *et al.*, 2005) and the physical exercise related with grazing. While in humans it is well documented that moderate physical exercise improves oxidative status (Cases *et al.*, 2005; Gordon *et al.*, 2008), the role of physical activity on oxidative status of animals is still controversial. Some studies reveal negative effects of strenuous exercise on oxidative status of horses (Art and Lekeux, 2005; Kinnunen *et al.*, 2005), while, according to other authors, animals free to move, as in pasture-based system, have better antioxidant status (Gatellier *et al.*, 2004; Venditti *et al.*, 2008).

No relationship between AI outcome and ROMs or BAP was found. Since no effects of these OS biomarkers on AI outcome were observed, it could be speculated that these systemic OS biomarkers may not reflect what actually happens within the uterus.

For example, it is well known that the main antioxidant system that protects embryo from excessive ROMs generation is within the embryo itself (e.g. superoxide dismutase and glutathione peroxidase) and the oviductal environment (e.g. taurine and ascorbic acid) (Guerin *et al.*, 2001). Therefore, it is possible that the systemic antioxidant status of the mother exerts less effects on the embryo than tubal and uterine environment. Unfortunately, tissue measurement of oxidative stress indicators was not performed and, therefore, no speculations about the possible local effects of oxidative stress can be made.

Oxidative status changed significantly during the post-partum (see table 6.1). Steady increase in plasma ROMs concentration ($P<0.01$) was observed. Plasma BAP decreased ($P<0.05$) between physiological phase 2 and 3, possibly as a direct consequence of ROMs increase. Indeed, changes in the components of antioxidants systems are often not the cause, but the consequence of the OS induced by higher free radical activity (Venditti *et al*, 2008).

Similar trend of OS biomarkers in the post-partum was observed also by Gabai *et al*. (2004): the activity of some oxidation products (e.g. MDA) and antioxidants (e.g. GSH, GPx) increased during the post-partum, suggesting that cellular metabolism of animals recovering from the negative energy balance shifts towards oxidative phosphorylation and, as a consequence, oxidative stress may increase throughout the post-partum.

Moreover, the decrease of BAP during the post-partum could be explained also by a combination of two factors: in winter the quality of pasture is not excellent and cows are given concentrate supplementation. Concentrate supplementation with the diet has been proved to either reduce pasture intake (Bargo *et al*, 2002) and to increase the degradation of some antioxidants (e.g. vitamin A) at ruminal level (Weiss *et al*, 1995).

It was therefore considered relevant to investigate which factors may have affected BAP levels of the animals during the post-partum.

The period of the year and the weather conditions were investigated in relation to oxidative status of the animals (i.e. ROMs and BAP) because of a different pasture quality and availability in relation to the season.

Rainfall, minimum and maximum temperatures registered during the experimental period were analysed by linear regression (GenStat) to check the effects on BAP level throughout the post-partum.

An effect of rainfall ($P<0.05$) and maximum temperature ($P<0.1$) during the post-partum on BAP was observed. The mean maximum temperature dropped considerably between May (22.3°C) and June (16.2°C) or July (16.7°C). Moreover, the maximum temperature in June and July 2007 was 1.5°C lower than the mean registered for these two months over the last 34 years (17.8°C and 17.2°C , respectively) (see graph 6.1). This might have affected antioxidants concentration of the pasture. Indeed, concentration of antioxidants in herbs and pastures varies from species to species and, within species, due to environmental effects, such as seasonal changes. In particular, air temperature and nutrient availability in the soil are considered to be major factors in this respect (Tamura and Nishibe, 2002; Al-Mamun *et al*, 2007).

Also the copious rainfall might have played a role on the antioxidant status of the cows by impairing pasture intake. It has been demonstrated that high rainfall reduces the dry matter content of pasture which, in turn, leads to a reduction in dry matter intake of cows (Butris and Phillips, 1987). Reduced dry matter intake may arise from behavioural limitations due to a palatability effect decreasing pasture acceptability (Butris and Phillips, 1987).

