Effects of dietary melatonin supplementation on total serum nitrites and antioxidant capacity of late gestating Holstein heifers

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Introduction

The hormone melatonin was first purified from a bovine pineal gland extract in 1958 (Chowdhury et al., 2008). Being a lipophilic molecule, it is able to easily pass through many cellular membranes, giving it the ability to act on cells all throughout the body (Chowdhury et al, 2008). This has made it a hormone of interest in several studies examining its effects on reproduction, cancer, sleep, immunity, blood flow, and its ability to scavenge free radicals. Melatonin is unique in that it can protect the body against free radicals via direct or indirect pathways (Reiter et al., 1995). Firstly, melatonin can bind directly to a cellular membrane and help stabilize that membrane against possible oxidative damage. Secondly, melatonin has the ability to protect cells indirectly by helping the body up-regulate its own antioxidant defense system.

Oxidative damage is something that every living organism experiences. While the body needs oxygen for normal metabolic processes and maintenance, up to 5% of the oxygen (O_2) intake is converted to free radicals. Free radicals and reactive oxygen species are formed naturally and continuously during metabolism and cause damage to cellular membranes. There are two specific free radicals that are common with oxidative damage; superoxide (O_2^-) andhydroxyl radical (OH^-;Saikumar et al., 2013). The body has a natural antioxidant defense

system in order to deal with this damage. Antioxidants include anything that helps protect against free radicals and reactive oxygen species such as vitamins, lipids, and proteins. These substances can be produced by the body or consumed through the diet. Two well-known antioxidants produced naturally by the body are superoxide dismutase (SOD) and glutathione. SOD acts specifically on O_2^-, converting it to H_20 and hydrogen peroxide (H_2O_2). H_2O_2 is not a direct threat; however, it is the precursor for OH^-. Glutathione acts on H_2O_2, converting it to H_2O and O_2, which eliminates the possible threat from OH^- (Reiter et al., 1995).

Nitric oxide (NO) is a powerful vasodilator that helps modulate blood flow during pregnancy (Beckman et al., 1996). Ample blood flow is important to make sure that the fetus is able to receive the necessary amount of nutrients during development. Some free radicals can bind with NO and decrease its bioavailability, which can lead to a decrease in blood flow. Specifically, O_2^- can react with NO to form peroxynitrite ($ONOO^-$) (Wink et al., 1996). Not only does this take away available NO, but also the new free radical formed can cause damage by itself, adding to the already present oxidative damage.

During pregnancy, there is an increase in the amount of O_2 needed by the body in order to maintain growth and development of the fetus. This, in turn, leads to an overall increase in the amount of free radicals and reactive oxygen species generated. Often in this situation the body's antioxidant defense system is not enough to keep up with the oxidative damage, which creates "oxidative stress" (Mutinati et al., 2013). Oxidative stress during pregnancy can lead to delay in fetal growth as well as a number of other complications.

A study conducted at North Dakota State University chronically infused melatonin directly into the pregnant uterus of ewes (Lemley et al., 2013). The results of this study showed an increase in umbilical artery blood flow, an increase in placental SOD activity, and in increase

in placental nitrite concentrations. One possible mechanism is that melatonin could increase NO and decrease oxidative stress leading to an increase in blood flow. These results brought up the question of whether or not melatonin would have an effect in dairy cattle. Seeing as dairy cows need to have a calf in order to lactate, pregnancy is a common process within the industry. Oxidative stress is a concern with the developing calf due to the fact that most calves are either put back into the facility or sold to other farms. Loss of pregnancy or an underdeveloped calf can result in profit loss for the facility (De Vries, 2006).

Hypothesis

This study examined the effects of dietary melatonin supplementation on total serum nitrites and antioxidant capacity in late gestating Holstein heifers. We hypothesized that dietary melatonin supplementation would lead to an increase in total serum nitrites and total antioxidant capacity. In order to obtain the needed results, the following experiment was designed.

