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15-F_{2t}-Isoprostane Concentrations and Oxidant Status in Lactating Dairy Cattle with Acute Coliform Mastitis

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Background: Severe mammary tissue damage during acute coliform mastitis in cattle is partially caused by oxidative stress. Although considered a gold standard biomarker in some human conditions, the utility of 15-F_{2t}-Isoprostanes (15-F_{2t}-Isop) in detecting oxidative stress in dairy cattle has not been validated.

Hypothesis: Concentrations of 15-F_{2t}-Isop in plasma, urine, and milk correlate with changes in oxidant status during severe coliform mastitis in cattle.

Animals: Eleven lactating Holstein-Friesian dairy cows in their 3rd–6th lactation.

Methods: A case–control study using cows with acute coliform mastitis and matched healthy controls were enrolled into this study. Measures of inflammation, oxidant status, and redox status in plasma and milk samples were quantified using commercial assays. Plasma, urine, and milk 15-F_{2t}-Isop were quantified by liquid chromatography/tandem mass spectrometry (LC-MS/MS) and ELISA assays. Data were analyzed by Wilcoxon rank sum tests ($\alpha = 0.05$).

Results: Plasma 15-F_{2t}-Isop quantified by LC-MS/MS was positively correlated with systemic oxidant status ($r = 0.83$; $P = .01$). Urine 15-F_{2t}-Isop quantified by LC-MS/MS did not correlate with systemic oxidant status, but was negatively correlated with redox status variables ($r = -0.83$; $P = .01$). Milk 15-F_{2t}-Isop quantified by LC-MS/MS was negatively correlated ($r = -0.86$; $P = .007$) with local oxidant status. Total 15-F_{2t}-Isop in milk quantified by a commercial ELISA (cbELISA) was positively correlated with oxidant status in milk ($r = 0.98$; $P < .001$).

Conclusions and Clinical Importance: Free plasma 15-F_{2t}-Isop quantified by LC-MS/MS and total milk 15-F_{2t}-Isop quantified by cbELISA are accurate biomarkers of systemic and mammary gland oxidant status, respectively. Establishing reference intervals for free and total 15-F_{2t}-Isops for evaluating oxidative stress in dairy cows should currently be based on the LC-MS/MS method.

Key words: Inflammation; Lipid peroxides; Oxylipids; Oxidative stress; Redox status.

Escherichia coli bacteria are a major cause of clinical coliform mastitis in dairy cattle.[1] Uncontrolled bacterial replication caused by dysfunctional immune responses results in severe mammary tissue damage and death.[2,3] Excessive production of reactive metabolites (RM) by phagocytic cells results in oxidative mammary tissue damage observed during coliform mastitis.[4] Increased metabolism of polyunsaturated fatty acids (PUFA) such as arachidonic acid (AA) generates lipid hydroperoxides that contribute to the RM pool.[5] Enhanced mitochondrial metabolism also contributes large amounts of superoxide ions to the toxic pool of RM during inflammation.[5] Oxidative stress occurs when elevated RM overwhelm the antioxidant defenses and induce tissue damage.[6] Antioxidants include enzymatic and nonenzymatic systems that quench the damaging effects of RM.[5] The role of oxidative stress in clinical coliform mastitis was demonstrated by the increased severity of disease associated with decreased vitamin C and increased lipid hydroperoxides. In addition, supplementation of vitamin E and selenium in the transition period decreased the severity of clinical disease.[7–9] Reliable biomarkers of oxidative stress in cattle are currently lacking despite experimental evidence supporting the critical role of RM in the pathophysiology of coliform mastitis in cattle.[10]

Lipids are particularly sensitive to RM attack resulting in generation of lipid hydroperoxides and isoprostanes.[6] Isoprostanes are prostaglandin-like metabolites of nonenzymatic peroxidation of AA.[11] Formation of these chemically stable peroxidation end-products starts with the free radical-mediated generation of an AA peroxyl radical followed by the cyclization into an F-pentane peroxyl ring that is then immediately reduced to F_2-isoprostanes.[11] A total of

Abbreviations:

15-F_{2t}-Isop	15-F_{2t}-isoprostane
AOP	anti-oxidant potential
GSH	reduced glutathione
GSSG	oxidized glutathione
RM	reactive metabolites

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64 F_2-isoprostane isomeric compounds from AA are generated and can be detected in biological samples by chromatography and mass spectrometry methods.[12] However, 15-F_{2t}-Isoprostanes (15-F_{2t}-Isop, also known as 8-isoprostanes) were the major isoform validated as gold standard markers of oxidative stress in humans.[12,13] Immune-based assays such as ELISAs that are specific to the common pentane ring in isoprostanes are used for 15-F_{2t}-Isop detection in some human biological samples, but are less accurate than the gold standard mass spectrometry techniques.[14,15]

The detection of oxidative stress in cattle utilizes lipid hydroperoxides that are quantified by measuring their low molecular weight degradation aldehyde products such as malondialdehyde (MDA) using the thiobarbituric acid reactive substances (TBARS) method.[16] The TBARS method, however, lacks specificity to the lipid-derived MDA because they can react with metabolites derived from other macromolecules including DNA and polysaccharides.[10] Therefore, determining the utility of 15-F_{2t}-Isop might provide an accurate marker for diagnosis of oxidative stress and monitoring treatment responses in veterinary medicine.[11]