However, these hypothesis can not be verified since neither pasture analysis nor feed intake measurements were performed in this study.

Moreover, in this experiment the effect of the physiological phase could not be incorporated from that of the environmental variables because in the animals object of this study the proceeding of the season was parallel to the physiological phase. Therefore, any impact of either the temperature or rainfall might be biological explained by the days postpartum more than the climate.

Milk yield was also investigated in relation to BAP levels and days post-partum since milk production has been positively associated with OS (Castillo *et al*, 2003). Indeed, antioxidants pass the milk barrier (Kay *et al*, 2005) and, therefore, it can be hypothesised that high milk yield leads to high antioxidants loss with milk. Monthly milk production per cow was divided into three classes: low >800 kg/month.cow; medium 800-1000 kg/month.cow; high > 1000 kg/month.cow.

Monthly milk yield was then analysed by linear regression (GenStat) to check the effects on BAP level throughout the post-partum, but no relationship between milk yield and BAP levels during the post-partum was found. However, it is important to notice that the animals object of this study were very homogenous in terms of milk yield.

6.3.3.2 Other oxidative stress biomarkers

During physiological phase 1, AOPP were higher in EM (61.91microM) than in AI+ (51.40 microM $\pm$ 1.72) and AI- (39.18 microM $\pm$ 4.44). This result seems to be in agreement with the findings of the previous experiment (see chapter 5), where AOPP concentrations were higher in animals experiencing EM. However, there was only one sample taken in the physiological phase 1 and belonging to an EM, therefore this result must be interpreted carefully.

6.3.4 Relationship between P4 and PAG concentrations

All the samples belonging to positive AIs and taken after 80 days post partum were analysed in order to investigate the relationship between P4 levels in early pregnancy and PAG production.

AIs were grouped on the basis of P4 concentrations observed on Day 30 into “Low P4 group” and “High P4 group”, according to whether their P4 concentrations at Day 30 were below or above the mean concentration ($4.66 \pm 0.43 \text{ ng/ml}$), respectively. Fifteen AIs were allocated to the low-P4 group and eleven AIs to the high-P4 group.

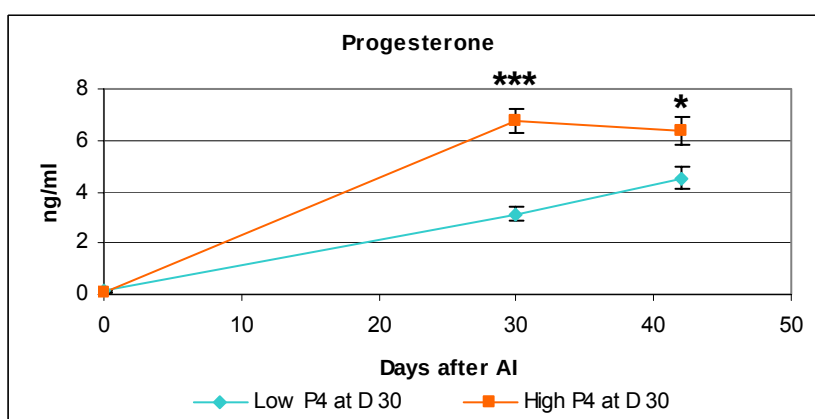


Figure 6.6 Mean P4 plasma concentrations in the two P4 subpopulations at Day 30: “Low P4 group” and “High P4 group” (***, $P < 0.001$; *, $P < 0.05$).