Materials and Methods

In January of 2013, all dairy heifers were artificially inseminated with sex-sorted semen at the Joe Bearden Dairy Research Center. Prior to day 170 of gestation, heifers (n=20) were trained to acquire their feed using the Calan feeding system (American Calan, Northwood, NH). On day 190 of gestation, heifers were blocked by their body weight and randomly assigned to one of two dietary treatments consisting of 1) 20 mg of dietary melatonin per day (MEL) or 2) no dietary melatonin supplementation (control; CON). At 0800 hours, the MEL heifers received 0.7 kg of grain top dressed with 2 mL of 10 mg per mL melatonin in ethanol. The CON heifers received 0.7 kg of grain top dressed with 2 mL of ethanol alone. The melatonin was supplemented in the morning hours in order to compensate for the bodies natural spike in melatonin production following the dark hours. After all heifers consumed their grain, they were

provided 17.5 kg of a total mixed ration. Blood samples were collected via venipuncture of the coccygeal vein on days 180 (baseline for comparison), 210, 240, and 262 of gestation. Treatment for both groups was terminated on day 262 of gestation at which point the heifers were transferred to a steam up maternity ration in preparation for parturition and onset of lactation. The collected blood samples were allowed to clot and then spun in a centrifuge at 2000 x g for 20 minutes. Following centrifugation, serum samples were collected. These serum samples were then analyzed for total serum nitrites and total antioxidant capacity using commercially available colorimetric kits.

Antioxidant assay

The antioxidant assay used throughout this study was purchased from the Cayman Chemical Company (Ann Arbor, MI). Total antioxidant capacity was defined as the sum of all endogenous and food derived antioxidants. The standard curve used a water-soluble tocopherol analogue, Trolox, for comparison in mM equivalents. The assay relies on the antioxidants ability to inhibit the oxidation of ABTS (2,2'–Azino-di-[3-ethylbenzthiazoline sulphonate]). Within the test, Metmyoglobin is added in order to initiate this oxidation. First, the Assay buffer, standards, and reagents were prepared according to the manufacturers instruction. All serum samples were diluted by 1:4 with the Assay buffer in new centrifuge tubes. Following dilution, 10 µL of each standard and sample were placed into duplicate wells in a 96 well plate provided with the assay kit. Using a multipipet, 10 µL of metmyoglobin were added to each well, followed by 150 µL of chromogen. Chromogen is converted into a colored compound when oxidized. The reaction was initiated by adding 40 µL of H_2O_2 to each well within 1 minute, using a multipipet. The plate was tapped for 5 minutes before being read at 750 nm room temperature using the Spectra Max Plus plate reader from Molecular Devices (Sunnyvale, CA).

Nitric Oxide assay

Total serum nitrite concentrations were determined using the Quantichrom™ Nitric Oxide Assay Kit purchased from BioAssay Systems (Hayward, CA). The standard curve used Sodium Nitrite in µM equivalents for comparison. NO is naturally oxidized to nitrite and nitrate within the body. This kit estimates NO production following the reduction of nitrate (NO_3^-) to nitrite (NO_2^-). First, the serum samples required deproteinization by adding 16 µL of zinc sulfate and 16 µL of sodium hydroxide to 300 µL each of sample. All samples and standards were vortexed following the addition of each substance. All samples and standards were then placed in a centrifuge for 10 minutes at 14,000 x g. After centrifugation, 200 µL of supernatant from each serum sample and standard was collected and placed into new centrifuge tubes. Each new tube received 400 µL of the working reagent before being placed into incubation for 1 hour at 37°C. Following the incubation period, tubes were centrifuged for 3 minutes at 1,000 x g. 250 µL of each sample and standard were then transferred into duplicate wells of a 96 well plate and read at 540 nm room temperature using the Spectra Max Plus plate reader from Molecular Devices (Sunnyvale, CA).

Statistical analysis

Dependent variables were analyzed using repeated-measures ANOVA of the mixed procedure of SAS (SAS software version 9.3, SAS Institute, Cary, NC) and means were separated using the PDIFF option of the LSMEANS statement. The model statement contained dietary treatment, gestational age, and the respective interaction. Main effects of dietary treatment or gestational day are discussed in the absence of significant ($P < 0.05$) treatment by day interactions. Lease square means and SEM are reported. Statistical significance was declared at $P < 0.05$.

