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Relationship between oxidative stress status and glycoprotein-associated pregnancy concentrations during the early pregnancy period in dairy cows

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ABSTRACT

Objective: This study was planned to assess the possible relationships with pregnancy-associated glycoproteins (PAG) concentrations by the determination of the biomarkers of oxidative stress in the plasma of dairy cattle during the early period of gestation.

Materials and Methods: Blood samples were collected from coccygeal vessels in pregnant (n = 54) and non-pregnant (n = 45) cows. Measurement of biomarkers of oxidative stress (LPO, GSH and SOD) was carried out in females using spectrophotometric method.

Results: Plasma PAG concentrations increased and continuously over the both periods investigated. There were significant differences between pregnant and non-pregnant groups ($P < 0.001$). The concentration of SOD were significantly lower ($P < 0.05$) in pregnant females from day 25 to 35 (7.08 ±0.31 U.ml^{-1}) and day 36 to 50 after AI (6.6 ± 0.29 U.ml^{-1}) compared with non-pregnant cows (7.59 ± 0.35 U.ml^{-1}). Concerning the concentrations of LPO and GSH, the values obtained were also significant lower ($P < 0.05$) in pregnant females in the period 25-35 days post AI (122.7 ±10.27 µM and 6.46 ±1.24 µmol/min.ml^{-1}, respectively) and 36-50 days post AI (108.05±6.17 µM and 6.2±0.77 µmol/min.ml^{-1}, respectively) than in the non-pregnant females (124.8 ± 12.16 µM and 6.96 ± 0.92 µmol/min.ml^{-1}, respectively).

Conclusion: It was observed that the markers of oxidative stress tended to be higher in non-pregnant females compared with pregnant females during the early period of gestation in dairy cattle. Our results suggest the existence of a relationship among the concentration of oxidative stress markers and PAG during early pregnancy.

Keywords: *Bovine; Pregnancy; Pregnancy-associated glycoproteins, Oxidative stress*

INTRODUCTION

During pregnancy, several hormones and proteins are synthesized and secreted in the maternal circulation by ovaries, the placenta and the foetus. Some of them are specific to gestation and being detected in maternal blood from the moment when the conceptus becomes more closely attached to the uterine wall and the formation of placentomes (Chavatte-Palmer and Tarrade, 2016).

Pregnancy-associated glycoproteins (PAG) constitute a large family of molecules specifically expressed in the outer epithelial cell layer of the placenta in eutherian species. Their functions are not fully understood and they tend to be enzymatically inactive because of a mature in their active site (Sousa *et al*, 2006). Some immune suppressive properties of bovine PAGs and PAG-like molecules as pregnancy specific protein-B (PSPB) have been suggested in cattle (Hoeben *et al,* 2000). In particular, diminished immune functions of peripheral blood polymorphonuclear neutrophils (PMN) have been detected after PAGs peak at parturition (Dosogne *et al,* 1999). In a recent study, a clear relationship between high plasma PAG levels and elevated total leukocyte and neutrophil counts has been shown (Abdelfatah-Hassan *et al,* 2012). Neutrophils play an important role in host defense mechanisms and are known to cause tissue damage at the inflammatory site (Sordillo and Aitken, 2009). The activation of PMN is characterized by the production of reactive oxygen species (ROS) causing oxidative stress (Nathan and Cunningham-Bussel, 2013). The last may result from increased production of free radicals and reactive oxygen species, and/or a decrease in antioxidant defense which leads to damage of biological macromolecules and disruption of normal metabolism and physiology (Trevisan *et al,* 2001). It is well known that toxic ROS and oxidative stress are of primary importance in immune and inflammatory mechanisms (Codoñer-Franch *et al,* 2011) involved in the majority of diseases (Poston *et al,* 2011; Tuuli *et al,* 2011). Several studies confirmed the occurrence of oxidative stress during pregnancy, parturition (Fainaru *et al,* 2002; Lopez-Gatius *et al,* 2007) as well as during post-parturient period (Wall *et al,* 2002).

Pregnancy is a physiological event characterized by a drastic increase in energetic demands, to ensure an adequate fetal development and growth, thus, both mother and fetus are likely to experience oxidative stress (Garrel *et al,* 2010) with increased oxygen free radicals production (Mohanty *et al,* 2006). In normal pregnancy, there is a physiological oxidative balance where vitamin C, vitamin D and E gradually increase leading to a maintained oxidative balance throughout pregnancy (Bomba-Opon *et al,* 2014). There are several studies to support the concept that oxidative stress is a significant underlying factor to dysfunctional host immune and inflammatory responses that can increase the susceptibility dairy cattle to a variety of health disorders (Castillo *et al,* 2005; Wilde, 2006). Very early embryo mortality depends on blastocyst development capacities and uterine environment features (Gilbert, 2011) and it is well known in humans, ruminants and other animal species, that reactive oxygen species ROS are involved in embryo/fetal loss (Talukder *et al,* 2014).

