Oxidative stress and trace elements before and after treatment in dairy cows with clinical and subclinical endometritis

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SUMMARY
The objective of this study was to investigate if changes in oxidative stress and trace element concentrations in postpartum cows can promote endometritis and may influence the issue of treatment. For that, oxidative stress markers and trace element concentrations were determined in sera from cows suffering from clinical or subclinical endometritis (CE and SE groups, n = 30 in each group) at the time of clinical and cytological diagnosis and 7 days after treatment and compared with healthy cows (n = 30). Compared to the controls, serum TAS (total antioxidant status) values were significantly decreased in all diseased cows whereas MDA (malondialdehyde) concentrations were dramatically increased, mainly in animals with clinical endometritis. Additionally, serum copper and zinc concentrations were significantly decreased in cows with clinical endometritis. Significantly higher initial TAS values were observed in CE cows for which treatment was successful than in affected cows not cured 7 days after. After successful treatment for clinical endometritis, significantly increased copper concentrations were observed and serum MDA concentrations have also significantly decreased when treatment was successful in subclinically and clinically affected cows 7 days after. These results suggest that decreases in antioxidant capacity and in trace elements (copper and zinc) could be associated with subsequent oxidative stress and increased susceptibility to endometritis and that endometritis resolution was related to the antioxidant and copper status and to decrease in oxidative stress.

Keywords: Oxidative stress, trace elements, copper, zinc, endometritis, treatment, dairy cows.

Introduction

Endometritis is defined as the inflammation of the endometrium that occurs from day 21 after calving and is not associated with systemic illness [4]. Endometritis has been sub-divided into clinical and subclinical categories [31]. Clinical endometritis is characterised by the presence of purulent (> 50% pus) uterine discharge detectable in the vagina 21 days or more after parturition, or muco-purulent (approximately 50% pus, 50% mucus) discharge detectable in the vagina after 26 days post partum [31]. Subclinical endometritis is defined as the presence of > 18% polymorphonuclear cells (PMN) in uterine cytology samples collected 21-33 days postpartum, or > 10% PMNs in samples collected at days 34-47 [31]. Cows with subclinical endometritis do not have uterine discharge; however, the severity of the disease is still considered sufficient to impair reproductive performance [31]. Clinical and subclinical endometritis are common causes of infertility and subfertility in high producing dairy cows, delaying the onset of ovarian cyclic activity after parturition, extending luteal phases and reducing conception rates [32].

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During the peripartum period, physiological changes occur that depress the defence mechanisms of the cow and render her more prone to uterine and mammary infections [23]. One of these changes is enhanced generation of reactive oxygen species (ROS). ROS are physiologically used by cells in intracellular signalling and redox regulation [27]. Physiological ROS play an important role in the regulation of reproductive processes such as folliculogenesis, oocyte maturation, corpus luteum, uterine function, embryogenesis, embryonic implantation and foeto-placental development [1]. However, an imbalance between ROS production and their safe removing lead to oxidative stress. Exceeding amounts of ROS can modify cell functions and endanger cell survival. Reactive oxygen species are generated during prostaglandin synthesis and increases number of PMNs to kill ingested microorganisms [13]. In PMNs, oxygen is converted to superoxide by NADPH oxidase. The ROS are in turn reduced to hydrogen peroxide by superoxide dismutase and then converted to hypochlorite free radical (HOCl) by myeloperoxidase [13]. Because of the involvement of prostaglandins and PMNs in inflammatory processes, generation of ROS may increase during inflammation [24]. Important inflammatory diseases in cattle, such as pneumonia, mastitis and endometritis are thought to be associated with the oxidative stress [2, 7, 14, 20, 21, 29, 41].

To protect against adverse effects of free radicals and their derivatives to the body, there is a group of antioxidants divided into enzymatic and non-enzymatic substances. Enzymatic antioxidants are represented mainly by enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase [27]. Glutathione, thioredoxin, vitamins, melatonin, polyphenols, trace elements, albumin, and others function as non-enzymatic free radical scavengers [12]. Trace elements such as zinc, copper, and selenium are essential components of the antioxidant defence of the body that play an important role in the prevention of free radical-induced damages to tissue for maintenance of health and production [11]. Trace element deficiencies have been reported during inflammatory disorders. There was a significant decrease in mean blood zinc concentration in dairy cows with mastitis [10, 29]. Selenium deficiency resulted in increased incidence of various infections and immunological reactions in dairy cows and the clinical signs were alleviated with dietary supplementation [22].