Results

Following the analysis of the total antioxidant capacity data, it was clear that no melatonin treatment by gestational day interaction ($P = 0.21$) was observed (Figure 1). However, there was a main effect of gestational day (Figure 2). Between days 180 and 210 of gestation there was not a significant increase in total antioxidant capacity ($P = 0.06$), but when comparing days 240 and 262 to the baseline at day 180, a significant increase in total antioxidant capacity was observed ($P < 0.01$). The main effect of treatment is illustrated in Figure 3. When comparing the antioxidant capacity in the MEL heifers to that of the CON heifers, there is about a 40% increase in the capacity of the MEL group ($P < 0.0001$).

Data analysis for the total nitrites showed no treatment by day interaction ($P = 0.11$; Figure 4) as well as no main effect of treatment ($P = 0.83$). However, a main effect of gestational day was observed in the study ($P < 0.0001$; Figure 5). A significant increase in total nitrites was observed between day 180 and 210 ($P < 0.0001$). Total nitrites continued to increase at day 240 ($P < 0.0001$) and day 262 ($P < 0.0001$). By the time day 262 was reached, there was a three-fold increase in total nitrites when compared to our baseline.

Conclusion

While dietary melatonin supplementation did not have an effect on total serum nitrites, a main effect of gestational day was observed with NO concentrations increasing naturally as gestation progresses. The third trimester of pregnancy is related to the final period of exponential growth for the fetus. There is a higher requirement for nutrient transport and O_2 exchange with the dam and fetus, which leads to an increase in blood flow to the uterus. The increase in NO concentrations coincides with the increase in blood flow to the uterus during the third trimester of gestation (Huang et al., 2012). It is possible that increasing NO concentrations could lead to

an overall increase in blood flow during a pregnancy. The increase in blood flow could result in more nutrients being supplied to the fetus and a healthier offspring being born.

Dietary melatonin supplementation resulted in a 40% increase in total antioxidant capacity in the MEL heifers compared to the CON heifers. This proves that supplementing melatonin can have a significant effect against oxidative damage and stress. It is possible that melatonin could be supplemented in order to further protect the dam and fetus against oxidative stress during a compromised pregnancy. A compromised pregnancy can be determined by a decrease of blood flow to the uterus. This decrease in blood flow results in less nutrients getting to the fetus when they are essential for development. The next step for this study would be to supplement dietary melatonin to late gestating dairy heifers during a compromised pregnancy. In order to attain a compromised pregnancy, heat stress or nutrient restriction models could be implemented. There are parts of the country where hot summers and heat stress are a common issue for dairy producers. Dietary melatonin could provide a cheap and easy way for producers to protect their dams and developing fetuses from cellular damage during a pregnancy where heat stress and oxidative stress are experienced (Rensis et al., 2003).

References

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Figure Legends

Figure 1. Total antioxidant capacity in MEL heifers (dotted line and open box) and CON heifers (solid line and closed circle) during the last third of pregnancy. The melatonin treatment by gestational day interaction ($P = 0.21$) was not significant; therefore, treatment means within a given day were not separated.

Figure 2. Main effect of gestational day ($P = 0.003$) on total antioxidant capacity of all Holstein heifers (solid line and closed box). Statistical differences between gestational days are represented by different lower case letters.

Figure 3. Main effect of treatment ($P < 0.0001$) on total antioxidant capacity in MEL heifers (open bar) compared to CON heifers (closed bar).

Figure 4. Melatonin treatment by gestational day interaction ($P = 0.11$) for total nitrites in MEL heifers (dotted line and open box) and CON heifers (solid line and closed box).

Figure 5. Main effect of gestational day ($P < 0.0001$) on total serum nitrites of all Holstein heifers (solid line and closed box). Statistical differences between gestational days are represented by different lower case letters.