Oxidative stress can be monitored with several biomarkers which can be assessed in plasma such as lipid peroxidation (LPO) and antioxidants including reduced glutathione (GSH) and superoxide dismutase (SOD). Whether oxidative status (LPO, GSH and SOD concentration) is related to trophoblast secretory properties (PAG secretion) during embryonic and early fetal development remains to be elucidated. To verify our hypothesis, we evaluated oxidative status and assessed the possible relationships with PAG concentrations by measuring LPO, GSH and SOD in Holstein Friesian dairy cattle during the early period of gestation.

MATERIALS and METHODS

Animals

The experiment was carried out from March to August 2011 in different dairy herds in Bass Kabylie area (36°34'N, 5°04'E), Algeria. This research was approved by the Scientific Council of the Faculty of Nature and Life Sciences (Report of Faculty Scientific Council Nbr. 07 dated December 14, 2010), University of Bejaia, Algeria). Concerning the ethical aspects, the experimental procedure was performed *in vitro* and the blood sampling of females was performed according to good veterinary practice under farm conditions. A total of 99 Holstein Friesian dairy cattle with mixed age (06 months and 12 years) and parity (0–9) were used in this study. The body condition score (BCS) was determined during the blood sampling period using a 5-points scale as described previously (Green *et al,* 2014). The BCS of experiment females was between 2.5 to 4.5. Fifty-four and forty-five Holstein Friesian females constituted the pregnant and non-pregnant groups, respectively. The pregnancies of females were diagnosis by ultrasonography (AgROSCAN A14, sondebi frequency 3.5 and 5.0 MHz) at 35-40 days post-artificial insemination (AI) (Wéré *et al,* 2012) or by rectal exploration approximately at 2 to 3 months after AI.

Blood sampling

Blood samples were collected in the in pregnant females between 25 to 50 days. For the control group, samples were randomly collected during a stabling period and in the absence of males. Samples collected from coccygeal vessels were transferred into a tube containing EDTA (Sarstedt®, Numbrecht, Germany). Plasma was obtained by centrifugation (1,500 × *g* for 15 min) immediately after collection and was stored at -20 °C until assay.

Measurement of pregnancy-associated glycoprotein (PAG) concentrations

The PAG concentrations in pregnant and non-pregnant groups were determined in plasma by Enzyme-linked immunosorbent assay (ELISA) performed in duplicates, as previously validated (Ayad *et al*, 2014). The detecting antibody used was a rabbit biotin-conjugated anti-PAG IgG. A spectrophotomer reader was used according to the kit instruction (Ref. Code E.G.7. CER. Marloie, Belgium). The enzyme substrate was avidin-horseradish peroxidase (HRP). The standard curve ranged from 0 to 2 ng/ml.

The basis of the test is a sandwich reaction involving two antibodies raised against PAG: the first one is coated on a 96 micro-plate whereas the second one is conjugated to biotin and detected using avidin-horseradish peroxidase (HRP). Briefly, dilution buffer is added just before adding PAG standards and serum samples. Afterwards, it is followed by an overnight incubation at room temperature. Micro titer wells are washed before addition of biotinylated anti-PAG. The washing step is followed by an incubation of 20 min at 37 °C with avidin-HRP. After the second washing, the substrate/chromogen solution is added to the wells and incubated 30 min at room temperature. The addition of the stopping reagent transforms the blue coloration into a yellow compound. Finally, the absorption at 450 nm was measured and the optical density was found to be proportional to the PAG concentration.

The threshold of 0.8 ng/ml was used to discriminate between pregnant and non-pregnant females (Ayad *et al*, 2009). The intra- and inter-assay coefficients of variation of PAG-ELISA were 2.78 and 6.08 %, respectively.

Measurement of the levels of superoxide dismutase (SOD), lipid peroxidation (LPO) and reduced glutathione (GSH) in plasma blood

Measurement of SOD was carried out in pregnant and non-pregnant females using spectrophotometric method with commercially available kits (Cayman Chemical). NADPH-dependent membrane lipid peroxidation was measured as thiobarbituric acid reactive substance using malonedialdehyde as standard (Sigma-Aldrich Fine Chemicals, St Louis, MO) (Nemmar *et al,* 2013). Measurement of GSH concentrations was carried out in pregnant and non-pregnant females according to the method described by commercially available kit (Sigma-Aldrich Fine Chemicals, Munich, Germany).

