Paraoxonase-1 activity and lipid profile in dairy cows with subclinical and clinical mastitis

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ABSTRACT

The aim of this study was to investigate serum paraoxonase-1 (PON1) activity and lipid status in cows with subclinical and clinical mastitis in order to evaluate systemic inflammatory and oxidative stress responses. The study was conducted on a total of 90 Holstein–Friesian dairy cows kept in farms in eastern Croatia. Cows were assigned into three groups: the cows suffering from clinical mastitis (CLM), the cows with subclinical mastitis (SCM) and control (CTL) group. In collected sera, PON1, lipid status and calcium concentration were measured. Total cholesterol (CHOL), high-density lipoprotein cholesterol (HDL-C) and calcium concentrations were significantly lower in the CLM group of cows compared to the SCM and CTL groups (P < 0.05). There were no significant differences in the lipid status and calcium level between the CTL and SCM groups. PON1 activity was significantly lower in both the SCM and CLM groups compared with CTL indicating that PON1 could be considered as a potential biomarker for the diagnosis of subclinical form of the disease.

Introduction

Bovine mastitis is one of the most frequent diseases of dairy cows which causes the greatest economic losses in dairy cattle industry on the global level. Reduced milk production, increased costs of medication, reduced fertility, early culling of animals and the value of discarded milk are the main losses caused by the mastitis (Barlow et al. 2013; Cvetnić et al. 2016). In dairy herds, subclinical mastitis (SCM) is the dominant form affecting cows, often under-diagnosed and, thus, not treated for long periods by the majority of dairy farmers (Hiller et al. 2003; Oliver et al. 2004; Barlow et al. 2013). The SCM can be detected by an elevated milk somatic cells count (SCC) (Schukken et al. 2003; Djuricic et al. 2014). Following pathogen invasion of the mammary gland through the teat cistern, the innate immune system responds by leukocytes infiltration to facilitate bacterial clearance through phagocytosis and production of reactive oxygen species (ROS) and antibacterial peptides resulting in increased SCC secreted into milk (Wellnitz and Bruckmaier 2012). During an inflammatory response, raised amount of ROS can overcome the antioxidant system and compromise the immune functions of cows (Turk et al. 2012). The oxidative fragmented lipids and ROS generated during oxidative stress are pro-inflammatory compounds and provoke acute phase response (APR, Steinberg 1997) indicating a strong relationship between oxidative stress and inflammation. Paraoxonase-1 (PON1) is a mammalian anti-oxidative/anti-inflammatory enzyme which is synthesized in the liver and secreted into the blood (Mackness et al. 1996). It has been shown that PON1 is able to hydrolyse lipid hydroperoxides and oxidatively fragmented phospholipids produced during oxidative stress (Turk et al. 2015, 2016; Folnožić et al. 2015). Oxidative stress is defined as a shift in the balance between cellular oxidation–reduction reactions towards increasing oxidation and to the state of excessive release of ROS, when their removal by antioxidants is impaired and even insufficient (Kumar et al. 2011). Several groups reported decreased PON1 activity related to inflammatory conditions (Bionaz et al. 2007; Bossaert et al. 2012; Escirbano et al. 2015; Tecles et al. 2015; Tvarijonaviciute et al. 2015) suggesting PON1 as a negative acute phase protein (APP). In addition, Feingold et al. (1998) found decreased PON1 mRNA expression in the liver and reduced serum PON1 activity in rodents challenged by cytokines, what might indicate that PON1 responded to inflammatory condition as a negative APP.

In dairy cows, particularly during the periparturient period lipid metabolism is challenged to ensure the increasing energy needs (Grummer 1993; Turk et al. 2013). Changes in serum lipid profile are a key aspect of the physiology and energy metabolism during the transition (Gross et al. 2013). The plasma concentrations of total and free cholesterol (total CHOL), cholesteryl esters, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) decreased from 3 weeks before to 1 week after parturition and increased thereafter steadily until reached maximal values at the 14th week of lactation (Kessler et al. 2014). In order to evaluate the oxidative stress response in the cows with SCM and CLM (clinical mastitis),
PON1 activity and lipid profile have been investigated in this study.