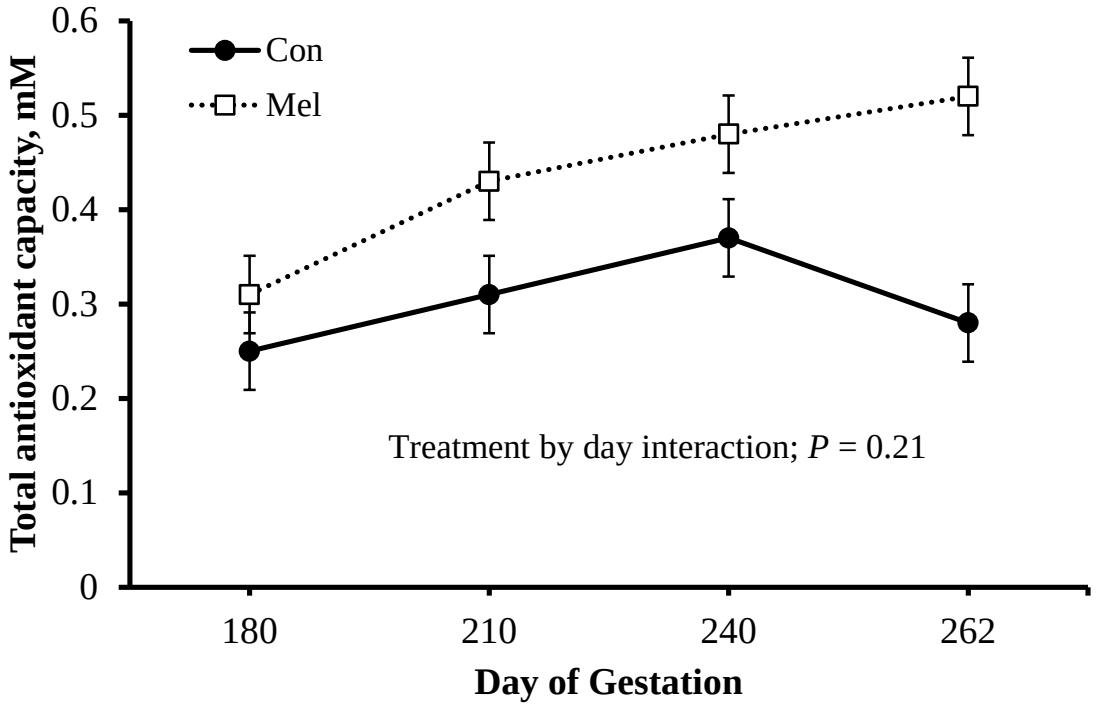
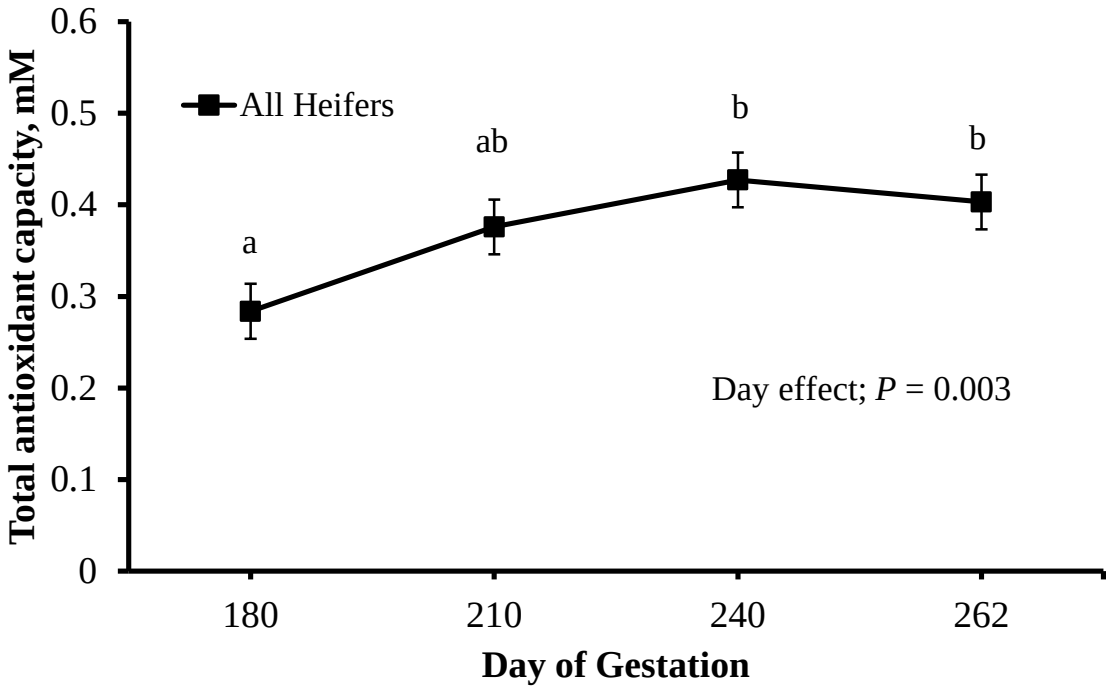

