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Evaluation of serum analytes in pregnant and non-pregnant dairy cows as indicators of pregnancy

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Abstract

This study was conducted to reveal the dynamics of haematological indicators in pregnant and non-pregnant dairy cows. Sixty multiparous healthy dairy cows were divided into four groups based on the length of time they had been pregnant, namely first, second and third trimesters, and non-pregnant (n=15 each). Blood was collected from each animal, and serum was harvested and stored at -20 °C for biochemical profiling. Concentrations of serum total oxidants (TOC), ceruloplasmin oxidase (CpO) and triiodothyronine (T_3) were higher (P <0.05) during the third trimester compared with non-pregnant cows. Serum arylesterase (Ary) concentration was lower (P <0.05) during the second and the third trimesters compared with the non-pregnant cows. The concentration of serum total homocysteine (tHcy) was higher (P <0.05) the third trimester compared with the first and the second trimesters. The concentrations of serum total antioxidants (TAC), paraoxonase 1 (PON1), thyroxine (T_4), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were not different in the pregnant and the non-pregnant cows. Thus, TOC, Ary, CpO, tHcy and T_3 could be taken as biological markers to assess the progression of pregnancy and to develop management tools to improve health status during late gestation in dairy cows.

Keywords: ceruloplasmin, hepatic enzymes, late gestation, triiodothyronine
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Introduction

Pregnancy is characterized by increased metabolic demand owing to the changes in female physiology and the requirements of the growing foetus (Đuričić *et al.*, 2020). The dynamic changes that occur in various body systems during the gestational period result in increased demand for oxygen. Other changes include consumption of the energy substrate by the dam, especially in the feto-placental unit (Baimishev *et al.*, 2019). Pregnancy, therefore, is a condition that favours oxidative stress and results in an imbalance between the pro-oxidant and antioxidant systems of the body, leading to potential damage (Abuelo *et al.*, 2015; Kleczkowski *et al.*, 2017; Szenci *et al.*, 2018; Folnožić *et al.*, 2019). The body develops a number of antioxidant mechanisms (Serdar *et al.*, 2002) to counteract this stress through enzymatic induction and activity of enzymes such as arylesterase (Ary) and paraoxonase (PON) (Turk *et al.*, 2007), ceruloplasmin oxidase (CpO) and apolipoprotein A-I (ApoA-I) (Folnožić *et al.*, 2015). These defence mechanisms are easily disrupted in pregnancy, as evidenced by increased oxidative stress (Castillo *et al.*, 2006; Turk *et al.*, 2018), low total antioxidant capacity (TAC) and decreased PON activity (Turk *et al.*, 2005a) in dairy cattle.

At higher concentrations, homocysteine (Hcy), a naturally occurring sulfur-containing intermediate product of methionine-cysteine metabolism, can increase the formation of reactive oxygen species leading to oxidative stress (Abud *et al*., 2016). In normal human pregnancy, plasma Hcy concentrations decrease to their lowest level during the second trimester (Hague *et al.*, 1997). Belo *et al.* (2004) reported a significant

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decrease in the serum TAC from the first to the third trimester. In another study, it was reported that serum TAC was reduced during the first trimester and then started to increase during the second and the third trimesters of pregnancy in humans (Toescu *et al.*, 2002).

Hormonal changes during pregnancy exert complex and profound effects on the biomarkers of metabolism such as thyroid hormones and hepatic enzymes. The changes in concentrations of free thyroxine (T_4) and triiodothyronine (T_3) remain controversial. In humans, some authors reported a decrease in free thyroid hormones (Kurtz *et al.*, 1979; Müller *et al.,* 2019), whereas others reported no change or an increase (Guillaume *et al.*, 1985). Similarly, the liver synthetic and metabolic functions are affected by increased serum oestrogen and progesterone levels during pregnancy (Van Thiel & Gravaler, 1987). The activity of aspartate aminotransferase (AST) shows occasional irregular small changes, whereas alanine aminotransferase (ALT) decreases significantly during the seventh and eight months of pregnancy in cattle (Aladrović *et al.*, 2018).