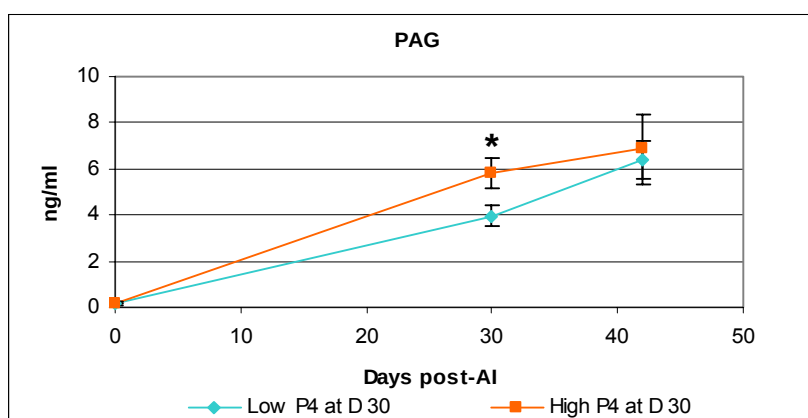


Figure 6.7 Mean PAG plasma concentrations in the two P4 subpopulations at Day 30: “Low P4 group” and “High P4 group” (*, $P < 0.05$).

Mean plasma concentrations of the two groups were significantly different one another, both at Day 30 (Low P4 group: $3.14 \pm 0.28 \text{ ng/ml}$; High P4 group: $6.73 \pm 0.47 \text{ ng/ml}$;

P<0.001) and Day 42 (Low P4 group: 4.52±0.44 ng/ml; High P4 group: 6.36±0.57; P<0.05) (graph 6.6).

PAG production at Day 30 was higher in High P4 group than in Low P4 group (P<0.05) (figure 6.7). At Day 42 the difference was no longer evident.

The relationship between P4 and PAG observed in this trial suggests that high P4 levels during the very first stages of pregnancy promote embryo development. Possibly, as suggested by several authors (Mann and Lamming, 2001; Green *et al*, 2005b; Mann *et al*, 2006), high P4 concentrations in early pregnancy provides a more suitable uterine environment for the developing embryo. As a consequence, the conceptus grows faster and it is larger, thus enhancing trophoblastic cells secretions (Mann *et al*. 2006). Since this positive effect of high P4 concentrations on PAG production is no longer evident at Day 42, it could be concluded that delayed embryo development due to low P4 levels and represented by lower PAG concentrations, is recovered earlier in this trial compared to the situation observed in experiment 1 of the present thesis (chapter 4.3.6).

6.4 Take-home message

This study provides a first picture of some OS biomarkers modifications during the post-partum in dairy cows at pasture. On pasture, oxidative status seems to be not related with AI outcome. In particular, the lack of relationship between OS biomarkers and EM may support the hypothesis that high antioxidants content of fresh pasture allows a better antioxidant defence at uterine level with positive effects on embryo survival and development. Positive effects of pasture feeding on milk and meat quality has extensively been demonstrated (see chapter 6.1), whereas this is the first study that investigates the relationship between OS and reproduction in dairy cows reared on a pasture-based system, which obviously requires deeper investigation.

General discussion

The present thesis aimed to investigate causes and factors involved in embryo mortality (EM), in particular the role played by oxidative stress (OS). Therefore, the first step was to find out a reliable method to diagnose EM. In Experiment 1 (chapter 4) the pregnancy-associated glycoproteins (PAG) assay, a new method for bovine pregnancy/EM diagnosis never applied in Italy before, was tested in a commercial dairy farm. The relationship between OS and EM was then investigated in two different rearing systems: intensive dairy production system (chapter 5) and pasture based system (chapter 6).

7.1 Reliability of the PAG test as pregnancy/EM diagnosis method

The PAG test turned out to be a reliable pregnancy/EM diagnosis method. Although it allowed to perform pregnancy diagnosis correctly in most cases (92.3% in experiment 2, see chapter 5.3.2), some false positive or false negative were found. For example, in Chapter 4, 6 cases of false-positive during early post partum period were observed (see chapter 4.3.5). Indeed, before 80 days post-partum, PAG belonging to the previous gestation are still detectable in the maternal serum (Zoli *et al*, 1992; Mialon *et al*, 1993). Moreover, PAG were not detectable in all pregnant cows before Day 28-30 post-AI, likely due to individual variation in PAG production (see table 4.2). These findings confirm the warning of several authors (Zoli *et al*, 1992; Szenci *et al*, 1998; Sousa *et al*, 2006) about the results of any PAG assay performed before Day 28-30 after AI or in cows bred before 80 days post-partum. In light of all these findings, it could be concluded that it is not possible to use this method for pregnancy diagnosis in the dairy cow before Day 28-30 after AI or before 80 days post-partum.