To the author knowledge, no study regarding the comparison of oxidant/antioxidant and trace elements status of cows with clinical and subclinical endometritis was available. Such studies could provide a better insight into the processes of establishment and persistence of endometritis. The objectives of this study were (i) to evaluate the oxidant/antioxidant and trace elements status of cows with clinical and subclinical endometritis and to compare them with healthy animals, and (ii) to compare these parameters in cows that recovered from endometritis after treatment with cows suffering from persistent endometritis.

Material and Methods

COWS AND GROUP CONSTITUTION

The study was conducted from April to September 2010 in a large commercial dairy herd ( Mashhad, North-east Iran), using multiparous Holstein cows. Cows were housed in free-stall barns. Artificial insemination was used exclusively after a voluntary waiting period of approximately 45 days. During scheduled weekly visits, all healthy cows between 21 and 33 days in milk (DIM) were identified and were enrolled into the study. If cows had history of systemic or intrauterine antibiotic therapy within 7 days prior to enrolment, reproductive hormone administration in the current lactation prior to enrolment or abnormal genitalia including adhesions, laceration and pyometra, they were excluded from the study. During the study period all animals were kept under identical conditions.

The presence of clinical endometritis (CE) was determined by finding pus in the lumen of the vagina by withdrawing its content with hand. First, the perineum and vulva were cleansed with paper towel, and a clean, lubricated, gloved hand was inserted through the vulva. The vagina was assessed for any signs of damage or injury by palpation of the lateral, dorsal, and ventral walls. Animals with palpable vaginal injury were excluded from the study. The mucus content of the vagina was withdrawn manually for examination. The vaginal mucus was characterized using an endometritis scoring system [43]. Score was assigned as follows: unaffected animals with clear or translucent mucus (0), affected animals with mucus containing flecks of white or off-white pus (1), muco-purulent exudates containing ≤ 50% white or off-white muco-purulent material (2) and exudate containing > 50% purulent material, usually white or yellow, but occasionally sanguineus (3). In 30 cows, CE was defined by the presence of > 50% purulent uterine discharge (score 3) detectable in the vagina 21 days or more post partum, or muco-purulent discharge (score 2 or 3) detectable in the vagina after 26 days post partum [31].

In cows with no abnormal discharge, endometrial cytology samples were collected using the modified cytobrush technique as described by KASIMANICKAM et al. [17]. Smears were prepared by rolling the brush on a microscopic slide, dried and fixed immediately after collection. Samples were stained with Giemsa at a dilution of 5% in buffer solution and evaluated by 400 X magnification (Zeiss, Germany) in the laboratory. A total of 300 cells were counted under the microscope to determine the proportion of PMNs. The endometrial cytology slides were assessed twice by a clinician. Totally, 30 cows with >18% PMNs were diagnosed as subclinical endometric (SE) cows.

Treatment of the cows with either CE and/or SE was done by the farm veterinarians with standard protocols. At the conclusion of the first examination, the SE cows were received one of two treatments: cloprostenol (Estroplan, Parnell, Australia, 500 mg, IM) on days 1 and 14 or benzathine cephapirin ( 500 mg in 19.6 g ointment base intra-udder, Metricure®, Intervet, Canada) and then cloprostenol (500 mg, IM) 7 days after. Treatment protocols for CE cows were as below: cloprostenol (Estroplan, Parnell, Australia, 500 mg IM) on days 1 and 14 or Na-ceftiofur (Excenell®, Pfizer, Animal health S.A., USA, Kalamazoo, Michigan, 1 mg/kg, IM) in 3 consecutive days and then cloprostenol (500 mg IM) 7 days after the last injection.

After treatment and taking second samples 7 days after treatment by cytobrush from SE cows, the animals were divided into two groups: successful treatment (ST group, n = 15; PMNs less than 18% in endometrial samples) and unsuccessful treat-
The concentrations of iron, zinc, copper and TAS (Total antioxidant status) in serum samples were measured by commercial kits [Pars Azmoon, Iran for iron; Giesse Diagnostics, Italy for zinc; EliTech diagnostics, France for copper; Randox, Antrim, UK for TAS] using an autoanalyser (Biotecnica, Targa 3000, Rome, Italy). Control serum (Randox control sera, Antrim, UK) was used for controlling measurement accuracy.