Statistical analysis

Data were analyzed using a mixed model for repeated measurements (Statview Software, Version 4.55) taking into account an autocorrelation between data obtained successively on the same animal. The data (± SE) were expressed as values of the PAG concentration and biomarkers of oxidative status. The Fisher's exact test was used to determine whether there was a significant difference between pregnant (P) cows (P-1: 25-35 days post AI; P-2: 36-50 days post AI) and non-pregnant (NP) cows. The values were statistically different when the *P*-value was ≤ 0.05.

RESULTS

Plasma PAG concentrations increased and continuously over the both periods investigated. Pregnant cows had the lowest levels of PAG (3.24±0.25 $ng.ml^{-1}$) in the period 25-35 days post-AI (Fig. 1). Thereafter, PAG concentrations increased significantly (*P*=0.05) in the period 36-50 days after fertilization (3.92±0.21 $ng.ml^{-1}$). The results of this experiment showed a very high PAG concentration (3.59±0.16 $ng.ml^{-1}$) in pregnant compared with non-pregnant cows (0.53±0.03 $ng.ml^{-1}$). There were significant differences between pregnant and non-pregnant groups ($P < 0.001$, Fig. 1).

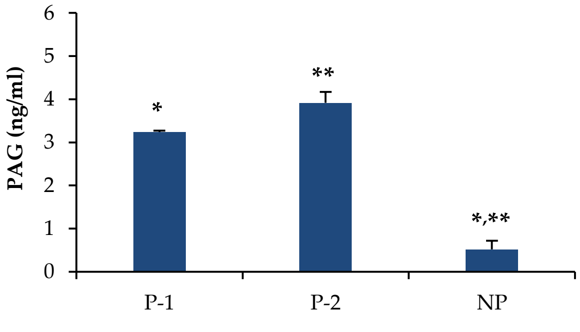

Figure 1. Plasma pregnancy associated glycoprotein concentration (mean ± SE) at early pregnancy period after IA in pregnant (P) cows (P-1: 25-35 days post AI; P-2: 36-50 days post AI) and non-pregnant (NP) cows. *,**: Significant difference in mean concentrations between P-1, P-2 and NP groups ($P<0.05$)

The levels of SOD (Fig. 2A) were significantly lower ($P≤0.05$) in pregnant cows at day 36 to 50 after AI (6.6±0.29 $U.ml^{-1}$) compared with non-pregnant cows (7.59±0.35 $U.ml^{-1}$). Concerning the concentrations of

LPO (Fig. 2B), the values obtained were significant lower ($P \leq 0.05$) in pregnant cows in the period 25-35 days post AI (122.7±10.27 µM) and 36-50 days post AI (108.05±6.17 µM) than in the non-pregnant cows (124.8±12.16 µM). On the other hand, GSH (Fig. 2C) concentrations were also significantly lower ($P \leq 0.05$) in pregnant cows during the period 36-50 days post AI (6.2±0.77 µmol/min.ml^{-1}) than in the non-pregnant cows (6.96±0.92 µmol/min.ml^{-1}).