To date, 15-F_{2t}-Isop was not evaluated as biomarkers of oxidative stress during acute coliform mastitis. The purpose of this study, therefore, was to determine whether 15-F_{2t}-Isop could predict systemic and local mammary gland oxidant status during coliform mastitis using liquid chromatography/tandem mass spectrometry (LC-MS/MS). The potential utility of using commercial ELISA assays**,** validated for use in samples from humans, also were evaluated as alternative 15-F_{2t}-Isop quantification techniques on bovine samples. The hypothesis for this study was that 15-F_{2t}-Isop in plasma, urine, and milk correlate with the oxidant status associated with severe bovine coliform mastitis. This study determined both the utility of 15-F_{2t}-Isop as a biomarker of oxidative stress and provided a basis for defining reference intervals for evaluating oxidative stress associated with clinical and subclinical coliform mastitis.

Materials and Methods

Animals

Control and coliform mastitis cows in this study (n = 4/group) were randomly selected from larger groups with housing and diet information published elsewhere.[17] Another group of healthy cows (n = 3) was selected for assessment of ELISA assays before utilizing them in analyses of samples from the control and coliform mastitis study groups. This study was approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC) and all cows were enrolled from the same herd with client consent.

Study Design

All cows enrolled in this study were multiparous Holstein dairy cows that ranged from 3rd to 6th lactation and averaged 69 (3–105) days in milk. Cows affected with acute coliform mastitis (n = 4) and matched healthy control (n = 4) made up the 2 experimental groups. The average body condition score was 3.06 (range: 2.75–3.50) for control cows and 3.00 (range: 2.50–3.50) for coliform mastitis cows. Cows in the coliform mastitis group had positive *E. coli* milk cultures (>100 colony forming units) and exhibited at least 2 signs of systemic clinical disease. Signs of acute systemic coliform mastitis included increased rectal temperature (>39.2°C), tachycardia (heart rate > 80 beats/minute), tachypnea (respiratory rate > 30 breaths/minute), episcleral injection, local signs of mammary gland inflammation including discoloration, swelling, heat and pain on palpation, and typical serum-like watery milk. By the time of sampling, coliform mastitis affected cattle had received at least a single dose of flunixine meglumine (2.2 mg/kg IV), ceftiofur sodium (2.2 mg/kg SC), and oral electrolyte fluids after standard farm treatment protocols. Healthy control cows had negative bacterial milk cultures, absence of overt clinical signs and a somatic cell count of <250,000 cells/mL on the last test day before the start of the study. Bacterial milk cultures were performed according to the National Mastitis Council guidelines.[18] Milk and blood samples were collected at the same time from mastitis and healthy control cows within 12 hours following a clinical diagnosis of systemic coliform mastitis.

Sample Collection and Analyses

Blood samples were collected in serum-separator and EDTA tubes, whereas urine and milk samples were collected in plain 15 mL tubes and processed on the day of collection and stored at −80°C until analyzed. Whole blood aliquot from EDTA tubes was processed, and analyzed for reduced (GSH) and oxidized (GSSG) glutathione[a] as previously described.[19] Plasma and serum were harvested after centrifuging at 711 × *g* for 15 minutes. Plasma, milk, and urine for 15-F_{2t}-Isop quantification were mixed with an antioxidant reducing agent (AOR, 4 μL/mL) as described previously.[17] Using commercial assays, RM[b] were analyzed in plasma and milk with no AOR, whereas, serum amyloid A (SAA)[c] and haptoglobin (Hp)[d] were analyzed in serum. Serum albumin and nonesterified fatty acids (NEFA) were analyzed at the Diagnostic Center for Population and Animal Health (Lansing, MI). Plasma and milk antioxidant potential (AOP) were measured in samples collected without AOR agent as described previously.[20] Briefly, the AOP of a sample was standardized to the reduction capacity of trolox[e] (synthetic vitamin E analog) in 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)[f] solution.

15-F_{2t}-Isoprostane Quantification: LC-MS/MS

Details for chemicals, sample processing, sample extraction and the LC-MS/MS protocol for the detection of 15-F_{2t}-Isop in plasma, urine, and milk were as previously described with some modifications.[17] Briefly, modifications included urine sample preparation where the first step was mixing 4 mL of sample with 24 μL of formic acid. Thereafter, urine samples were processed in the same way as plasma samples. Prostaglandin E_2-d_9 ($PGE_2\_d_9$) was the single deuterated standard used as a reference for the quantification of 15-F_{2t}-Isop. Multiple reaction monitoring parameters for 15-F_{2t}-Isop (Cone voltage = 21, collision voltage = 22, parent ion m/z = 353.2, and product ion m/z = 193) and $PGE_2\_d_9$ (Cone voltage = 21, collision voltage = 16, parent ion m/z = 360.2, and product ion m/z = 280) were optimized using MassLynx software (Waters Corp, Milford, MA USA).

