Original Article

Effect of vitamin C on pregnancy rate and 8-OHdG levels during heat stress in post-partum dairy cattle

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Received December 2, 2021

Revised December 7, 2021

Accepted December 7, 2021

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ABSTRACT In this study the effect of vitamin C administration on pregnancy rates during summer heat stress in dairy cows was examined. A total of 80 Holstein-Friesian cows were divided into control and treatment groups (n = 40 each). Control group animals were given 10 mL isotonic normal saline, and treatment group, Vitamin C (4 mg/kg) on artificial insemination day (day 0) and 4th, 8th and 12th day post insemination. Pregnancy diagnosis was performed on 30th day post insemination by ultrasonography. Blood samples were randomly taken from 11 animals from each group. Serum P₄, GSH, MDA and plasma 8-OHdG levels were determined by using ELISA method. Results showed that 8-OHdG levels were lower in treatment group on day 4, 8 and 12 ($p < 0.05$) compared with the control group. Similarly, pregnancy rate was higher in treatment group (32.5%) than control (22.5%), respectively. However, MDA, P₄ and GSH levels were similar in both groups at 4th, 8th and 12th day. A gradual increase in P₄, and MDA levels, and a strong positive correlation between 0, 4th ($r = 0.54$), 4, 8th ($r = 0.59$) and 8, 12th ($r = 0.51$) day was found. Similarly, GSH levels also showed positive correlation at days 0, 4th ($r = 0.47$) and 4, 8th ($r = 0.56$). However, a strong negative correlation ($r = -0.56$) between MDA day 0, and GSH day 8 was found. In conclusion, vitamin C application during insemination period in postpartum cows increases pregnancy rate, and reduces oxidative stress metabolite 8-OHdG levels.

Keywords: conception rate, dairy cows, heat stress, vitamin C, 8-OHdG

INTRODUCTION

The impact of environmental stress on dairy cows is getting severe with global warming and changes in climate. The increase in average temperature of the earth by 1.8–4°C has been reported between 1990 and 2100 accord-

ing to the intergovernmental climate change report 2007. Similarly, Dikmen et al. (2012) in a work between 2002 and 2008 reported that there was an annual increase of 0.07°C in the body temperature of the cows. The optimal environment conditions for the milking cows are 13–18°C, 60–70% proportional humidity, 5–8 km/hr wind speed,

and medium degree solar radiation (Hansen, 2007). As environmental temperature rises, the heat stress starts to begin between 26.92 and 32.2°C air temperature, and 50-90% humidity (Fidler and VanDevender, 2013).

Various indexes have been developed using environmental conditions such as solar radiations, wet thermometer temperature (°C), dry thermometer temperature, humidity rate, and wind speed in determining heat stress. As rule of thumb the most updated index is known as “temperature humidity index” (THI) and it helps to calculate the heat stress as per recently developed indexes (Dikmen and Hansen, 2009). It is believed that cows are under the stress or out of stress $72 < \text{THI}$, and between 72 and 79 THI values light stress starts, and 80-89 THI shows medium level of stress, and $90 > \text{THI}$ values is fatal heat stress (Armstrong, 1994).

The free radicals are produced as a consequence of heat stress, and harm the body by changing the structure and function of target molecules at cell level. This further affects genetic material DNA and RNA, which subsequently alters the cell membrane and different enzymatic events. The level of oxidative stress, and the damages in the cells are determined by using lipid peroxidation, DNA damage and metabolic products during oxidative alteration of the proteins (Valko et al., 2006). As a result of oxidative stress lipid peroxidation starts removing hydrogen ions away from unsaturated fat chain, thus changing the membrane structure. Ultimately, this leads to increase in malondialdehyde (MDA) levels (Yerer and Aydoğan, 2000).