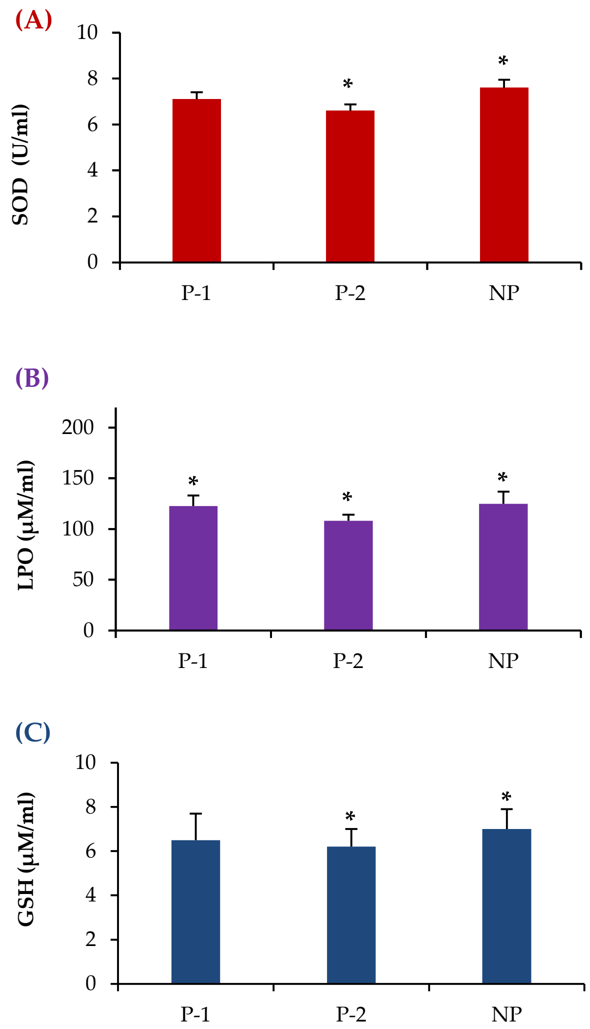

Figure 2. Plasma concentration (mean ± SE) of SOD (2A), LPO (2B) and GSH (2C) levels at early pregnancy period after IA in pregnant females (P-1: 25-35 days post AI; P-2: 36-50 days post AI) and non-pregnant females. * Significant difference in mean concentrations between P-1, P-2 and NP groups ($P<0.05$)

Positive correlation between LPO concentrations and SOD levels was r=0.6 ($P \leq 0.05$, Table 1). There was no significant correlation between PAG and other oxidative stress parameters.

Table 1. Correlation coefficients (r) between of pregnancy associated glycoprotein, SOD, LPO and GSH concentrations at early pregnancy period from day 25 to 50 after AI in pregnant females ($^{*}P < 0.05$)

	SOD	LPO	GSH
LPO	<u>0.6*</u>		
GSH	-0.25	-0.14	
PAG	0.12	0.05	-0.11

DISCUSSION

This study investigated whether the concentrations of markers of oxidative stress during the early period of pregnancy in dairy cattle could be related to trophoblastic secretory function based on the measurement of PAG concentrations in pregnant cows.

Bovine pregnancy-associated glycoproteins are mainly secreted by trophoblastic binucleate cells (Wooding *et al*, 2005) and they have been detected in the blood pregnancy cows from day 25 after AI (Green *et al*, 2014). Therefore, these glycoproteins have been used as a biochemical marker of pregnancy (Gajewski *et al*, 2014), monitoring of placental secretory function in ongoing pregnancies (Breukelman *et al*, 2012), detection of placental abnormalities (Constant *et al*, 2011) and investigation of embryonic and fetal mortalities (Karen *et al*, 2014). Detection of PAG by radioimmunoassay method (García-Ispierto *et al*, 2015) or ELISA (Piechotta *et al*, 2011) is currently used as a specific serological method for pregnancy diagnosis in cattle from 28 day after breeding, with a threshold level for pregnancy of 0.8 ng/ml (Ayad *et al*, 2009).

The results of this experiment showed that the mean PAG concentrations measured at early gestation period by ELISA method in the pregnant cows agreed with those of Friedrich and Holtz (2010). There was only a significant difference at period 25-30 days after AI. The lack of significant difference in PAG values from 30 to 50 of gestation appears to be probably attributed to the high individual variation in PAG concentrations. A high variability of individual PAG levels has been documented in the literature. Lobago and co-workers (2009) reported that the PAG profiles were considerably influenced by factors such as breed, weight and parity status of the dam. Likewise, it was reported that PAG concentrations during the early fetal pregnancy period are correlated with some factors such as milk production in high producing dairy cows (Lopez-Gatius *et al,* 2007). In other study, Ayad *et al.* (2009) observed that PAG concentrations tended to be higher in primiparous than nulliparous or multiparous females. However, according to Ledezma-Torres *et al* (2006) parity had no significant effect in plasma PAG levels. As shown in figure 1, the difference between pregnancy and non-pregnant cows were very significant. In the non-pregnant group, ELISA method detected PAG concentration in all females was lower than 0.8 ng/ml (Ayad *et al,* 2009).

Oxidative stress corresponds to an imbalance between the rate of oxidants production and that of their degradation (Sorg, 2004). All mechanisms linked to pregnancy are under control of steroid hormones, prostaglandins as well as other biologically active factors (Kindahl *et al,* 2004). These mechanisms are based on metabolic pathways which are usually aerobic and may be related to the production of certain amounts of ROS. The blastocyst losses the zona pellucid 9-10 days before implantation in the bovine specie (Ayad *et al,* 2006). This is accompanied by an increase in ROS generation, related to an augment in the cell to cell contact and to NADPH oxydase activation. Mitochondrial ROS generation increases during embryogenesis because an imbalance exists between ROS generation and its modulation, with ROS generation being overwhelming (Aurousseau *et al,* 2004).