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The concentrations of malondialdehyde (MDA) were estimated in serum according to the method of PLACER et al. [28]. The reaction mixture consisted of 0.2 mL of serum, 1.3 mL of 0.2 M Tris and 0.16 M KCl buffer (pH 7.4) and 1.5 mL of thiobarbituric acid reagent. The mixture was heated in a boiling water bath for 10 minutes. After cooling, 3 mL of pyridine/n-butanol (3:1, v/v) and 1 mL of 1N sodium hydroxide were added and mixed by vigorous shaking. A blank was run simultaneously by incorporating 0.2 mL distilled water instead of the serum. The absorbance of the test sample was read at 548 nm. The concentration of MDA (nmol/L of serum) was calculated using 1.56 x 10^5 as extinction coefficient.

Results

Table I shows the oxidative status markers and blood concentrations of trace elements in healthy, CE and SE cows. The index of serum lipid peroxidation assessed by the MDA concentration was dramatically increased in CE and SE groups compared to the controls (P < 0.001) and the mean serum MDA concentration was also higher in the CE group than in the SE group (P < 0.05). Mean serum TAS values were significantly decreased (P < 0.001) in both CE and SE groups compared to the control animals. In addition, significant and marked decreases in serum copper and zinc concentrations (P < 0.05) were noticed in cows with clinical endometritis compared to the healthy controls whereas intermediate mean values (but non significant compared to the control values) were recorded in cows with subclinical endometritis. By contrast, iron concentrations tended to increase in diseased cows but not significantly compared to the controls.

Pearson’s correlation analysis of the paired data revealed the existence of a significant positive correlation between serum TAS and zinc concentrations in the SE group (r = 0.412, p = 0.024; Table II). However, the concentration of serum MDA was inversely correlated with zinc concentration in the SE group (r = -0.496, p = 0.005; Table II). No significant correlations were evidenced in the control and CE groups.

Table III summarizes the evolution of the oxidative status and serum element concentrations after successful or unsuccessful treatments in cows suffering from clinical and subclinical endometritis (CE and SE groups). It was noticed that in cows with clinical endometritis, the mean serum TAS value at the time of diagnosis (before treatment) was significantly higher in animals that have been cured with treatment than in untreated in endometrial samples). The same procedure was applied for CE cows and they were divided into two groups: successful treatment (ST group, n = 15; no abnormal discharge and PMNs less than 18% in endometrial samples) and unsuccessful treatment (UST group, n = 15; with abnormal discharge and PMNs more than 18% in endometrial samples).

BIOCHEMICAL ANALYSES

Blood samples were taken from all cows with CE and SE at the time of diagnosis (before treatment) and on day 7 after treatment. After clotting for 1 hour at room temperature (18-22°C), sera were separated after spinning at 1800 g for 10 minutes at room temperature (18-22°C) and stored at -20°C until analysis. Blood samples were also taken from control group (healthy cows with no clinical or subclinical endometritis) on the day of the first examination.

The amounts of iron, zinc, copper and TAS (Total antioxidant status) in serum samples were measured by commercial kits [Pars Azmoon, Iran for iron; Giesse Diagnostics, Italy for zinc; EliTech diagnostics, France for copper; Randox, Antrim, UK for TAS] using an autoanalyser (Biotecnica, Targa 3000, Rome, Italy). Control serum (Randox control sera, Antrim, UK) was used for controlling measurement accuracy.

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<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 30)</th>
<th>SE group (n = 30)</th>
<th>CE group (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper (μmol/L)</td>
<td>18.29 ± 2.73a</td>
<td>16.65 ± 3.85ab</td>
<td>14.97 ± 3.80b</td>
</tr>
<tr>
<td>Iron (μmol/L)</td>
<td>27.33 ± 5.46</td>
<td>29.25 ± 6.62</td>
<td>30.70 ± 6.57</td>
</tr>
<tr>
<td>Zinc (μmol/L)</td>
<td>14.54 ± 3.78ab</td>
<td>12.11 ± 4.57ab</td>
<td>11.13 ± 7.51b</td>
</tr>
<tr>
<td>TAS (mmol/L)</td>
<td>4.65 ± 0.97a</td>
<td>3.55 ± 1.14b</td>
<td>3.67 ± 1.22b</td>
</tr>
<tr>
<td>MDA (nmol/L)</td>
<td>17.03 ± 4.83a</td>
<td>29.76 ± 12.36b</td>
<td>36.45 ± 12.44c</td>
</tr>
</tbody>
</table>

TAS: total antioxidant status; MDA: malondialdehyde.
Different superscripts a,b,c in the same row indicate significant differences (P < 0.05) between groups.