**Material and methods**

**Animals and blood sampling**

Prior to the start of the study, an ethical and legal approval was obtained from the Ethical committee at Faculty of Veterinary Medicine, University of Zagreb, Croatia. The research protocol and animal management were in accordance with Directive 2010/63/EU (European Union 2010) on the protection of animals used for scientific purposes. The study was conducted on a total of 90 Holstein–Friesian (HF) dairy cows kept in farms in eastern Croatia. All cows on the farm were clinically examined by experience practitioner and California mastitis test (CMT) was done. Milk samples were taken from each quarter of each CMT positive cow to determine SCC in Central Laboratory of Milk Quality Control in Križevci, Croatia. Bacteriological examination of milk samples was not followed-up and not analysed. According to the results, the cows were divided into three groups: the cows suffering from CLM, the cows with SCM and control (CTL) cows. The CLM group consisted of cows with the clinical signs of mastitis (n = 30) including changes in milk composition, udder inflammation and disturbances of general health status (e.g. elevated body temperature, increased heart and respiratory rate, decreased ruminal contraction and decreased appetite, depression, relaxed cold ears, dehydration, etc.). The SCM group comprised the cows without clinical signs of mastitis (n = 30), but with a positive CMT and SCC above 200,000 cells/mL. The CTL group (n = 30) consisted of healthy cows with a negative CMT and SCC below 200,000 cells/mL and without any clinical sign of mastitis.

Blood samples were taken from *v. coccygea* and after clotting for 2 hours at room temperature centrifuged at 700 g for 15 minutes.

**Analytical procedures**

Serum triglycerides (TG), total cholesterol (CHOL), HDL-cholesterol (HDL-C) and Ca concentrations were assayed using standard commercial kits (Beckman Coulter Biomedical Limited, Lismeehan, O’Callaghan’s Mills, Co, Clare, Ireland) on an automatic Beckman Coulter AU 680 analyser (Beckman Coulter Biomedical Ltd, Ireland). Serum PON1 activity was measured spectrometrically by the method of hydrolysis of paraoxon (Mackness et al. 1991; Schiavon et al. 1996). Serum was added in the reaction mixture of 0.1 M Tris–HCl buffer pH 8.0 containing 2.0 mM of paraoxon (O,O-diethyl-O-p-nitrophenyl phosphate; Sigma Chemical Co, London, UK), 2.0 mM of CaCl and 1 mM of NaCl at 37°C. The P-nitrophenol generation was monitored bicomatically at 410/480 nm on Beckman Coulter AU 680.

**Statistical analyses**

Statistical analyses of data were performed using SAS 9.3. Software (SAS Institute Inc., Cary, NC, USA). The mixed model (PROC MIXED) with the repeated measure statement was used to analyse the measured parameters. The multiple comparison test of least-square means was performed using the Tukey–Kramer correction and Spearman’s correlation coefficient was calculated for the parameters recorded (TG, CHOL, HDL-C, PON1 and PON1/HDL-C) using the CORR module (PROC CORR). The values differing at *P* < 0.05 or lower were considered as significant.

**Results**

Serum PON1 activity was significantly lower in the SCM and CLM groups (Table 1) as compared to that in the CTL group (*P* < 0.05). Total CHOL, HDL-C and calcium (Ca) concentrations were significantly lower in the CLM group as compared to those found in the SCM and CTL groups. There were no significant differences in lipid status and calcium level between the CTL and SCM groups (Table 1). The ratio PON1/HDL-C was significantly higher only in CLM compared to CTL and SCM (Table 1). A high positive correlation (*r* = 0.98, *P* < 0.001) was found between total CHOL and HDL-C (Table 2). Significant positive correlations were found between PON1 and PON1/HDL-C (*r* = 0.47, *P* < 0.0001) while significant inverse correlation (*r* = −0.51 and *r* = −0.53, respectively) of PON1/HDL-C with total CHOL and HDL-C was calculated (*P* < 0.0001).