15-F_{2t}-Isoprostane Quantification: ELISA assays

The ELISA assays validated for human plasma and urine samples from Cell Biolabs (cbELISA)[g] and Cayman Chemicals (ccELISA),[h] were used for quantifying 15-F_{2t}-Isop in milk, plasma and

urine samples. Preliminary performance of the ELISA assays was first determined on samples (milk, plasma, and urine) from healthy third lactation cows (n = 3) in the first 30 days in milk. Samples were pooled by type, split into 3 aliquots and assayed independently. Samples were assayed for free (unbound) and for total (free + esterified) 15-F_{2t}-Isop. Total 15-F_{2t}-Isop were measured after alkaline hydrolysis for milk and plasma, and acidic hydrolysis for urine. Hydrolysis of samples was based on the Cell Biolabs protocol with some modifications. Briefly, 200 μL of milk or plasma were combined with 50 μL of 10N sodium hydroxide and incubated at 45°C for 2 hours. For cbELISA analyses, after incubation, 55 μL of 6N hydrochloric acid (HCl) were added and samples were centrifuged for 5 minutes at 4,816 × *g* and room temperature. Finally, a 1 : 2 sample dilution with neutralization solution to achieve a pH between 6 and 8 was performed. For ccELISA analyses, after incubation, plasma and milk samples were diluted to 1 : 6 using acetonitrile with 1% formic acid and centrifuged (4,816 × *g*, room temperature for 5 minutes). The supernatant was eluted through 1 mL phenomenex solid phase extraction columns and dried in a SpeedVac (55°C, 2 hour). Residues were suspended in ccELISA buffer. For acidic hydrolysis, 200 μL of urine were combined with 50 μL of 6N HCL until a pH < 3.0 was reached and then diluted 1 : 6 with phosphate buffered saline. A final 1 : 3 urine sample dilution was performed with neutralization solution. In addition, a 10 ng quantity of a 15-F_{2t}-Isop standard was added to samples to assess the effect of the various sample preparation methods on 15-F_{2t}-Isop recovery. All ELISA assays were performed in duplicate following manufacturers' recommendations.

Statistical Analyses

No assumptions for normality of data were made because of the small sample size. All variables were expressed as median (range) concentrations and analyzed using the Wilcoxon rank sum procedure ($\alpha = 0.05$) using the SAS[i] software. Spearman correlations between plasma and urine 15-F_{2t}-Isop concentrations to systemic inflammatory, oxidant status and redox status parameters were calculated. Similarly, Spearman correlations between milk 15-F_{2t}-Isop and the oxidant status and redox status parameters for the local environment in the mammary gland were calculated. For correlations, all data were combined to obtain a range of values for a given variable from normal to severely diseased cows. The ELISA assays were also compared for the detection and quantification of 15-F_{2t}-Isop concentrations in similarly processed samples.

Results

All 4 coliform mastitis cows exhibited local mammary gland signs of inflammation as well as signs of systemic involvement including tachycardia, tachypnea, pyrexia, and scleral injection. Within the coliform mastitis group, 3 cows died on days 1, 3, and 8 post sampling and 1 cow was still present in the herd at 105 days post sampling. Cows with naturally occurring coliform mastitis, in this study, had significant differences in the acute phase proteins with greater SAA and Hp ($P = .014$) and lower albumin ($P = .043$) concentrations than control cows. Serum NEFAs also were increased significantly ($P = .029$) in coliform mastitis cows compared to control cows (Table 1). Although AOP did not differ between experimental groups in milk or plasma, RM concentrations were greater in plasma ($P = .029$) and milk ($P = .014$) from coliform mastitis than control cows (Table 2). Concentrations of reduced glutathione (GSH) were lower ($P = .014$), whereas concentrations of the oxidized form (GSSG) were greater ($P = .057$) in coliform mastitis than control cows. The ratio of reduced to oxidized glutathione (GSH : GSSG) was lower in mastitis than control cows ($P = .051$) (Table 2). Plasma RM correlated positively with SAA ($r = 0.69$, $P = .058$) and Hp ($r = 0.67$, $P = .071$) and NEFAs ($r = 0.31$, $P = .456$) but negatively with serum albumin ($r = -0.85$, $P = .008$).

Table 1. Median (range) concentrations of acute phase proteins and non-esterified fatty acids from coliform mastitis and control cows (n = 4/group).

Variable	Control	Mastitis	*P*-Value
Acute phase proteins			
Serum Hp, mg/mL	0.003 (0.0005–0.01)	0.49 (0.05–1.7)	.014
Serum SAA, μg/mL	68.0 (12–218)	1395.9 (1,139–1853)	.014
Serum albumin, g/dL	3.0 (2.9–3.2)	2.8 (2.6–2.9)	.043
Lipid mobilization			
Serum NEFA, mEq/L	0.08 (0.06–0.13)	0.62 (0.12–1.2)	.029

Hp, haptoglobin; SAA, serum amyloid A; NEFA, non-esterified fatty acids.

Statistical analyses: Wilcoxon rank sum test, $\alpha = 0.05$.

LC-MS/MS Based 15-F_{2t}-Isop Concentrations in Plasma, Urine and Milk

Cows with coliform mastitis had relatively greater 15-F_{2t}-Isop in plasma and urine ($P = .057$) than control cows (Table 2). Plasma 15-F_{2t}-Isop concentrations correlated positively with plasma RM ($P = .010$). The plasma RM were not significantly correlated with urine 15-F_{2t}-Isop concentrations (Table 3). Both plasma and urine 15-F_{2t}-Isop concentrations negatively correlated with GSH levels, however, only urine 15-F_{2t}-Isop significantly correlated with the GSH : GSSG ratio (Table 3). Milk 15-F_{2t}-Isop concentrations were lower in coliform mastitis than control cows ($P = .014$, Table 2) and negatively correlated with the RM ($P = .007$) (Table 3).