0.6
0.5
0.4
0.3
0.2
0.1
0
Con
Mel
Total antioxidant capacity, mM
Treatment by day interaction; P = 0.21
180
210
240
262
Day of Gestation

Figure 1.


0.6
0.5
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Total antioxidant capacity, mM
All Heifers
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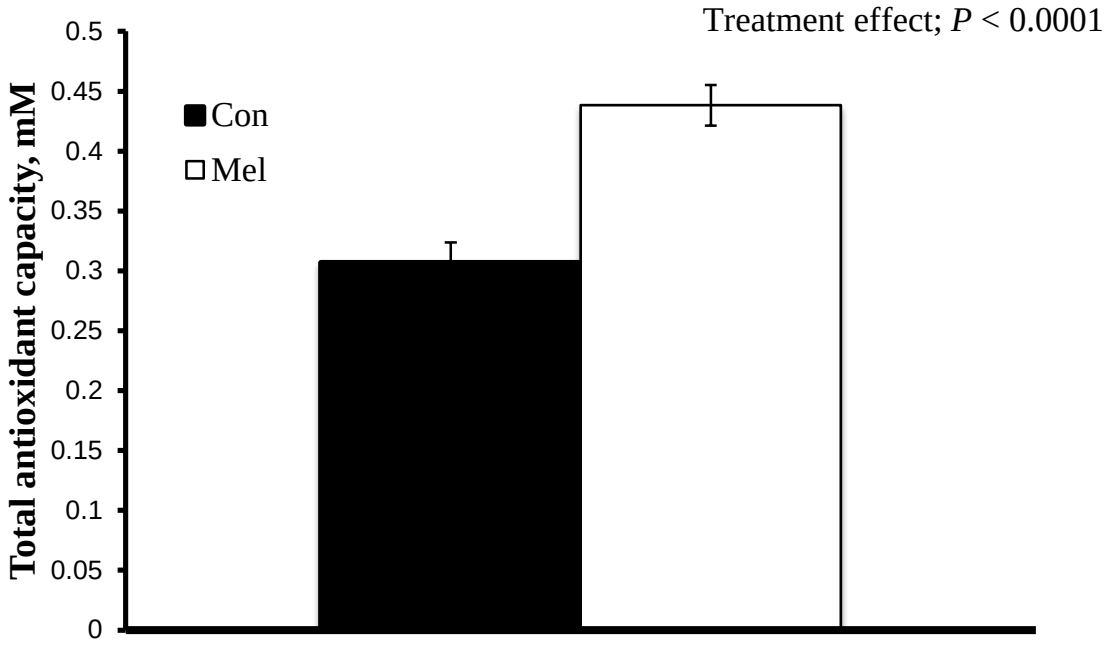
b
b
Day effect; P = 0.003
180
210
240
262
Day of Gestation

Figure 2.