The substances and biomolecules which prevent oxidations and neutralize the free radicals are known as antioxidants. Numerous studies have reported the benefits of antioxidants like reduction in lipid peroxidation by preventing chain reaction of peroxidation, and MDA production (Gürbüz et al., 2014). Oxidative stress causes body damage by various mechanisms, base and sugar modifications in DNA, and single chain breaks (Yokuş and Çakır, 2002). Hydroxydeoxyguanosine (8-OHdG) is widely known oxidative DNA damage biomarker. 8-OHdG levels in peripheral blood can indicate a long-term response to oxidative stress, and has been commonly used in a research related with DNA, protein and lipid peroxidation (Harri et al., 2007). Antioxidants protect the cells against oxidative stress by reacting with GSH, free ions and peroxides, and serve as defense line against oxidative stress. Similarly, vitamins also play an important role in the body

as multifunctional antioxidant (Du et al., 2012). In ruminants, vitamin C deficiency is seen in cases of ketosis, hypoglycemia, and stress related diseases (Haliloğlu et al., 2002). Moreover such decrease in level of plasma vitamin C is also reported in mastitis occurring lactation periods, in calving periods, and especially in summer months (Padilla et al., 2006). Such low levels are treated by administering high dose vitamin C from outside sources (Weiss, 2001). Similarly, the application of vitamin C supports milking cows by stabilizing the productivity even at high temperatures (Padilla et al., 2006). Therefore, in this study it was aimed to explore the potential efficacy of vitamin C administration on pregnancy rates and oxidative stress markers (MDA, 8-OHdG) in milking cows under heat stress.

MATERIALS AND METHODS

Animals and management

In this study, a total of 80 Holstein - Friesian cows were included in the experiment, 45-80 days postpartum, with age 3-7, and one parity, in Milas county of Muğla province. Animals located in half-open freestanding barn and were given daily ration of 8kg milk silage (Abal ı oğlu Yem® % 19 Protein - 2700 kcal), oat, barley, dry alfalfa hay and corn silage. Milk productivity per cow was 28.3 kg. No precautions were taken against heat and study was conducted in during months of July-August (highest air temperature). All 80 cows were subjected to estrus synchronization with CIDR® (Pfizer). Following 6th day PGF₂α was administered intramuscularly and CIDR® was taken out in 7th day. Blood samples were taken on each day of experiment (0, 4, 8, 12th days). During the study all animals were artificially inseminated at the same time and exposed to same THI values. These artificially inseminated cows were randomly divided into two groups (control and treatment).

Cows of control group (n = 40) were administered 10 mL isotonic NaCl intramuscularly on the day of artificial insemination (0th day) and following 4th, 8th, 12th days between 09:00 and 10:00. Similarly cows of treatment group (n = 40) were administered 10 mL (250 mg/mL) Ascorvet® injection solution including 250 mg ascorbic acid (Vitamin C) per milliliter intramuscularly following the schedule of control group. Blood samples were taken from randomly selected (n = 11) cows from both group 2 mL in order to

measure 8-OHdG, and 8 mL for measuring P_4 , GSH, MDA values on the day of artificial insemination (0th day) and following 4th, 8th, 12th days between 12:00 and 13:00. Serum and plasma extracted from taken blood samples were centrifuged at 3000 rpm along with 10 minutes. Extracted serum and plasma were kept in deep freezer at -20°C to 10 days, till analysis. Cows were examined for pregnancy on 30th days of AI by using trans-rectal ultrasonography (KX5100VET®).

Meteorological data

Air temperature values (°C) and relative humidity rates (%) data were taken from Ministry of Water Affairs and Forestry, General Directorate of Meteorology, Turkish state Meteorological Data Archive System's (TUMAS). The meteorological station was located 5 km away from the study place. THI values were calculated by using dry bulb temperature (T_{db}) and relative humidity (RH) according to the formula below provided by Mader et al. (2006).

$$THI = (0.8 \times T_{db}) + [(RH/100) \times (T_{db} - 14.4)] + 46.4$$

Biochemical analysis

Biochemical analyses were performed by spectrophotometry (Shimadzu UV-1601) according to kit procedures. In order to determine P_4 levels, Bovine progesterone ELISA kit (catalog number: CSB-E08172b), GSH levels by Glutathione Assay Kit (catalog number: 703002), MDA levels by Bovine Malondialdehyde (MDA) ELISA Kit (catalog number: CSB-E14000B), and 8-OHdG levels Bovine by 8-hydroxydeoxyguanosine (8-OHdG) ELISA Kit catalog number: CSB-EQ02916BO) manufactured by Cusabio firm were used. Competitive inhibition enzyme technique was used of all parameters and results were calculated by Curve Expert software v. 1.3.