**Discussion**

The present study demonstrates decreased PON1 activity and alteration in lipid and calcium concentrations in cows with subclinical and clinical mastitis indicating oxidative stress and inflammatory response in the mastitis development. Diagnosis of mastitis in its subclinical form when there is no clinical

| Parameter            | Units   | CLM    | SCM    | CTL    |
|----------------------|---------|--------|--------|--------|
| PON1                 | U/L     | 235.53 ± 11.33a | 263.90 ± 7.99a | 304.60 ± 11.57b |
| HDL                  | mmol/L  | 2.97 ± 0.13a   | 4.03 ± 0.13a   | 4.25 ± 0.15b   |
| PON1/HDL             | –       | 81.06 ± 4.05a  | 67.17 ± 2.66a  | 73.59 ± 3.32a  |
| TGL                  | mmol/L  | 0.15 ± 0.01a   | 0.12 ± 0.01a   | 0.11 ± 0.00a   |
| TCH                  | mmol/L  | 5.24 ± 0.27a   | 7.53 ± 0.31a   | 7.88 ± 0.29a   |
| Ca                   | mmol/L  | 2.39 ± 0.03a   | 2.49 ± 0.02a   | 2.54 ± 0.02a   |
| Days of lactation    | days    | 210.30 ± 21.97a | 226.00 ± 49.45a | 238.93 ± 20.63a |
| Daily milk yield     | L       | 26.28 ± 2.85a  | 27.34 ± 1.54a  | 27.69 ± 1.16a  |
| (average per week)   |         |         |         |         |

*Groups comprised 30 animals each. Significantly different values (*P* < 0.05) in the same column are marked with different superscript letters.

**Table 2.** Spearman correlation coefficient and *P* value between parameters of lipid metabolism (TCH, TGL, HDL) and PON1 activity.

| Parameter  | TCH | HDL | TGL | PON1/HDL |
|------------|-----|-----|-----|----------|
| PON1       | n.s. | n.s. | n.s. | <0.0001  |
| TCH        | 0.98 | n.s. | <0.0001 | <0.0001  |
| HDL        | 0.98 | n.s. | <0.0001 | <0.0001  |
| TGL        | n.s. | n.s. | n.s. | n.s.     |
| PON1/HDL   | 0.47 | −0.51| −0.53| n.s.     |

n.s., non-significant.
signs of disease is challenging, and searching for suitable and reliable biomarkers should be measurable in milk and should report pathogen-specific changes at an early stage of the subclinical disease is crucial for early diagnosis (Kusebauch et al. 2018). New potential method for accurate detection of clinical mastitis in an automatic milking system using electronic data from the new develop support software (Khatun et al. 2018).

During pathogen invasion of the mammary gland, bacteria release toxins which activate leukocytes and epithelial cells in the mammary gland to release inflammatory mediators which attract numerous polymorphonuclear leukocytes (PMN) and macrophages functioning as phagocytic cells at the site of infection (Paape et al. 2003). Bacteria invading the mammary gland can cause pathogen-dependent differences in the permeability of the blood–milk barrier leading to the differential paracellular transfer of blood and milk components. Glucocorticoids are known to increase the integrity of the blood–milk barrier and quickly restore the decreased milk quality associated with mastitis (Wall et al. 2016b). During an inflammatory response, leukocytes, macrophages and other inflammatory cells produce ROS by which they promote the elimination of bacteria, and also cause damages of surrounding tissue (Pham 2006). Because of ROS accumulation, oxidative stress could develop. An excess amount of ROS and other products of oxidative stress, such as lipid hydroperoxides, could additionally contribute to cell and tissue destruction (Ryman et al. 2015). Additionally, increased accumulation of ROS in milk impairs its organoleptic properties and directly reduces milk quality (Lykkesfeldt and Svedsen 2007). PON1 is an anti-oxidative/anti-inflammatory which serum activity decreased during oxidative stress and inflammation (Turk et al. 2008, 2012, 2013). Its reduced activity in both subclinical and clinical mastitis in the present study could indicate oxidative stress and inflammatory response already in the subclinical form of disease what could predispose PON1 as a putative marker for early diagnosis of mastitis.