Although measures of oxidant status were significantly correlated between plasma and milk (RM: $r = 0.83$, $P = .010$), the 15-F_{2t}-Isop in plasma and milk were inversely correlated ($r = -0.76$, $P = .028$).

ELISA Based 15-F_{2t}-Isop Quantification in Plasma, Urine and Milk

Using the cbELISA, total 15-F_{2t}-Isop were greater ($P = .05$) in milk and plasma than the free 15-F_{2t}-Isop in samples (Fig 1A and B). For free 15-F_{2t}-Isop, spiking resulted in greater 15-F_{2t}-Isop ($P = .05$) compared to

Table 2. Median (range) concentrations and ratios for oxidant status, redox status and 15-F_{2t}-isoprostanes in samples from coliform mastitis and control cows (n = 4/group).

Variable	Control	Mastitis	*P*-value
Milk RM,[a] RFU × 1,000/μL	4.1 (3.5–4.8)	26.6 (14.0–36.7)	.014
Milk AOP[b]	7.7 (4.9–10.2)	4.8 (4.4–11.4)	NS
Plasma RM,[a] RFU/μg protein	0.30 (0.28–0.51)	0.67 (0.46–0.88)	.029
Plasma AOP[b]	5.5 (4.7–6.2)	4.9 (4.1–5.8)	NS
Blood GSH, μM	471.5 (455.1–577.4)	403.9 (141.2–417.4)	.014
Blood GSSG, μM	3.2 (2.1–22.9)	15.8 (4.7–38.7)	.057
GSH : GSSG ratio	147.5 (17.9–268.6)	47.5 (1.6–87.3)	.051
Urine 15-F_{2t}-Isop, ng/mg Cr	0.05 (0.02–0.89)	1.5 (0.7–5.2)	.057
Plasma 15-F_{2t}-Isop, ng/L	2.2 (1.0–6.3)	10.0 (5.1–27.6)	.057
Milk 15-F_{2t}-Isop, ng/L	1177.5 (436–1,535)	182.4 (87–317)	.014

RM, reactive metabolites represent reactive oxygen species and reactive nitrogen species; AOP, antioxidant potential; 15-F_{2t}-Isop, 15-F_{2t}-isoprostane; Cr, creatinine; GSH, reduced glutathione; GSSG, oxidized glutathione; NS, not significant.

[a]RFU, relative florescence units per μL (milk) and per μg of protein (plasma).

[b]AOP expressed as sample antioxidant concentrations with ABTS- radical reducing power equivalent to standard vitamin E (Trolox) concentrations.

Statistical analyses: Wilcoxon rank sum test, $\alpha = 0.05$.

Table 3. Correlations of non-esterified fatty acids, oxidant status and redox status with the LC-MS/MS quantified 15-F_{2t}-isoprostane concentrations in samples from coliform mastitis and control cows (n = 4/group).

	15-F_{2t}-Isop		
Variable	Milk	Plasma	Urine
Lipid mobilization			
Serum NEFA	−0.48	0.14	0.38
Oxidant status			
Plasma RM	–	0.83*	0.52
Plasma AOP	–	−0.57	−0.17
Milk RM	−0.86*	–	–
Milk AOP	0.50	–	–
Redox status			
Blood GSH	–	−0.71*	−0.81*
Blood GSSG	–	0.55	0.83*
GSH : GSSG ratio	–	−0.55	−0.83*

NEFA, non-esterified fatty acids; RM, reactive metabolites represent reactive oxygen species and reactive nitrogen species; AOP, antioxidant potential; GSH, reduced glutathione; GSSG, oxidized glutathione; 15-F_{2t}-Isop, 15-F_{2t}-isoprostane.

*$P < .05$ (Spearman correlation, $\alpha = 0.05$).

non-spiked and was associated with recovery rates of 73, 69 and 118% in milk, plasma, and urine, respectively. In spiked milk and plasma samples, hydrolysis resulted in greater total 15-F_{2t}-Isop ($P = .05$) with recovery rates of 106 and 291%, respectively.

Using the ccELISA, there was no difference between free and total 15-F_{2t}-Isop ($P = .20$) in both milk and plasma (Fig 2A and B). For free 15-F_{2t}-Isop, spiking resulted in greater 15-F_{2t}-Isop ($P = .05$) compared to non-spiked and was associated with recovery rates of 102, 362 and 154% in milk, plasma, and urine, respectively. In spiked samples, hydrolysis resulted in greater total 15-F_{2t}-Isop ($P = .05$) in plasma with a recovery rate of 552%. Interestingly, hydrolysis of spiked milk samples resulted in significant ($P = .05$) lower 15-F_{2t}-Isop concentrations representing a 60% loss of the spiked amount.

Based on significant differences between free and total 15-F_{2t}-Isop, milk and plasma samples were analyzed for both free and total 15-F_{2t}-Isop for the cbELISA. Only free 15-F_{2t}-Isop were analyzed by ccELISA in plasma and milk based on the lack of differences from total 15-F_{2t}-Isop. Following manufacturers' recommendations, urine samples for cbELISA were analyzed for both free and total 15-F_{2t}-Isop, whereas only free 15-F_{2t}-Isop were quantified by ccELISA.