Table I: Trace elements and oxidative stress markers in cows with clinical endometritis (CE, n = 30) or subclinical endometritis (SE, n = 30) at the time of diagnosis and in healthy cows (n = 30). Results are expressed as mean ± standard deviation.
those in which the treatment was unsuccessful ($P < 0.001$) but treatment has not significantly affected this parameter. In cows with subclinical endometritis, no difference in mean TAS values between the 2 subgroups was recorded before treatment. In the same way, although differences were not significant between ST and UST subgroups, the serum zinc concentrations tended to be increased in the ST subgroup of cows with clinical endometritis compared to the UST group. In cows with subclinical endometritis, this parameter was quite similar in the 2 subgroups before treatment. Seven days after successful treatment for CE, mean serum copper concentration significantly increased ($P < 0.05$), whereas MDA concentrations dramatically dropped ($P < 0.01$). Similarly, the oxidative status significantly declined in cows with subclinical endometritis 7 days after treatment only when it was successful ($P < 0.05$). If treatment was not successful, no significant difference was seen in these parameters before and after treatment.

**Discussion**

Considerable increase in oxygen requirements at the time of increased metabolic demands results in augmented production of ROS. During the periparturient period, antioxidants are required to reduce ROS accumulation. An imbalance between increased production of ROS and availability of antioxidants may expose cows to increased oxidative stress. The possibility that oxidative stress during the transition period may be a major underlying cause of inflammatory and immune dysfunction in dairy cows is supported by several studies [3, 6, 8, 34, 42].

The key feature of the present study is that the serum concentrations of oxidative stress markers and trace elements in cows affected with endometritis was estimated before and after treatment. In the present study, development of endometritis occurred against the background of a lower functional potential of the antioxidant system (i.e. decreased serum TAS and trace elements). Therefore, the function of antioxidant system is probably relevant to the establishment and persistence of endometritis. Similar finding was reported by others [2, 7, 14] in cows and buffalos suffering from endometritis. The decrease in serum TAS values in the present study might be due to overutilization or sequestration of antioxidants to neutralize the overproduction of ROS during inflammatory condition of the uterus.

Lipid peroxidation is a well-established mechanism of oxidative damage caused by ROS, and the measurement of the MDA provides a convenient index of lipid peroxidation [26]. This study provides evidence of an increase in serum MDA in

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**Table II:** Correlation between oxidative stress markers and trace elements in cows with clinical (n = 30) and subclinical (n = 30) endometritis (CE and SE groups, respectively) and in healthy cows (Controls, n = 30).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SE group (n = 30)</th>
<th>CE group (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ST (n = 15)</td>
<td>UST (n = 15)</td>
</tr>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Copper (μmol/L)</td>
<td>17.20 ± 3.71</td>
<td>14.89 ± 2.83</td>
</tr>
<tr>
<td>Iron (μmol/L)</td>
<td>28.76 ± 5.96</td>
<td>24.56 ± 5.34</td>
</tr>
<tr>
<td>Zinc (μmol/L)</td>
<td>13.06 ± 3.49</td>
<td>12.96 ± 4.29</td>
</tr>
<tr>
<td>TAS (mmol/L)</td>
<td>3.79 ± 1.54</td>
<td>4.64 ± 0.89</td>
</tr>
<tr>
<td>MDA (nmol/L)</td>
<td>29.2 ± 12.0b</td>
<td>16.1 ± 5.4b</td>
</tr>
</tbody>
</table>

TAS: total antioxidant status; MDA: malondialdehyde; ST: cows with successful treatment; UST: cows with unsuccessful treatment. Different superscripts a,b,c in the same row indicate significant differences ($P < 0.05$) between subgroups within a given group (CE or SE group).