Statistical analysis

The data of present study belonging to animals of control and treatment group were determined by using SPSS 22.0 software package. Weather data was homogeneous or not according to Komorrov-Smirnov test, was determined by making homogeneity tests in groups. The data demonstrating abnormal standard distribution was standardized by logarithmic transformation. Mann-Whitney U test was used to determine the differences between blood parameters at 0th, 4th, 8th, 12th days belonging both groups, and Chi-square test was used to check whether time dependent change in treatment group is present or not. The results were given in means $\pm$ SE and statistical significance was considered at $p < 0.005$.

RESULTS

The results showed that day THI values for this study were higher than the threshold value and caused heat stress in cows. THI values calculated by using TUMAS data were as follows 83, 84, 84, and 82 for days 0, 4, 8 and 12, respectively. So it was determined that the weather conditions in study days were be able to cause medium level of stress.

The data of pregnancy rates of both groups (control and treatment) are presented in Fig. 1. The pregnancy rate was higher (32.5%, 13/40) in treatment group then in control group (22.5%, 9/40), respectively.

The blood parameters like P_4 , GSH, MDA serums, and plasma 8-OHdG levels of control and treatment groups are presented in Fig. 2 and 3. It was found that 8-OHdG levels were lower in treatment group on day 4, 8 and 12 ($p < 0.05$) compared with the control group. However, MDA levels were similar in both group and there was no effect

Fig. 1. Represents the pregnancy rate in control and treatment group, BS (blood sampled), NS (no blood sample). Each groups animal number has been shown (n = 40) in each group. Total pregnancy % refers to percentage of animals pregnant out of 40 animals. * $p < 0.05$

Fig. 2. Represents 8-OHdG (pg/mL) in control and treatment groups, similarly, MDA (pg/mL) level are given at day 0, 4, 8 and 12. Different superscripts show significant differences ($p < 0.05$).

was found on P_4 and GSH levels.

No statistical significant difference ($p > 0.05$) was found between control and treatment in terms of P_4 , GSH, MDA. Although 8-OHdG levels showed no statistical difference within groups, when both groups were compared significant ($p < 0.05$) differences at 4th, 8th, 12th days were found.

The scatterplots in Fig. 4 and 5 represent the correlation of MDA with P_4 and GSH, respectively. Progesterone has strong positive correlation at day 0 and 4 ($r = 0.54$), 4 and 8 ($r = 0.59$), 8 and 12 ($r = 0.51$). This shows increase in P_4 level from day of AI to last day of blood sampling. MDA levels also showed weak positive correlation with increase in days after AI. GSH levels, like P_4 showed strong positive correlation between day 0, and 4 ($r = 0.47$), 4 and 8 ($r = 0.56$), respectively. Interestingly, GSH day 8 showed strong negative correlation with MDA day 0. 8-OHdG also showed positive correlation between days 4 and 8 ($r = 0.43$), 8 and 12 ($r = 0.43$) (Fig. 6).

DISCUSSION

The importance of the heat stress is obvious from day to day increase in heat stress due to global warming and it severely affects cows. Moreover, heat stress is considered as main factor reducing breeding performance especially in summer months (De Rensis and Scaramuzzi, 2003; Jordan, 2003; Morton et al., 2007). In one study Lucy (2001) indicated that pregnancy rate decrease by 35% in high milk producing cows since last 60 years all over the world. Similarly, García-Ispuerto et al. (2007) reported that heat

Fig. 3. Represents GSH (μmol/mL) in control and treatment groups, similarly, P_4 (ng/mL) level are given at day 0, 4, 8 and 12. Different superscripts show significant differences ($p < 0.05$).

stress decreases the breeding rates by 23% in dairy cows. To improve the breeding performance of dairy cows different synchronization protocol are applied during heat stress in order to overcome difficulties related to estrus detection. The synchronization protocol of the present study improved the rate of estrus detection as suggested by Aréchiga et al. (1998).

In subtropical climates, the conception rates of 90 to 135 postpartum cows diminished proportionally to 20%-30% in summer months from 46%-76% in winter, respectively (De Rensis and Scaramuzzi, 2003), moreover, various studies also concluded that pregnancy rates were lower in summer than in winter (Alnimer et al., 2002; López-Gatius, 2003; García-Ispuerto et al., 2007; Dirandeh, 2014; Schüller et al., 2014). The environmental temperature and relative humidity are commonly used to calculate THI and in this study, the values were 83, 84, 84, and 82 and such conditions are considered as medium level of heat stress (Schüller et al., 2014). Studies have reported that pregnancy rates decrease as THI values increase (Dirandeh, 2014; Schüller et al., 2014). Although the THI values in this study were higher than the THI values of earlier studies, pregnancy rates in cows were higher.