Total 15-F_{2t}-Isop in plasma and urine samples quantified by cbELISA did not differ significantly between control and mastitis cows, whereas greater 15-F_{2t}-Isop concentrations were detected in milk from coliform mastitis than control cows (Table 4). Free 15-F_{2t}-Isop quantified by cbELISA in urine and milk did not differ significantly between the mastitis and control group (Table 4). The cbELISA estimated significantly greater ($P = .014$) free 15-F_{2t}-Isop concentrations in urine relative to the ccELISA within each of the mastitis and control cow groups (Table 4).

Free 15-F_{2t}-Isop concentrations quantified by both cbELISA and ccELISA in nonhydrolyzed urine samples did not correlate with plasma oxidant or redox status parameters (Table 5). Similarly, free 15-F_{2t}-Isop concentrations quantified by cbELISA in milk did not correlate with milk oxidant status parameters (Table 5). Total plasma and urine 15-F_{2t}-Isop concentrations quantified by cbELISA in hydrolyzed samples did not correlate with oxidant status or redox status in plasma (Table 6). Total milk 15-F_{2t}-Isop concentrations quantified by cbELISA were positively correlated with milk oxidant status variables (Table 6).

Discussion

Plasma and urine 15-F_{2t}-Isop concentrations are well established oxidative stress markers in several human diseases.[13] Cows suffering from naturally occurring

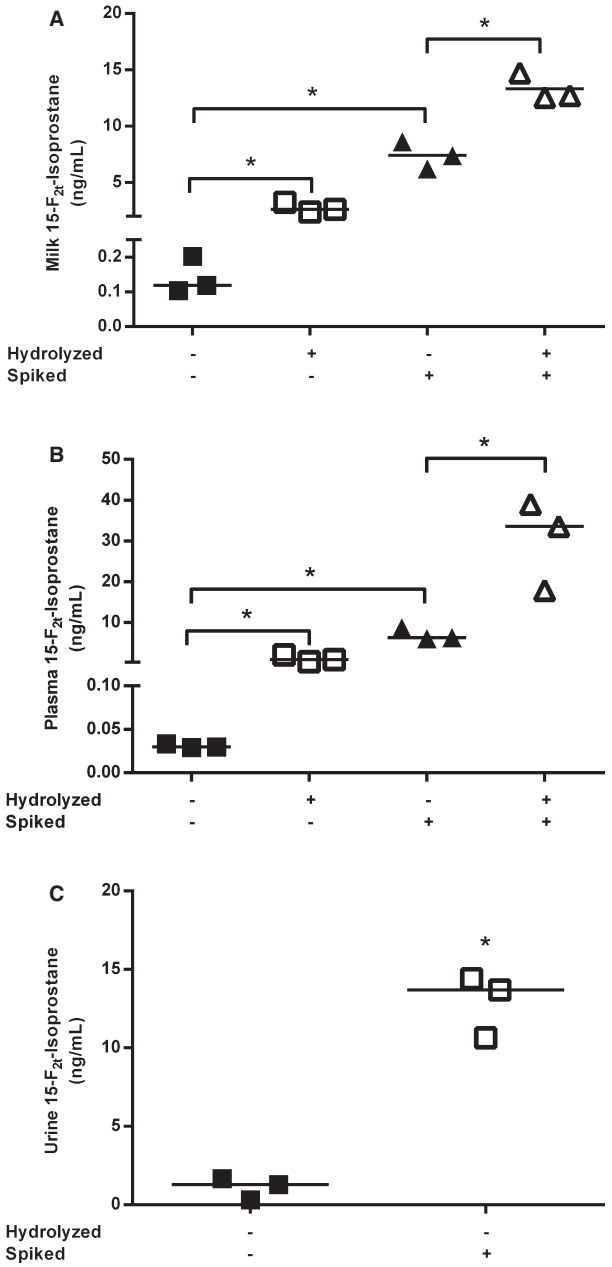

Fig 1. Concentrations (+Median) of 15-F_{2t}-isoprostane in pooled samples from healthy cows (n = 3) quantified by cbELISA (Cell Biolabs, San Diego, CA). Milk (**A**), plasma (**B**) and urine (**C**) samples from cows (n = 3) less than 30 days in milk were pooled by sample type and divided into 3 replicates. Replicates were either treated by alkaline hydrolysis (10 N sodium hydroxide) or not, and spiked with 10 ng/mL 15-F_{2t}-isoprostane (standard) or not as indicated below each graph. The limit of detection (LOD) was 10 pg/μL of sample and limits for accuracy were recovery of standard 15-F_{2t}-Isop was ±20%. Data linked by a horizontal line indicate comparisons of interest and the asterisks label indicates significant difference (Wilcoxon rank sum, $\alpha = 0.05$).