Since several members of the PAG family exist, which have different temporal pattern of expression during pregnancy, to avoid either false negative before Day 30 after breeding or false positive before Day 80 post-partum, the assay should ideally detect a member of the PAG family having the following characteristics: (a) it should be expressed abundantly in early pregnancy; and (b) it should be absent or decline rapidly in maternal blood after parturition, as proposed by Green *et al*. (2005a).

The results reported in chapter 5.3.2 confirm also that the PAG assay is very useful for diagnosing late embryo mortality using serial plasma samples from the same cow. Indeed, an embryonic loss can be diagnosed when, after a first positive pregnancy diagnosis, a steady decrease in PAG concentrations is observed at subsequent examinations. In case of EM, PAG production and secretion start to decrease steadily, reaching levels indicative of a non-pregnant animal within 15 (Humblot, 2001) or 8 days (Szenci *et al*, 2003). The presence of PAG in the maternal serum for some days after embryo death is due in part to the long half-life of these proteins but also to the fact that, likely, embryonic membranes (in particular the trophoblast) function with a certain independence from the embryo, allowing PAG production for several days after embryo death, as hypothesised by Lopez-Gatius *et al*. (2007b) and Serrano *et al*. (2008). For instance, in chapter 5.3.2 of the present thesis, in some cases ultrasonography allowed to identify EM while PAG concentrations were still high. Cases of PAG detection in the maternal circulation after embryonic death occurred are reported in the literature (Humblot *et al*, 1988; Szenci *et al*, 2000; Lopez-Gatius *et al*, 2007b; Serrano *et al*, 2008). This leads to the hypothesis that PAG may be a better indicator of trophoblastic function rather than embryo viability (Serrano *et al*, 2008).

The main advantage of PAG test in serial samples is that it allows to discriminate between late EM and AI that do not result in pregnancy. In this way investigations could aim to establish differential diagnosis of pathologies affecting pregnancy and the pathogenetic mechanisms involved in embryo losses. These aspects could be very useful for research purposes but also for practical applications on the field. For example, PAG assay could be used in conjunction with progesterone (P4) profiles as pregnancy test in farms affected by poor reproductive performance. This could help to restrict the field of investigation about the causes of low fertility (failure of fertilization, embryonic losses; see chapter 4).

7.2 Relationship between P4 and PAG concentrations

In order to investigate whether P4 produced by corpus luteum (CL) in early pregnancy could affect the secretion of PAG, in chapters 4.3.6, chapter 5.3.3, and chapter 6.3.4, positive AIs were divided into two subpopulations: “Low P4 group” and “High P4 group”, according to whether their P4 concentrations at Day 21, 15 or 30 (in experiment 1, 2 or 3, respectively) were below or above the mean concentration. The interesting aspect of these P4 subpopulations is that the higher or lower mean P4 concentrations between the

two groups were maintained throughout the sampling period (figure 7.1, which is a combination of figures 4.18, 5.10 and figure 6.6).