During APR, the lipid metabolism changes, particularly changes in the structure of HDL particle. HDL is an anti-oxidative/anti-inflammatory particle which structural proteins and enzymes provide a non-specific defence of a host. During inflammatory and oxidative stress condition, there is a remodelling of HDL when several antioxidant proteins are depleted from HDL (including PON1) while pro-inflammatory proteins enrich HDL making it a pro-inflammatory particle (Link et al. 2007; Feingold and Grunfeld 2010). The use of LDH in combination with SCC may be used as a marker to differentiate between gram-positive and gram-negative bacteria, but does not allow differentiating the immune response between different gram-positive bacteria (Hernández-Castellano et al. 2017). Endocrine profiles change and lipolysis and lipogenesis are regulated to increase the lipid reserve during pregnancy, which will be utilized following parturition and initiation of lactation (Roche et al. 2009). Insute that, lipid metabolism and changes in serum lipid and lipoprotein profiles are not a key aspect only of the physiology and energy metabolism during transition (Gross et al. 2013) or the peripartum period (Arfuso et al. 2016), but also during peak of lactation and later, as in our study, especially in some pathological conditions such as subclinical or clinical mastitis. The plasma concentrations of total and free cholesterol and HDL-C decreased few weeks before and 1 week after parturition and increased until a maximum at 98 days of lactation (Kessler et al. 2014). In our study, average days of lactation were 220. Total cholesterol, in our study, was lower in CLM than in CTL or SCM group (−33.50% and −30.41%) but not as in dairy cows with fatty liver disease and control (−66.45%) according to Farid et al. (2013). The calcium level, i.e. physiological range in healthy dairy cows is from 2.43 to 3.10 mmol/L (Kaneko et al. 1997), but in CLM group average calcium level was below. In HF cows from Poland, there were significant differences in PON1/HDL ratio between postpartum and lactation peak values (71.91 ± 31.06 and 72.11 ± 28.59, vs. 49.23 ± 20.40 and 40.77 ± 14.75, respectively) during two production cycles (Kulka et al. 2016). In our study, opposite to Kulka et al. (2016), PON1/HDL ratio was higher in all three groups of cows during lactation, but the highest and significantly different in the CLM group. Lower PON1/HDL was measured by Kulka et al. (2016) on the 63rd day of lactation in comparison to 220th day in our study. Average HDL concentrations during lactation in HF were similar to our results, but in Polish Red and Norwegian breed of dairy cows (Kulka et al. 2016) were lower than in our study with exception of CLM group. In our study, total cholesterol and HDL-cholesterol were decreased only in clinical mastitis.

During inflammation, there is a decrease in total cholesterol and HDL-C, probably because of remodelling of lipoprotein particles and transport of cholesterol from HDL to other lipoprotein particles. Feingold and Grunfeld (2010) reported that during APR reverse transport cholesterol is reduced. Additionally, some inflammatory mediators, such as LPS, TNF, IL-2 and others, reduce cholesterol concentration in blood (Khovidhunkit et al. 2004). However, the exact mechanism of this reduce is still scarce. Oxytocin treatment is likely most effective when there is little or no transfer of immunoglobulin G, such as in SCM cases (Wall et al. 2016a). The ratio between PON1 and HDL-C (PON1/HDL-C) was higher in clinical mastitis compared to the control and cows with SCM. This discrepancy between changes in PON1 and PON1/HDL-C could be explained with the decrease of PON1 activity in both subclinical and clinical mastitis while lipid changes occurred only in clinical mastitis indicating that PON1 is more influenced by inflammatory response than lipids. In the present study, calcium concentration was significantly lower in clinical mastitis compared to the control and cows with SCM. The Merck Veterinary Manual defines normal blood calcium concentration in the cow as 2.1–2.8 mmol/L. Although the calcium level in CLM was not below 2.0 mmol/L, what is considered a limit for subclinical hypocalcemia, cows with lower calcium concentration in blood are more susceptible to inflammation and disease development (Reinhardt et al. 2011).

Our results demonstrated oxidative stress and inflammatory response in cows with subclinical and clinical mastitis. A significant reduction of PON1 in both SCM and CLM might be a contributing factor to inflammation in the mastitis development. Moreover, decreased PON1 activity in SCM suggests PON1 as a potential biomarker for early diagnosis of a subclinical form of the disease.

Disclosure statement
No potential conflict of interest was reported by the authors.