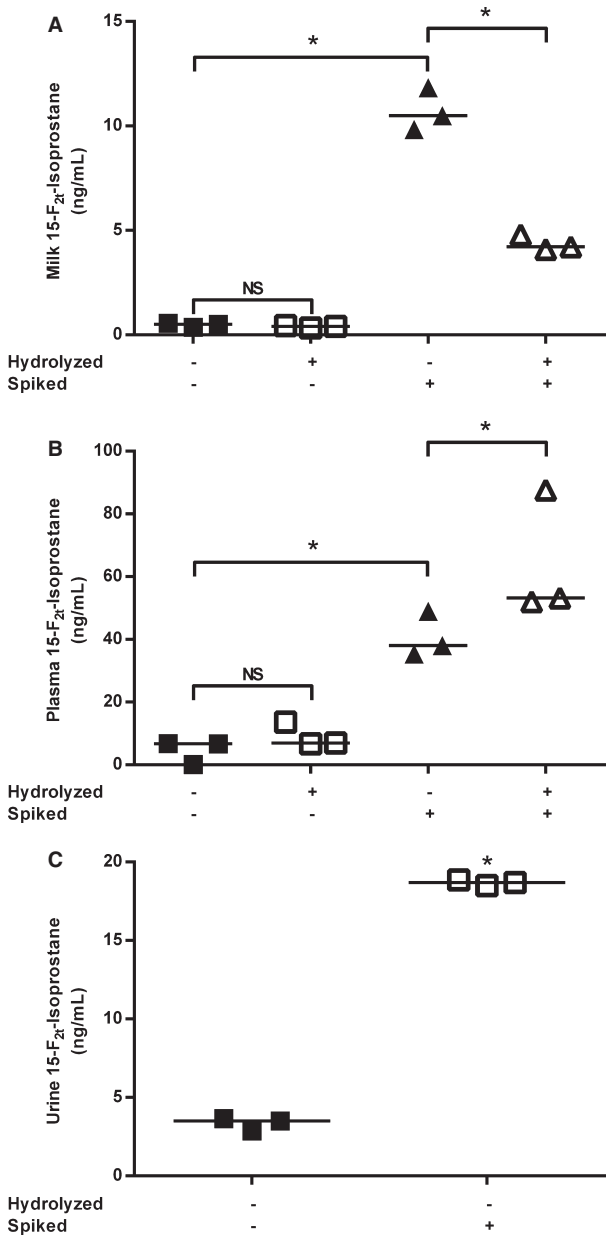

Fig 2. Concentrations (+Median) of 15-F_{2t}-isoprostane in pooled samples from healthy cows (n = 3) quantified by ccELISA (Cayman Chemicals, Ann Arbor, MI). Milk (**A**), plasma (**B**) and urine (**C**) samples from cows (n = 3) less than 30 days in milk were pooled by sample type and divided into 3 replicates. Replicates were either treated by alkaline hydrolysis (10 N Sodium hydroxide) or not, and/or spiked with 10 ng/mL 15-F_{2t}-isoprostane (standard). The limit of detection (LOD) was 2.7 pg/μL of sample and limits for accuracy were recovery of standard 15-F_{2t}-Isop was ±20%. Data linked by a horizontal line indicate comparisons of interest and the asterisks label indicates significant difference (Wilcoxon rank sum, $\alpha = 0.05$); NS, not significant

acute systemic coliform mastitis in the current study provided an effective model, as supported by the changes in acute phase proteins and NEFAs, to evaluate the utility of 15-F_{2t}-Isop as a biomarker of oxidative stress. Using the gold standard method of LC-MS/MS for quantifying 15-F_{2t}-Isop, both plasma and urine 15-F_{2t}-Isop increased during mastitis. These findings agree with animal models of oxidant injury and severe sepsis

Table 4. Median (range) concentrations of 15-F_{2t}-isoprostanes quantified by ELISA in samples from coliform mastitis and control cows (n = 4/group).

Variable	Control	Mastitis	*P*-Value
cbELISA[1]			
Plasma 15-F_{2t}-Isop, μg/L	24.6 (21.4–27.3)	30.5 (7.6–45.2)	NS
Urine 15-F_{2t}-Isop, ng/mg Cr	11.0 (7.1–19.4)	16.2 (8.1–34.9)	NS
Milk 15-F_{2t}-Isop, μg/L	5.0 (4.6–6.8)	20.6 (7.5–42.8)	.014
cbELISA[2]			
Milk 15-F_{2t}-Isop, μg/L	0.81 (0.77–0.81)	0.74 (0.15–1.10)	NS
Urine 15-F_{2t}-Isop, ng/mg Cr	11.8 (7.9–14.3)[a]	20.5 (7.7–45.1)[a]	NS
ccELISA[2]			
Urine 15-F_{2t}-Isop, ng/mg Cr	1.8 (1.3–3.3)[b]	3.2 (2.5–4.4)[b]	.056

ccELISA, Cell Biolabs ELISA; ccELISA, Cayman Chemical ELISA; 15-F_{2t}-Isop, 15-F_{2t}-isoprostane; Cr, creatinine; NS, not significant.

[1]Total 15-F_{2t}-Isop in hydrolyzed samples.

[2]Free 15-F_{2t}-Isop in non-hydrolyzed samples.

statistical analyses: Wilcoxon rank sum test, $\alpha = 0.05$.

Values with letter superscripts (a,b) denote the only comparison in the same column (group) and different superscripts denote statistical difference (Wilcoxon rank sum, $P = .014$).

Table 5. Correlations of nonesterified fatty acids, oxidant status and redox status with ELISA quantified free 15-F_{2t}-Isop concentrations in samples from coliform mastitis and control cows (n = 4/group).