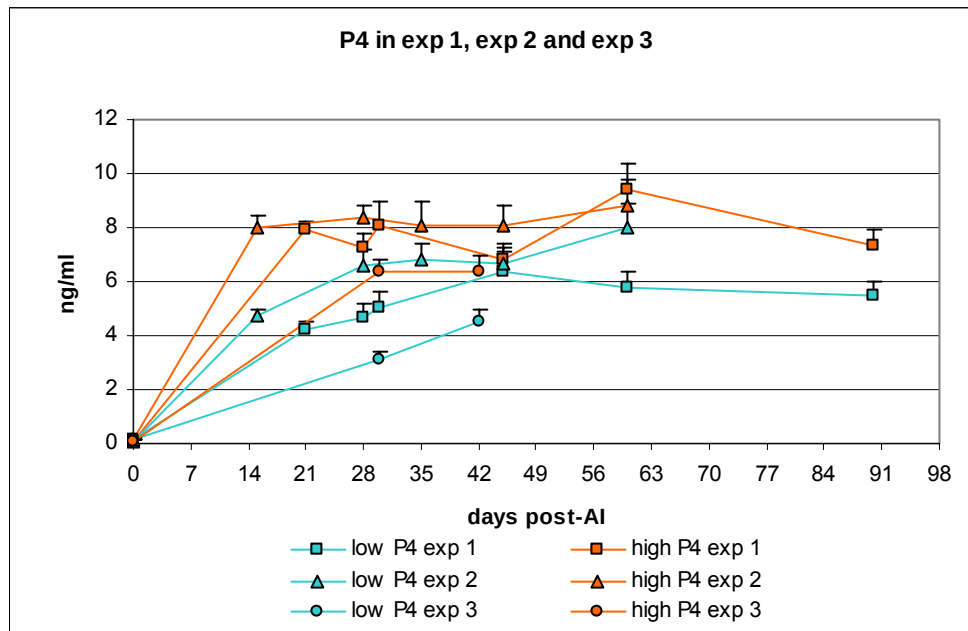


Figure 7.1 Plasma P4 profiles in “Low P4 group” and “High P4 group” in the three experiments object of this thesis.

This suggests that P4 concentration in early stages of pregnancy may affect CL development and function with a positive feedback involving the cross-talk between the embryo, endometrium and CL. A possible mechanism is that high P4 levels result in greater stimulation of endometrial secretions, necessary for embryo growth. As a consequence, a dramatic increase in the trophoblast size occurs resulting in higher IFN τ production. Increased IFN τ production leads to stronger antiluteolytic and luteotrophic signals, thus enhancing P4 production (see chapters 2.1.3 and 2.1.4).

This positive effect of P4 on trophoblastic function seems to be confirmed also by the positive relationship between P4 levels and PAG production found in the present thesis, being PAG a product of trophoblastic cells, like IFN τ . PAG production is strictly correlated to the trophoblast size (Wallace *et al*, 1997), which, in turn, is the result of adequate signalling between the mother and conceptus regulated by P4. Therefore, it could be speculated that PAG, as well as other compounds produced by the trophoblast at different stages of pregnancy (e.g. IFN τ and placental lactogen), can be considered an indicator of the degree of interaction between the mother and conceptus.

The concentrations of P4 produced in early stages of pregnancy were positively associated with PAG secretion by trophoblastic cells in experiment 1 and 3 but not in experiment 2. An interesting observation arose from the comparison of P4 and PAG patterns within the three experiments. It seems that around the time of implantation (Day 21 up to Day 30) there is a threshold P4 concentration (around 6 ng/ml in these experiments) above which no further beneficial effects on PAG production can be observed. This can be appreciated observing P4 pattern at this stage of pregnancy in experiment 2. On Day 28 both P4 groups had P4 concentrations higher than 6 ng/ml and no difference in PAG production between low and high P4 group were observed (see figure 5.11). A positive effect of high P4 levels on PAG secretion can rather be appreciated when compared to P4 levels lower than the postulated threshold (see, for example, experiment 1). However, an effect of P4 levels higher than the threshold concentration on PAG production can not be excluded since overall PAG concentrations in experiment 2 were higher than those observed in the other two experiments (figure 7.2. which is a combination of figures 4.19, 5.11 and figure 6.7).