The role of biological health markers of oxidative and metabolic stress (Castillo *et al.*, 2006) is gaining importance because they can provide useful information about the progression of pregnancy and early detection of complications. The literature about human beings illustrates that the oxidative status of a pregnant individual can be monitored with several biomarkers including pro-oxidants and antioxidants (Toescu *et al.*, 2002). However, many biomarkers, such as PON1 and ApoA-I, have been studied to evaluate the oxidative status in dairy cows during pregnancy (Turk *et al.*, 2005; Castillo *et al.*, 2006; Turk *et al.*, 2012; Turk *et al.*, 2016; Kovačić *et al.*, 2019; Nedić *et al.*, 2019). Therefore, the aim of the current study was to investigate and identify the changes in biochemical profile, enzymic antioxidants, and enzymes of hepatic and thyroid origin during known stages of pregnancy of dairy cattle and compare them with non-pregnant crossbred dairy cattle. It was hypothesized that any change in these blood analytes in pregnant dairy cattle provide evidence of pregnancy status.

Materials and Methods

The experimental procedure was approved by the Institutional Animal Care and Use Committee of the University of Veterinary and Animal Sciences, Lahore. The study was carried out with 60 clinically healthy crossbred dairy cows (Sahiwal x Holstein-Friesian) maintained at Breeding Farm, Department of Animal Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan. The ages of the cows ranged between 4 and 7 years and they were divided into four groups, based on the stages of pregnancy, namely first, second and third trimester and non-pregnant, with 15 cows in each group. The diets were formulated to meet or exceed all of the NRC (1994) recommendations for pregnant dairy cows.

Blood was collected in plane vacutainers by puncturing the *Vena jugularis*. Blood was allowed to stand at room temperature for two hours and then centrifuged at 1500 x g at 4 °C for 15 minutes to harvest serum (Beckman Instruments, Inc., USA). Serum samples were stored at -20 °C for further serum biochemical profiling.

The activity of serum TOC was assayed (Erel, 2005). Briefly, 35 µL of serum sample was added to 225 µL of Reagent 1 (150 µM Xylenol Orange, 140 mM NaCl, 1.35 M Glycerol, pH 1.75), and the first absorbance was taken at primary wavelength of 800 nm and secondary wavelength of 560 nm. Thereafter, 11 µL of Reagent 2 (5 mM ferrous ammonium sulfate, 10 mM o-Dianisidine dihydrochloride) was mixed and the final absorbance was taken after four minutes. The delta change in absorbance was used to calculate the final concentrations. The concentration was expressed in terms of micromolar hydrogen peroxide equivalent per litre (µM H_2O_2 EquivL^{-1}).

Serum TAC was assayed (Erel, 2005). Briefly, 5 µL of the serum sample was added to 200 µL of Reagent 1 (75 mM Clark and Lubs solution, pH 1.8 containing 10 mM o-Dianisidine as substrate and 45 µM ferrous ion) and the first absorbance was taken at wavelength of 444 nm. Thereafter, 10 µL of Reagent 2 (7.5 mM H_2O_2 in Clark & Lubs solution) was mixed and the final absorbance was taken after four minutes. The delta change in absorbance was used to calculate the final concentrations. The concentration was expressed in terms of millimolar vitamin C equivalent per litre (mM Vit C EquivL^{-1}).

In the absence of sodium chloride, serum PON1 was assayed as described by Juretic *et al.* (2006) and expressed in units per litre (UL^{-1}). Briefly, 10 µL of serum sample was added to 350 µL of basal assay mixture (0.1 M Tris-HCl buffer, pH 8.0 containing 2.0 mM paraoxon as substrate and 2.0 mM $CaCl_2$). The initial rate of hydrolysis of paraoxon was determined by measuring liberated p-nitrophenol at a wavelength of 405 nm and at 37 °C.

Serum Ary was assayed (Juretic *et al.,* 2006) when 875 µL assay mixture (20 mM Tris-HCl buffer, pH 8.0 containing 1.0 mM phenylacetate as substrate and 0.9 mM calcium chloride ($CaCl_{2)}$) was mixed with 125 µL of diluted serum sample (1:10; deionized water).The initial rate of hydrolysis of phenylacetate was determined at a wavelength of 270 nm and at 37 °C. Ary was expressed in (UL^{-1} of serum.

Serum CpO was assayed (Schosinsky *et al.*, 1974) when 12 µL serum sample was added to 238 µL of basal assay mixture (acetate buffer containing o-Dianisidine dihydrochloride as substrate). The mixture was incubated for 5 minutes and then 500 µL of H_2SO_4 (9.0 molL^{-1}) was added. The initial rate of hydrolysis of o-Dianisidine dihydrochloride was determined at a wavelength of 540 nm and at 37 °C. CpO was expressed in UL^{-1}.