	15-F_{2t}-Isop		
	cbELISA		ccELISA
Variable	Milk	Urine	Urine
Lipid mobilization			
NEFA	−0.26	0.24	−0.60
Oxidant status			
Plasma RM	–	0.02	−0.57
Plasma AOP	–	−0.405	−0.17
Milk RM	−0.43	–	–
Milk AOP	0.66	–	–
Redox status			
Blood GSH	–	−0.55	0.36
Blood GSSG	–	0.55	−0.07
GSH : GSSG ratio	–	−0.55	−0.07

NEFA, nonesterified fatty acids; RM, reactive metabolites represent reactive oxygen and nitrogen species; AOP, antioxidant potential; GSH, reduced glutathione; GSSG, oxidized glutathione; 15-F_{2t}-Isop, 15-F_{2t}-isoprostane; cbELISA, Cell Biolabs ELISA; ccELISA, Cayman Chemicals ELISA.

Spearman correlations, $\alpha = 0.05$.

Table 6. Correlations of nonesterified fatty acids, oxidant status and redox status with ELISA quantified total 15-F_{2t}-Isop concentrations in samples from coliform mastitis and control cows (n = 4/group).

	cbELISA 15-F_{2t}-Isop		
Variable	Milk	Plasma	Urine
Lipid mobilization			
NEFA	0.67	−0.31	0.52
Oxidant status			
Plasma RM	–	0.14	0.29
Plasma AOP	–	0.02	0.26
Milk RM	0.98***	–	–
Milk AOP	−0.19	–	–
Redox status			
Blood GSH	–	−0.21	−0.54
Blood GSSG	–	0.31	0.54
GSH : GSSG ratio	–	−0.31	−0.55

NEFA, nonesterified fatty acids; RM, reactive metabolites represent reactive oxygen and nitrogen species; AOP, antioxidant potential; GSH, reduced glutathione; GSSG, oxidized glutathione; cbELISA, Cell Biolabs ELISA; 15-F_{2t}-Isop, 15-F_{2t}-isoprostane.

***$P < .001$ (Spearman correlation, $\alpha = 0.05$) .

with multi-organ damage in humans.[13,21] Plasma 15-F_{2t}-Isop are validated as indicating endogenous production in different tissues and the renal excretion closely reflects plasma levels.[22] In humans with acute respiratory distress syndrome (ARDS), exhaled breadth condensate and plasma 15-F_{2t}-Isop concentrations correlated significantly with urinary levels.[23] Plasma and urine samples are thus acceptable for evaluating 15-F_{2t}-Isop during oxidative stress in humans. In this study, both plasma and urine 15-F_{2t}-Isop correlated negatively with GSH, however, only plasma 15-F_{2t}-Isop significantly correlated with the systemic oxidant status. Reduced glutathione (GSH) is an important donor of thiol groups involved in scavenging of oxidants and maintaining redox balance.[5,24] A decrease in the GSH : GSSG ratio indicating depletion of the thiol donor is considered a useful measure of oxidative stress in sepsis-induced ARDS in humans.[25] On the basis of increased 15-F_{2t}-Isop that correlated positively with oxidant status and negatively with GSH, plasma is ideal for quantifying 15-F_{2t}-Isop reflective of systemic oxidant status in bovine coliform mastitis.

The lack of significant correlation of urine 15-F_{2t}-Isop with oxidant status was unexpected as the renal excretion of 15-F_{2t}-Isop is related directly to its plasma concentrations. For example, a murine model of ischemia-reperfusion injury showed that renal excretion of 15-F_{2t}-Isop was increased by up to 300%.[26] In the same study, intrarenal infusion of 15-F_{2t}-Isop was associated with diminished glomerular filtration rate in a dose dependent manner suggesting a possible contribution of 15-F_{2t}-Isop in the pathogenesis of renal failure.[26] Renal failure is common in humans with sepsis and the association with plasma 15-F_{2t}-Isop is direct evidence for oxidative stress mediated damage.[21] Renal failure frequently occurs in cows with severe coliform

mastitis[27] and would be expected to increase the rate of 15-F_{2t}-Isop excretion. The observation that increases in plasma free 15-F_{2t}-Isop lag behind the rise in esterified 15-F_{2t}-Isop in murine models of oxidative stress[28] might explain the lack of significance in urine 15-F_{2t}-Isop and oxidant status in this study. The strong correlations of urine 15-F_{2t}-Isop to redox status parameters (GSH, GSSG, and GSH : GSSG ratio) might indicate depletion of an early line of antioxidant defense before overall changes in AOP as suggested by lack of differences in plasma AOP in this study. Evaluating the temporal changes in 15-F_{2t}-Isop excretion as the disease progresses might uncover the relationship between plasma and urine 15-F_{2t}-Isop during coliform mastitis.