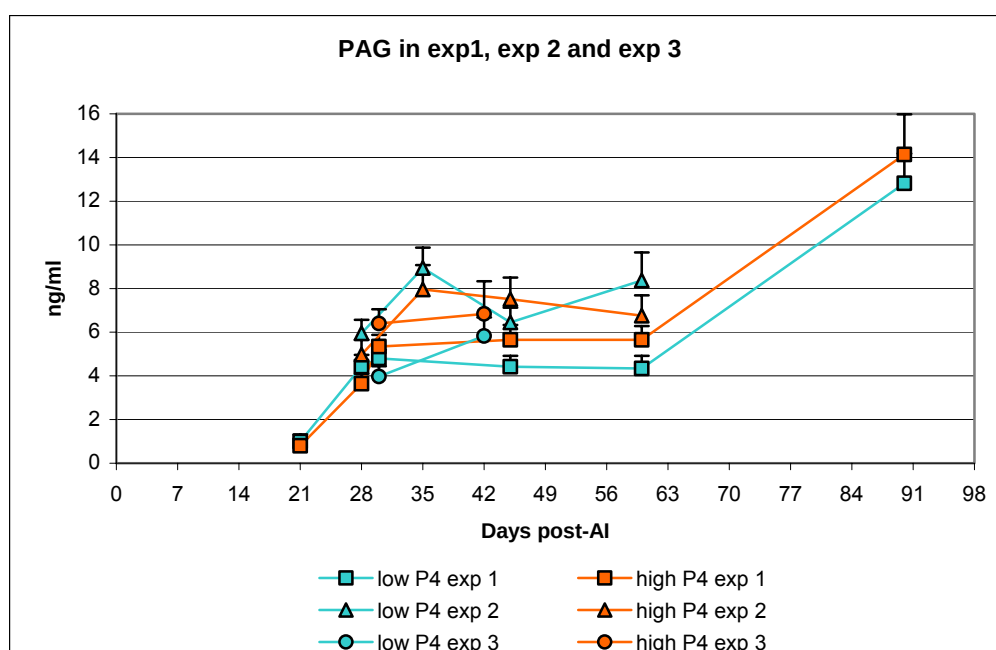


Figure 7.2 Plasma PAG profiles in “Low P4 group” and “High P4 group” in the three experiments object of this thesis.

These findings suggest that factors other than P4 are responsible for further PAG production. Since P4 acts upon trophoblast by means of P4 receptors on endometrial cells, once the mechanism activated by P4-receptor are fully operating, no further

increase of PAG release can be achieved. The present experiment, however, was not design to study this mechanism that needs further investigation.

7.3 Oxidative status: intensive vs. pasture-based system

Several OS biomarkers were measured in experiment 2 and 3. In both experiments OS biomarkers were related to the physiological phase (see tables 5.8 and 6.1), showing an increase during the post-partum. However, a specific comparison between intensive system and pasture-based system in terms of oxidative status of animals was not possible since the two experiments measured different OS biomarkers. In spite of this, it could be speculated that pasture feeding is probably a more favourable condition for the oxidative status of animals. Indeed, fresh pasture is particularly rich in natural antioxidants such as vitamins from group A, C, and especially E, and so it offers a great protection against lipids and proteins oxidation (Gatellier *et al*, 2004; Realini *et al*, 2004; Descalzo *et al*, 2005). Moreover, differently from animals reared on a feedlot, pasture fed animals are free to move, and exercise has been shown to have positive effects on oxidative status (Gatellier *et al*, 2004; Venditti *et al*, 2008).

7.4 Relationship between OS and EM

In the present thesis very few modifications of the OS indicators were observed in case of EM suggesting a weak involvement of OS in the aetiology of EM. However, the relationship observed between advanced oxidation protein products (AOPP) levels and EM is very interesting, because it suggests a greater degree of protein oxidation in case of EM.

In this study, the variations of AOPP in the cow plasma were reported for the first time. AOPP are terminal products of proteins damaged by free radicals. AOPP have been studied only recently in humans. Therefore, how they are produced and their function are still poorly understood. The most accredited hypothesis is that AOPP arise from the reaction between plasma proteins and chlorinated oxidants mediated by the enzyme myeloperoxidase (MPO) (Witko-Sarsat *et al*, 1998; Fialova *et al*, 2006; Noyan *et al*, 2006). MPO is released by activated neutrophils during early stages of acute inflammation.