Commercial kits were used to determine the concentrations of serum tHcy (DZ 568A-K, Diazyme Laboratories, USA), AST, ALT (Randox Laboratories, UK) spectrophotometrically, and T_3 and T_4 (EIA kit, Biocheck Inc. USA) by ELISA reader (Statfax-303 plus, Awareness Technology, Inc., USA). All the spectrophotometric readings were taken with a temperature-controlled biochemistry analyser (Biosystems, BTS-330, Barcelona, Spain). All chemicals, excluding commercial kits, were of analytical grade and were purchased from Merck, Darmstadt, Germany.

SPSS for Windows (version 10.0.1, SPSS Inc., Chicago, Illinois, USA) was used for data analysis. Data were expressed as means ± SE. The Kolmogorov-Smirnov test was used to test the normal distribution of the data. The data were analysed using one-way analysis of variance. Differences between treatments were evaluated using Duncan's multiple range test. Differences that occurred by chance at P <0.05 were considered significant.

Results and Discussion

The results of the current study revealed that serum TOC, CpO and T_3 were higher (P <0.05) during the third trimester compared with the non-pregnant cows. Serum T_3 was also higher (P <0.05) in the third trimester compared with the first and the second trimesters. Serum Ary concentration was lower (P <0.05) during the second and the third trimesters compared with the non-pregnant cows. The concentration of the serum tHcy was higher (P <0.05) during the third trimester compared with the first and the second trimesters. The tHcy concentration, however, was not different during the various stages of pregnancy compared with the non-pregnant cows. The concentrations of the serum TAC, PON1, T_4, AST, and ALT remained unchanged (Table 1).

Table 1 Serum analytes (means ± SE) in pregnant and non-pregnant crossbred dairy cows

Serum analytes	1st Trimester	2nd Trimester	3rd Trimester	Non-Pregnant
TOC (µM H_2O_2 EquivL^{-1})	0.484 ± 0.057^{ab}	0.504 ± 0.083^{ab}	0.696 ± 0.082^{a}	0.360 ± 0.041^{b}
TAC (mM Vit C EquivL^{-1})	0.188 ± 0.051	0.174 ± 0.022	0.167 ± 0.028	0.219 ± 0.056
PON1 (UL^{-1})	198 ± 10.57	221 ± 49.18	208 ± 19.38	201 ± 13.58
Ary (UL^{-1})	38.40 ± 5.04^{ab}	30.87 ± 3.49^{b}	24.60 ± 3.15^{b}	49.75 ± 7.04^{a}
CpO (UL^{-1})	81.89 ± 21.55^{ab}	83.57 ± 17.22^{ab}	130.90 ± 26.23^{b}	50.62 ± 7.75^{a}
tHcy (µmolL^{-1})	5.85 ± 0.58^{b}	6.52 ± 0.30^{b}	10.5 ± 0.45^{a}	8.00 ± 1.23^{ab}
AST (UL^{-1})	13.17 ± 1.87	17.65 ± 2.27	17.55 ± 2.92	11.03 ± 1.32
ALT (UL^{-1})	9.20 ± 2.53	10.1 ± 2.85	10.43 ± 3.15	9.87 ± 3.34
T_3 (ngmL^{-1})	3.40 ± 0.50^{b}	3.99 ± 0.30^{b}	7.37 ± 0.62^{a}	2.73 ± 0.22^{b}
T_4 (µgdL^{-1})	12.75 ± 1.20	14.09 ± 1.24	16.57 ± 1.24	12.66 ± 1.33

Different letters $^{a, b}$ in a row differ (P <0.05). TOC: total oxidants; TAC: total antioxidants; PON: paraoxonase; Ary: arylesterase; CpO: ceruloplasmin oxidase; tHcy: total homocysteine; AST: aspartate aminotransferase; ALT: alanine aminotransferase; T_3: triiodothyronine; T_4: thyroxine