To our knowledge, this is the first report on the relationship between markers of oxidative stress and PAG concentrations from bovine plasma at early stages of pregnant cows. The activities of the LPO is one the most important expression of oxidative stress induced by ROS, and as well as antioxidant enzymes, namely SOD and GSH. In the present study, LPO, SOD and GSH were determined in plasma sample of pregnant and non-pregnant cows.

Oxidative stress seems to have a role in the cause and progression of a number of reproductive events in both humans and animals, such as fertilization and early embryo development (Al-Gubory *et al,* 2010). Oxidative stress during early placental development is associated with pregnancy-related disorders in humans (Agarwal and Allamaneni, 2004; Gupta *et al,* 2007). The depletion of placental antioxidant systems has been suggested as a key factor in early human pregnancy failure (Liu *et al,* 2006). Some data published suggested that enhanced activities of key antioxidant enzymes with gestational ages may act as protective mechanism against oxidative stress during early human (Qanungo *et al,* 2000) and sheep (Garrel *et al,* 2010) placental development and growth.

Antioxidant enzymes such as superoxide dismutase and glutathione peroxidase could be beneficial in enhancing implantation and maintaining pregnancy by antagonizing the harmful oxygen free radical (Smith *et al,* 1998). Superoxide dismutase enzyme is believed to play a major role in the first line of antioxidant defense by catalyzing the dismutation of superoxide anion radicals to from hydrogen peroxide (H_2O_2) and molecular oxygen (Soehnlein *et al,* 2009). Glutathione peroxidase is a selenoprotein that reduce lipidic or non lipidichydroperoxides as well as H_2O_2 while oxidizing GSH (Sordillo *et al,* 2009). It is important to note that mitochondria are the major site of endogenous O_2^- production (Wallace, 2005). $O_2^-$is converted to H_2O_2 by mitochondrial matrix SOD.

The conceptus develops a good defense mechanism against free radicals, possessing high concentrations of antioxidants at 5 weeks of gestation such as vitamin A and E; and increased expression of catalase, Cu, Zn-SOD, Mn-SOD in villous placenta at twelve weeks (Jauniaux *et al,* 2003). The plasma markers of oxidative stress measured in the present study were statistically different between non-pregnant than in pregnant group. These differences could play a role of successful pregnancy during the early period in dairy cattle. It is difficult to compare these results with others reported in literature because of the lack of studies on oxidative stress during the early pregnancy period in dairy cattle. Preliminary results obtained in the present study suggest that PAG concentrations seem have an indirect effect on markers oxidative stress levels in pregnant females during the early period of gestation. Previous studies suggesting that advanced oxidative protein product (AOPPs), as markers of protein oxidation is not recent (Witko-Sarsat *et al,* 1996). The AOPPs are higher in dairy cows suffering from embryonic mortality than in pregnant females (Celi *et al,* 2012). These results are in disagreement with those reported in literature which showed that a SOD level was significantly higher in pregnancy positive than in pregnancy negative in women (Younis *et al,* 2012).

It is known that the polymorphonuclear (PMN) leukocytes play an important role in host defense mechanisms and are known to cause tissue damage at the inflammatory site. The activation of PMN is characterized by the production of reactive oxygen species (ROS) such as superoxide anion radical (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (OH) (Sordillo *et al*, 2009). Considering that the activity of monocytes and macrophages is increased during pregnancy, and that the concentration of several markers of oxidative stress is concomitantly increased, it could be argued that pregnancy is characterized by a pro-inflammatory state (Ness, 2004). It is interesting that decreased SOD, LPO and GSH activities in pregnant females. In this study, during the early period of pregnancy was associated with an increase of PAG concentrations compared with non-pregnant group. Several investigations carried out in order to detect relations between PAG or PSPB and a local immunological function. Dosogne and associated (2000) reported the succession of the very high concentrations of PAG and the decrease of the oxidation activity of the polymorphonuclear neutrophils. In other study, the results indicate that bPAG may directly or indirectly affect the respiratory burst activity of bovine neutrophils in the periparturient period (Hoeben *et al*, 2000).

CONCLUSION

In conclusion, this study presents a first report on the relationship between oxidative stress and PAG concentrations during the first trimester of pregnancy in dairy cattle. It was observed that the markers of oxidative stress tended to be higher in non-pregnant females (PAG concentration 0.53±0.03 ng.ml^{-1}) compared with pregnant females (PAG concentration, 3.59±0.16 ng.ml^{-1}) during the early period of gestation in dairy cattle. However, further studies including large-scale investigations are needed to confirm our results, as well as to investigate maternal concentrations of both oxidative stress parameters and PAG in pregnant females experiencing interrupted pregnancies.

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