Once produced, AOPP may have proinflammatory activities (Kalousová *et al*, 2005; Shi *et al*, 2008) either activating the chemotaxis or stimulating the immune response. Indeed, several oxidation products are highly immunogenic, therefore they may activate the

immune reaction and be the target of autoantibodies, thus triggering the inflammatory process (Shanti *et al*, 1999; Palinski and Witztum, 2000; Kurien *et al*, 2006).

Although a role of AOPP in immune system activation has not been demonstrated so far, it is possible that the higher levels of AOPP observed in EM may reflect subclinical inflammatory events at uterine levels that can compromise the correct embryonic development.

It might be hypothesised that OS represent a link between inflammation/immune response activation and EM rather than being the primary cause of EM. OS in reproductive disorders might be seen as a secondary phenomenon, a consequence of disease activity rather than the primary cause of the disease. Thus, as proposed by Halliwell (1994), the main question to be asked in evaluating OS role in human and animal disease should not be “can we demonstrate OS?” but rather “does the OS that occurs make a significant contribution to disease activity?”. In light of this different perspective, several questions may arise from the findings of the present thesis. For example, which role do OS biomarkers like AOPP play in the pathogenesis of EM? Do AOPP exert biological activity on their own or are they just a by-product of inflammatory/immune reactions?

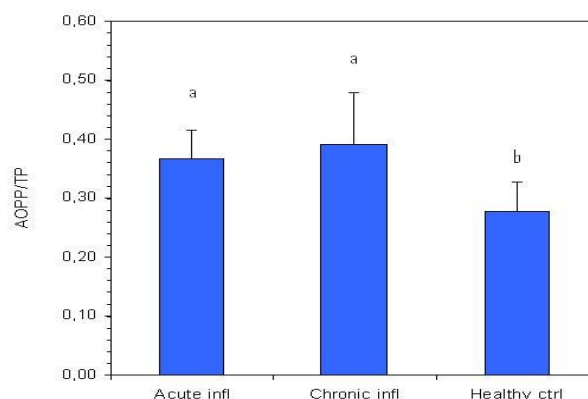


Figure 7.3 Ratio AOPP/Total Proteins in three experimental groups of cows: “acute inflammation”, “chronic inflammation”, and “healthy control” (Different letters indicate significantly different means; $P < 0.05$).

Preliminary results from current research (Da Dalt L, personal communication, figure 7.3) seem to indicate that AOPP are more concentrated in plasma of cows with chronic and acute inflammatory pathologies such as mastitis, lameness, and endometritis.

These findings lead to the hypothesis that AOPP are indeed by-products of the reactions against infections. However, further studies should be done to understand if AOPP may induce the formation of auto-antibodies.

Conclusions

The results obtained in this thesis suggest that the pregnancy-associated glycoproteins (PAG) test is a reliable method to perform pregnancy diagnosis in commercial dairy farms. However, sensitivity and specificity of PAG assay could be improved in order to avoid either false negative before Day 30 after breeding or false positive before Day 80 post-partum.

The determination of PAG in the maternal blood is a very efficient tool for diagnosing embryo mortality (EM) using serial plasma samples from the same cow. PAG test can also be used for pregnancy follow up, thus providing a valuable tool for further studies related to the bovine pregnancy.

In general, in the cow the involvement of oxidative stress (OS) on the EM is rather poor, as very few modifications of the OS indicators were observed. However, it is interesting the relationship between advanced oxidation protein products (AOPP) and EM observed in this thesis. This is the first time that AOPP modifications are described in the bovine, therefore the role they might play in EM needs further studies. The prevalent hypothesis is that AOPP may represent a by-product of inflammation derived from leukocyte activation. Therefore, inflammation and not OS might be the main responsible for embryo loss.

Thus, the main contribution of this PhD thesis was to provide new knowledge about the oxidative status of dairy cows. In particular, original contributions are in relation to OS and inflammation biomarkers, as AOPP, and their involvement in EM. As a result, new questions have arisen on this area that could constitute the objective of further research programs in the future.

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