The lower concentrations of 15-F_{2t}-Isop and its inverse correlation with the abundance of RM in milk was unexpected because oxidative stress mediates mammary gland damage, especially during coliform mastitis.[29] The presence of increased PUFA substrates in the same local environment of the mammary with excess RM could be expected to also generate increased 15-F_{2t}-Isop. A recent study found that several PUFA substrates were increased in milk during coliform mastitis in tandem with some nonenzymatic derived oxylipids including hydroperoxy acids from AA.[17] The formation of 15-F_{2t}-Isop, which occurs predominantly, while AA is esterified to phospholipids,[11] could explain the lack of prediction of oxidant status by free 15-F_{2t}-Isop quantified by LC-MS/MS in milk. The concentrations of free 15-F_{2t}-Isop in milk also were inversely correlated with free 15-F_{2t}-Isop concentrations in plasma suggesting that differential release from esterification sites might exist across compartments. The 15-F_{2t}-Isop initially formed in situ esterified to phospholipids are subsequently hydrolyzed to yield free 15-F_{2t}-Isop by phospholipase (PL) enzymes.[28] Differential PL activity was detected in humans where the activity in plasma was greater with more 15-F_{2t}-Isop hydrolysis compared to intracellular PL.[30] The concept of differential hydrolysis was supported by the use of a hydrolysis method and analyzing for total 15-F_{2t}-Isop by 1 of the 2 ELISA assays (cbELISA) in this study which yielded greater concentrations that positively correlated with milk oxidant status. Therefore, it appears that predictive ability of 15-F_{2t}-Isop concentrations on local mammary gland oxidant status during coliform mastitis can be improved by performing sample hydrolysis. Further research is required to understand the counterintuitive decrease in 15-F_{2t}-Isop concentrations in the presence of increased RM especially when other nonenzymatic oxylipid metabolites were detected in nonhydrolyzed samples.

An alternate approach to LC-MS/MS for the quantification of 15-F_{2t}-Isop is by use of immunoassays such as ELISA.[12] Despite the reported accuracies of plasma and urine 15-F_{2t}-Isop quantified by ELISAs of 95–101%, ELISAs are often affected by cross-reactivity from prostaglandin and other isoprostane metabolites because they target a single metabolite.[12,14] Free 15-F_{2t}-Isop in milk and urine as well as total 15-F_{2t}-Isop in plasma and urine, analyzed by cbELISA, failed to predict oxidant status. Only the total 15-F_{2t}-Isop quantified in milk by cbELISA demonstrated potential reliability that was supported by the accurate recovery (106%) of the spiked 15-F_{2t}-Isop standard (Fig 1A). Variable performances of ELISAs in quantifying 15-F_{2t}-Isop were previously reported in studies in veterinary species and humans that reported poor correlations with GC/MS or LC-MS/MS as well as between different ELISA assays.[31–33] Results of this study showed that, despite acceptable recovery rates by the ccELISA (102%) on free milk 15-F_{2t}-Isop and cbELISA (118%) on free urine 15-F_{2t}-Isop, there was no correlation with oxidant status. Further, the variable performances of the ELISAs as shown by both overestimation (plasma, cbELISA, Fig 1; plasma and urine, ccELISA, Fig 2) and underestimation (milk, ccELISA Fig 2) make it impossible for general recommendations for using ELISA in quantifying 15-F_{2t}-Isop in bovine samples. A recent study reported a positive linear correlation between milk and plasma 15-F_{2t}-Isop by utilizing an ELISA based (ccELISA) assay. The investigators of that study suggested that milk was a possible alternate route of 15-F_{2t}-Isop excretion.[34] The inverse correlation between free 15-F_{2t}-Isop in plasma and milk using LC-MS/MS in this study does not support the possibility of milk as a route of excretion for 15-F_{2t}-Isop. An expanded lipidomic profile during coliform mastitis showed lack of correlation among several oxylipids suggesting that oxylipid biosynthesis, including isoprostanes, between plasma and milk could be independent of each other.[17] The difference in the correlation with the present findings might be the use of a disease model associated with high degree of oxidative stress, as well as different sample extraction methods. It is unclear whether the study[34] analyzed free or total 15-F_{2t}-Isop in both milk and plasma. The performance of ELISA assays in quantifying 15-F_{2t}-Isop in bovine samples should be validated with the gold standard mass spectrometry-based methods such as the LC-MS/MS.

In conclusion, results of this study show that free plasma 15-F_{2t}-Isop concentrations are predictive of systemic oxidant status during acute coliform mastitis. Free 15-F_{2t}-Isop in urine and milk were not predictive of systemic or local mammary gland environment oxidant status, respectively. Quantification of total 15-F_{2t}-Isop in milk by cbELISA is an accurate alternative to LC-MS/MS and suggests that milk samples should be hydrolyzed when determining 15-F_{2t}-Isop associated with oxidant status in the mammary gland. The lack of significance for some variables in this study might have been because of the small number of experimental animals in this study. Findings of this study can thus be used as a basis for further studies designed to provide broader scope inferences. Establishing threshold concentrations for 15-F_{2t}-Isop during clinical coliform mastitis will provide a basis for formulation and application of practical interventions to control oxidative stress in dairy cows and currently should be based on gold standard methods such as LC-MS/MS.

Footnotes

[a] GSH/GSSG assay, OxisResearch, Portland, OR
[b] ROS and RNS assay, Cell Biolabs, San Diego, CA
[c] SAA, Tridelta Development Limited, Co., Kildare, Ireland
[d] Hp, Tridelta Development Limited, Co., Kildare, Ireland
[e] Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), Sigma, St. Louis, MO
[f] ABTS (2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), Sigma, St. Louis, MO
[g] OxiSelect 8-iso-Prostaglandin $F_{2\alpha}$ ELISA, Cell Biolabs, San Diego, CA
[h] 8-Isoprostane EIA, Cayman Chemical, Ann Arbor, MI
[i] SAS 9.4, SAS institute Inc., Cary, NC

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