In the present study, pregnancy rate of control group was 22.5%, which is lower than reported in earlier studies of (López-Gatius, 2003; García-Ispuerto et al., 2007) in summer, and higher than reported by (Alnimer et al., 2002). The pregnancy rates of vitamin C treatment group were higher than the control group and when compared

Fig. 4. Scatterplot correlation matrix of progesterone (ng/mL) and MDA levels (pg/mL) at day 0, 4, 8 and 12. The histogram on the diagonal axis shows the distribution and left lower diagonal section illustrates the density eclipses with the magnitude of linear association between variables (tighter eclipses show stronger correlation). The upper-diagonal section illustrates the significance of relationship between variables. * $p < 0.05$, ** $p < 0.01$.

with other studies, our treatment group pregnancy rates were partially higher (Alnimer et al., 2002; López-Gatius, 2003; García-Ispuerto et al., 2007; Dirandeh, 2014). It is possible that difference between pregnancy rates might be caused by number of cows used in study and feed, age, breed, milk productivity, continuous change in THI values during the study period. It might be attributed to difference in geographical region, adaption of cows to environmental conditions, and feeding. It is well known that if Holstein cows reduce milk production in warm and hot climate (Ravagnolo et al., 2000; Barash et al., 2001; West, 2003; Padilla et al., 2006).

The milk production of the cows in treatment and control groups was 23.2 ± 2.6 and 21.2 ± 6 , and such lower milk production in control shows heat stress pressure (Ravagnolo et al., 2000; Barash et al., 2001; West, 2003; Padilla et al., 2006). Besides no differences in average milk production between treatment and control groups was found, as both groups were exposed to same THI values, and were given same ration. Different results have been documented regarding P_4 hormone levels during summer heat stress. Studies have reported that serum P_4

concentrations were not affected by summer heat stress (Wilson et al., 1998; Guzeloglu et al., 2001). However, multiple studies have reported that serum P_4 levels are reduced during summer heat stress (Younas et al., 1993; Howell et al., 1994; Ullah et al., 1996; Wolfenson et al., 2002). In addition, lower P_4 levels affect the follicular development, cause abnormal oocyte maturation and early embryonic death. Besides change in morphology and function of endometrium due to change in P_4 levels has been reported (Korkmaz and Küplülü, 2014). In our results no difference in P_4 level was found between control and treatment group, which shows that vitamin C application might have balanced P_4 levels during the heat stress period (Younas et al., 1993; Howell et al., 1994; Wolfenson et al., 2002). Furthermore, the correlation analysis showed positive correlation of progesterone hormone levels ($r = 0.54, 0.59$) between day 0 and 4, 8 of the artificial insemination (Fig. 4). This shows that P_4 levels increased gradually after ovulation. Similarly, GSH at day 0 and 4, 8 of AI showed positive correlation ($r = 0.47, 0.56$), this shows rise in antioxidants in the blood serum level of cows treated with vitamin C (Fig. 5). This might be

Fig. 5. Scatterplot correlation matrix of MDA (pg/mL) and GSH levels ($\mu\text{mol/mL}$) at day 0, 4, 8 and 12. The histogram on the diagonal axis shows the distribution and left lower diagonal section illustrates the density eclipses with the magnitude of linear association between variables (tighter eclipses show stronger correlation). The upper-diagonal section illustrates the significance of relationship between variables. * $p < 0.05$, ** $p < 0.01$.