**Table III:** Comparison of the serum concentrations of trace elements and oxidative stress markers in cows with clinical and subclinical endometritis (CE and SE groups, respectively) at the time of diagnosis (before treatment) and 7 days after treatment. Results are expressed as mean ± standard deviation.
cows with clinical and subclinical endometritis. Similar finding in cows and buffalos with endometritis was reported by other [2, 7, 14]. The higher blood MDA concentrations in cows affected with endometritis as compared to healthy animals is apparently due to a marked increase in ROS leukocyte production occurring during development of the inflammatory process. Increased ROS production coupled with increased NEFA concentrations in cows at early lactation, increases lipid peroxidation [6]. ROS are especially harmful to immune cells and can decrease the ability of the immune system to respond to infections [36].

Diminished functional capabilities of leukocyte populations during the peripartum period also, may result from inadequate contents of trace elements that are needed for optimal antioxidant mechanisms during times of pro-oxidant challenge [5, 35, 37]. Marginal copper deficiency may cause reduced neutrophil killing and decreased interferon production by mononuclear cells [38, 39]. Copper supplementation of a diet marginal in copper, reduced the peak clinical response during experimental Escherichia coli mastitis [15]. Research in humans and laboratory animals has documented that zinc deficiency impairs immune responses and reduces disease resistance [30]. Some researches suggest that organic forms of zinc may affect mammary gland health status [29]. Supplementation of dairy diets with zinc methionine has reduced somatic cell counts in milk [18]. In the present study, mean serum copper and zinc concentrations in cows suffering from clinical endometritis were significantly lower than those in healthy cows. The concentrations of these elements in cows with subclinical endometritis were also lower than those in the control animals, but the differences were not statistically significant (P > 0.05). NARESH et al. [25] and RANJAN et al. [29] also reported significant reduction in blood zinc concentrations in cows affected by mastitis. These changes are part of the defence mechanisms of the body, induced by IL-1, IL-6 and TNF-α [19]. Utilization of essential elements increases during inflammation. The interaction between toxins and enzymes released by the causative agents may activate utilization of these elements [16]. It is also possible that, during inflammation, higher metabolic activity of cells might cause a deficiency of trace elements, which are major components of metabolic enzymes [16].

Copper and zinc are involved in destruction of free radicals (i.e. ROS) and these trace elements play an important role in the prevention of free-radical-induced damage to tissues for the maintenance of health and production. Superoxide radicals are reduced to hydrogen peroxide by superoxide dismutases in the presence of copper and zinc cofactors [9]. In a report, participation of zinc as a component of the oxidant defence mechanism was indicated. The contents of the report supported the relationship of in vitro antioxidant action of zinc as well as in vivo association of oxidative stress with zinc deficiency [44]. Both in animal and in cell models, zinc deficiency induces oxidative damage to cell components and enhances alterations in antioxidant enzymes [44]. In this way, it was observed here that zinc concentrations were significantly and positively associated with the TAS values whereas they were negatively and significantly correlated with MDA concentrations in the group of cows with subclinical endometritis. Copper is an integral part of several proteins i.e. ceruloplasmin and the superoxide dismutase enzyme. These proteins act as antioxidants by scavenging oxygen free radicals and could serve as an endogenous modulator of the inflammatory response [13]. Regarding the role of zinc and copper in destruction of free radicals, the oxidative stress (decreased serum TAS and increased serum MDA) observed in the cows affected with endometritis might be related to insufficient concentrations of these trace elements.

In the present study, cows affected with endometritis with higher TAS showed a better response to the treatment. Successful treatment in cows affected with endometritis was associated with improved oxidative stress and trace elements status (decreased MDA and increased copper concentrations after successful treatment). Antioxidant and trace element status may be indicators of reproductive function in farm animals. Moreover, establishment and persistence of the uterine infection are dependent, among others, on the antioxidant and trace element status of the cows. Multiple studies have shown that supplementing vitamin E and selenium (as antioxidant agents) in excess of traditional recommendations decreases the incidence and severity of clinical mastitis [33, 40]. Further research is required to investigate the effect of zinc and copper supplementation in the development of endometritis and other diseases of transition cows where oxidative stress may be a significant contributor of pathogenesis.

As a conclusion, results of the present study suggest that a decrease in the concentration of trace elements and antioxidants and subsequent oxidative stress could be associated with an increased susceptibility to endometritis. The later increase in the concentration of trace elements and antioxidants as well as the decrease in the oxidative stress might favour the resolution of endometritis after treatment.

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References