An elevated plasma lipid peroxide profile has been advocated by many researchers in pregnancy of human and domesticated animals (Belo *et al.*, 2004; Erisir *et al.*, 2009). The results of the current study are in line with those studies that revealed that TOC, an indicator of lipid per-oxidation, was significantly higher during the third trimester compared with non-pregnant cows. Nazari and co-workers (2019) found an elevated TAC in pregnant Holstein cows compared with non-pregnant ones. They also found that cows with an abortion history had a lower antioxidant profile than their healthy counterparts. The results of the current study revealed that the serum TAC was non-significantly altered. Landray *et al.* (1998) demonstrated that the major contributors to serum TAC were uric acid (70.3%), vitamin C (10.3%), vitamin E (8.6%), and vitamin A (10.7%). A number of studies showed that the concentrations of uric acid and vitamin C and vitamin E (Wang

et al., 1991) fluctuated during normal pregnancy. Moreover, increased TAC might be an adaptive response to increased oxidative stress and decreased TAC might be attributed to lower production of free radicals (Castillo *et al.,* 2006). This indicates that TAC is not an important parameter, at least in dairy cows, in evaluating oxidative status because uric acid is a weak antioxidant, and a number of enzymic antioxidants, namely superoxide dismutase, glutathione peroxidase, catalase, paraoxonase, arylesterase, and ceruloplasmin oxidase, play vital roles in the antioxidant system.

In addition to dietary antioxidants, the maternal body develops a number of enzymic antioxidant mechanisms to cope with the production of free radicals (Serdar *et al.*, 2002) and progression of normal gestation. Serum PON1, a high-density lipoprotein (HDL) associated antioxidant enzyme, is known to eliminate organophosphorus compounds such as paraoxon and carcinogenic lipid soluble radicals from lipid peroxidation. It also protects both low density and high-density lipoproteins against lipid peroxidation. Theoretically, increased oxidative stress can leads to reduced PON1 activity. Some studies revealed that PON1 activities are independent of HDL (Turk *et al*., 2005) Moreover, based on oxidative stress and metabolic adaptation on PON1 activity and MDA profile in transition dairy cattle, it has been shown that serum PON1 activity varies among animals (Turk *et al.*, 2007) and PON1 has at least five polymorphisms that affect its hydrolytic activity for certain substrates such as paraoxon and lipid peroxides and their expression (Suehiro *et al.*, 2000). Furthermore, PON1 gene expression is determined by both dietary and genetic factors. A decrease in PON1 concentrations (Turk *et al.*, 2005) has been reported in cows during pregnancy. Turk *et al.* (2005) suggested a possible role of increased oxidative stress for reduction in PON1 activity during pregnancy in dairy cows. However, Bionaz *et al.* (2007) suggested that inflammatory conditions may be responsible for the reduction of PON1 activity in transition dairy cows. Contrary to these findings, the current results revealed that serum PON1 concentrations did not change during the stages of gestation. This controversy may be attributed to genetic and individual variations and to the polymorphism feature. Another possible reason for these erratic findings probably stems from the differences in lipid and lipoprotein metabolism in pregnancy-oriented stressful physiological status, genetics, breed and nutritional plan. This variation may be the result of a negative energy balance at the terminal stage of pregnancy and may continue post partum (Turk *et al.*, 2007)

PON1 also has an arylesterase (Ary)-like activity that is not affected by polymorphism. The serum Ary esterase enzyme has lipophilic antioxidant characteristics. In the present study the authors found a lower (P <0.05) concentration of serum Ary during the second and third trimesters. Nus *et al.* (2007) reported a negative correlation between serum Ary activity and lipid peroxidation. The findings of the current study and those of Nus *et al.* (2007) demonstrated that Ary is utilized to combat the oxidative stress induced by the pregnancy. Moreover, the current study demonstrated that Ary is a potential oxidative stress marker compared with PON1 in crossbred dairy cows. Ceruloplasmin oxidase (CpO), a glycoprotein, acts as a free radical scavenger and an antioxidant owing to the prevention of metal ion-catalysed oxidation of lipids in the cell membrane. Increased CpO activity has been shown by many researchers (Kristensen *et al.*, 2009) in healthy females with uncomplicated pregnancy. It has been postulated that an elevated level of oestrogen is responsible for the increased concentration of CpO However, to the best of the authors' knowledge data are not available on CpO activity in dairy cows during pregnancy. The current study showed that serum CpO is higher (P <0.05) in the third trimester compared with non-pregnant dairy cows in which its concentration was just 38% of full-term pregnancy.