Treatment effect; P < 0.0001
Total antioxidant capacity, mM
0.5
0.45
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0.05
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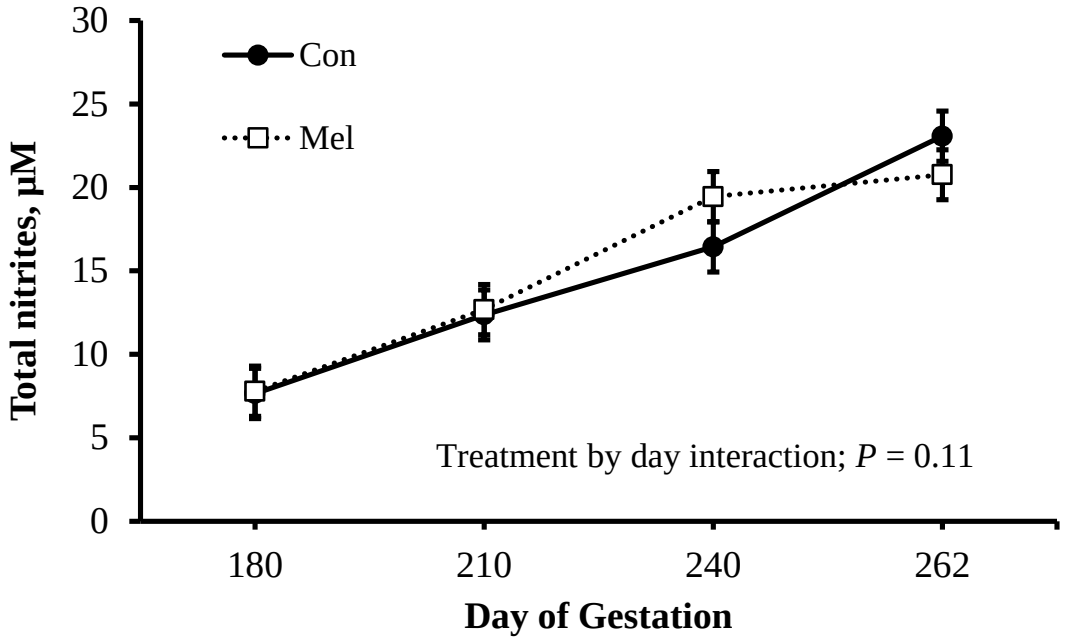
Con
Mel

Figure 3.


30
25
20
15
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Con
Mel
Total nitrites, µM
Treatment by day interaction; P = 0.11
180
210
240
262
Day of Gestation

Figure 4.

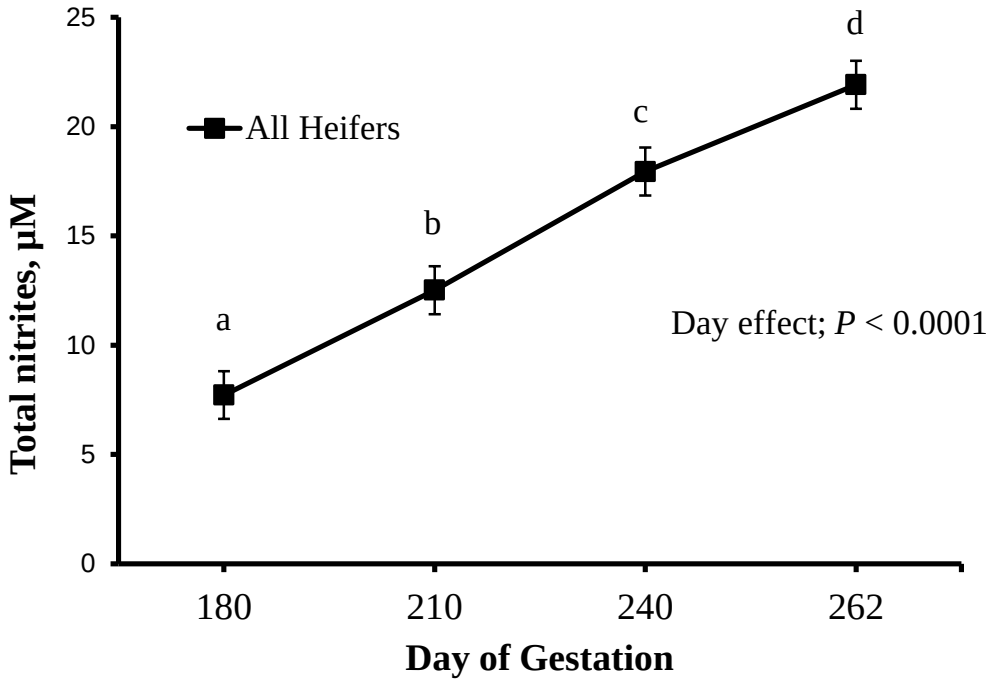
25
20
15
10
5
0
Total nitrites, µM
All Heifers
a
b
c
d
Day effect; P < 0.0001
180
210
240
262
Day of Gestation

Figure 5.