Fig. 6. Scatterplot correlation matrix of OHDG (8-OHdG) (pg/mL) and GSH levels ($\mu\text{mol/mL}$) at day 0, 4, 8 and 12. The histogram on the diagonal axis shows the distribution and left lower diagonal section illustrates the density eclipses with the magnitude of linear association between variables (tighter eclipses show stronger correlation). The upper-diagonal section illustrates the significance of relationship between variables. * $p < 0.05$, ** $p < 0.01$.

reason of high conception rate in the treatment group. As vitamin C has important functions in body as multifunctional antioxidant and cows are highly sensitive vitamin C deficiency (Padilla et al., 2006). It was determined that serious deficiency of vitamin C results in oxidative stress, and during excessive heat stress free radicals production doubles (Gündüz, 2000; Padilla et al., 2006). It has also been suggested that free -OH radicals levels increase during low concentration of GSH and this causes harm to embryo DNA (Takahashi et al., 1993; Yoshida, 1993; Yoshida et al., 1993). In a study GSH was added to culture medium in mouse and cow embryos, and it helped the embryo development (Nasr-Esfahani and Johnson, 1992; Gardiner and Reed, 1994; Luvoni et al., 1996). No statistical difference was found in GSH levels of cows from treatment and control group in terms of days and within groups ($p > 0.05$). It might be possible that high level of heat stress caused both groups to present very close values and only numeric differences could be found. Studies have documented that GSH deficiency in mice reduces ascorbic acid synthesis and inhibited conversion of the ascorbic acid into dehydroascorbate (Jain et al 1992).

MDA which is final product of lipid peroxidation, and is used to determine the harm caused by free radicals (Lykkesfeldt, 2007; Liu et al., 2013). As reported in previous studies we also found high level of MDA especially in summer period, along with the heat stress (Hozyen et al., 2014; Kumar et al., 2015). Though, there was neither day dependent nor group dependent statistical differences were found ($p > 0.05$). It is possible that vitamin C application decreased lipid peroxidation in treatment group, though no statistical differences were observed.

8-OHdG which is output product of the oxidized guanine base and is the most commonly identified as DNA base damage product. It can be easily measured and shows deep knowledge of mutagenic property of G:C→T:A trans-version (Mazlumoglu, 2014). It has been documented that administration of Vitamin C reduces levels of 8-OHdG (Tarng et al., 2004; Ekuni et al., 2009; Ryan et al., 2010; Ellah et al., 2014). During literature review no study describing the effect of heat stress in cows and its relation with 8-OHdG was found. However, one study by (Ellah et al., 2014) reported 8-OHdG levels in blood serum and saliva samples of cows during lactation and dry period. It was confirmed that oxidative stress harm was more, saliva and serum 8-OHdG increased explicitly in dry term than

in lactation term. It was detected that 8-OHdG increasing with the effect of oxidative stress and start of dry term decreased with the lactation (Ellah et al., 2014). Similar mechanism due to heat stress and oxidative DNA damage might be possible in our study Fig. 6. Although, no statistical difference was found between days of sampling but the correlation analysis showed its increase along with the days post AI. Moreover, weak negative correlation between GSH and 8-OHdG shows that high level of GSH decrease the oxidative stress during heat stress in dairy cattle. It was thought that similar stress factors continued to exist similarly in blood samples were taken. No statistical differences between control and treatment group on 0th day was found but at 4th, 8th, and 12th days ($p < 0.05$) shows vitamin C beneficial effects.

CONCLUSION

Taken together it can be concluded Vitamin C administration during heat stress period and AI has antioxidant properties, and it could reduce serum 8-OHdG and MDA levels. Results confirmed that MDA level increases post insemination as the level of progesterone increases after insemination. Therefore, it is suggested that Vitamin C has beneficial effects in an environment of medium level heat stress and it increases pregnancy rates. Similarly, it has no negative effect on level of P₄ hormone, GSH levels but reduces MDA level especially at 8th day post insemination. For future studies vitamin C can be administered at different dose rates on larger cow herds and its effect on different reproductive health parameters can be examined.

Author Contributions: Conceptualization, supervision, project administration and funding acquisition, H.Ç.; methodology, investigation, data curation; A.K., review of original draft preparation, writing, review and editing, S.R.

Funding: This work was supported by Adnan Menderes University Scientific Research Projects Council (project no: VTF-13012).

Ethical Approval: This study was carried out after getting approval from Animal Experiments Local Ethics Committee, Adnan Menderes University, dated 20/06/2012, number (050.04/2012/023).

Consent to Participate: Yes.

Consent to Publish: Yes.

Availability of Data and Materials: Yes.

Acknowledgements: This work has been published from thesis work of Dr. Armağan Kirdeci.

Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

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