Plasma tHcy concentrations fall in normal pregnancy, with the lowest occurring in the second trimester (Walker *et al.*, 1999). Many studies have shown that high levels of tHcy can increase ROS formation and lead to oxidative stress (Perna *et al.*, 2003). Furthermore, its increased concentration is associated with adverse pregnancy outcomes or early pregnancy losses (Walker *et al.*, 1999) in human beings. During normal pregnancy the blood concentrations of folate and cobalamin decrease (Steeger-Theunissen, 2004). The precise mechanisms are still not known, but it has been proposed that a shortage of vitamins, increased age, gender and polymorphisms in related enzymes could lead to an elevated tHcy concentration (De Bree *et al.*, 2001). The higher (P <0.05) tHcy concentrations during the third trimester in crossbred cows could be attributed to the higher metabolic demands as indicated by raised serum T_3 concentrations.

The effects of pregnancy on serum ALT and AST activity levels are controversial in humans. A few studies reported a slight increase in ALT and AST activity during the third trimester (Salgo & Pal, 1989). However, most of the human literature demonstrated that ALT and AST activities remained within normal limits during pregnancy. Data are not available in the literature about the stages of pregnancy in dairy cattle. There are certain studies in which hepatic enzymes have been evaluated under metabolic disorders (Wang *et al.*, 2018). Serum activities of AST and ALT were used as diagnostic tools for clinical ketosis and fatty liver disease during the peripartum period. Wang and co-workers (2018) demonstrated a significant increase in the AST profile in the group of cows with ketosis (160.28 ± 15.79) and fatty liver disease (348.13 ± 25.5). Similarly, the ALT profile was within normal limits in the control group (19.21 ± 0.45 for healthy cows) in

comparison with ketosis (22.29 ± 0.93) and fatty liver disease (74.30 ± 2.15). In the current study, the activities of both ALT and AST remained within the normal limits in the cows during pregnancy (Kaneko *et al.*, 1997). Statistically, the change in all groups was non-significant (P >0.05). because of the selection of healthy animals.

Pregnancy is also known to induce adaptations in the biochemical parameters associated with thyroid hormones. Some authors reported a decrease in humans, (Kurtz *et al.*, 1979) in thyroid hormone concentrations during pregnancy, while others reported no change or an increase (Guillaume *et al.*, 1985; Soldin, 2006). Thus, the data in the literature are controversial. To date, no study has evaluated the T_3 and T_4 profiles during bovine pregnancy. However, hyposecretion of T_3 after the induction of hypothyroid in lactating dairy heifers resulted in significant reduction of P_4 on the fourteenth day of the subsequent cycle in comparison with control animals, which had normal thyroid function (Thrift *et al.*, 1999). A rise in total thyroid hormone concentration was reported in early pregnancy, which remained high to form a plateau in the early second trimester (Guillaume *et al.*, 1985). The proposed mechanisms for this rise are i) increased serum thyroxine-binding globulin (TBG) concentration under the stimulatory effect of oestrogens (Glinoer, 1997), resulting in elevated concentration of thyroid hormones in the first and the second trimesters, which was continued up to the third trimester and then showed a decline till term (Soldin, 2006); ii) production of type III deiodinase from the placenta during the second half of gestation; and iii) greater metabolic demand during pregnancy increased the production of these hormones and of concentrations in the circulation (Glinoer, 1997). Similar to the findings of Guillaume *et al.* (1985), the current authors found a higher (P <0.05) serum T_3 concentration during the third trimester compared with other stages and non-pregnant animals. The concentration of T_3, however, was non-significantly higher during the first and the second trimesters compared with non-pregnant animals. Similar to Kurtz *et al. (*1979), the authors found that serum T_4 concentrations remained unchanged during pregnancy and non-pregnant stages.

Conclusion

The evaluation and monitoring of biological health and metabolic markers such as TOC, Ary, CpO and T_3 could provide useful information about the progression of pregnancy and be used to manage and improve the health status of dairy crossbred cows during late gestation. To this end, profiles of these serum analytes could offer practitioners and dairy farmers useful tools for strategic interventions.

Authors' Contributions

SA, MSY, IA, HR, and AKM conceived the project, designed the experiment, and analysed the data. SA and MSY performed the experiment. SA, AHS, RSB, DN, YN and MRY wrote the manuscript. IA, MSY and HR supervised the research. All authors read and approved the manuscript.

Conflict of Interest Declaration

The authors have no conflict of interest to declare.

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