Veterinary Clinical Pathology ISSN 0275-6382

ORIGINAL RESEARCH

Oxidant and antioxidant profile of hyperketonemic ewes affected by pregnancy toxemia

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Key Words
β-Hydroxybutyrate, cortisol, hyperketonemia, lipid peroxidation, oxidative stress, TBARS

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DOI:10.1111/j.1939-165X.2011.00284.x

Background: Negative energy balance during late pregnancy in ewes is an important cause of hyperketonemia. Ketone bodies can generate superoxide radicals and cause oxidative stress and cellular dysfunction, as noted in cows with subclinical ketosis or in diabetic people.
Objective: The aim of this study was to evaluate the role of hyperketonemia in initiating the process of lipid peroxidation.
Methods: The study included 10 pregnant ewes (aged 3.5–6 years) with pregnancy toxemia, 10 clinically healthy pregnant ewes, and 10 clinically healthy nonpregnant ewes. Serum concentrations of β-hydroxybutyrate (BHB), cortisol, and glucose, plasma activities of glutathione peroxidase, superoxide dismutase, and catalase, and plasma concentrations of thiobarbituric acid reactive substances (TBARS), markers of lipid peroxidation, and reduced glutathione were measured. Data from the 3 groups were statistically analyzed and compared.
Results: Serum concentrations of BHB, cortisol, and TBARS were significantly higher in ewes with pregnancy toxemia when compared with concentrations in healthy pregnant and nonpregnant groups ($P \leq .05$). In ewes with pregnancy toxemia, a strong positive correlation was found between concentrations of TBARS and BHB ($r = .80$; $P = .002$) and between concentrations of BHB and cortisol ($r = .76$; $P = .005$).
Conclusions: Oxidative stress and lipid peroxidation are involved in the development and complications of pregnancy toxemia. An association between hyperketonemia and the products of lipid peroxidation has also been demonstrated, suggesting that ketosis is a risk factor in the development of lipid peroxidation and oxidative stress in ewes affected by pregnancy toxemia.

Introduction

Pregnancy toxemia is a metabolic disorder characterized by hypoglycemia and hyperketonemia resulting from incapacity of the animal to maintain adequate energy balance. Clinically, the animals have neurologic signs and are weak. In general, pregnancy toxemia develops in the last third of pregnancy, with a greater incidence in animals carrying 2 or more fetuses; it can also be observed in poorly nourished sheep with only a single fetus.[1–3] Specifically, toxemia appears in the last 6 weeks of pregnancy when the fetuses have reached 2/3 of their prenatal development. Obese animals develop ketonemia because fat deposited in the liver interferes with metabolism.[4] However; nonobese animals can develop ketonemia as well. Symptoms of ketonemia include depression, anorexia, weakness, staggering gait, apparent blindness, recumbency, coma, and death. Risk factors include multiple fetuses, poor quality of ingested energy, decreased dietary energy level, genetic factors, obesity, lack of good body condition or high parasite load, and lack of exercise.[4–6]

Negative energy balance during the late pregnancy is considered key to the development of the disease and hyperketonemia.[7–9] In hyperketonemic animals in which body fuel is mainly derived from fat, the concentration of ketones begins to rise in blood and may reach 10 mmol/L, which is 10-fold higher than concentrations in normoketonemic animals.[10,11] In a previous study, it was reported that the ketone body, acetoacetate, can generate superoxide radicals that can then form hydroxyl radicals.[12] These free radicals exert their cytotoxic

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effect by initiation of membrane phospholipid peroxidation, resulting in accumulation of the final products of lipid peroxidation, such as thiobarbituric acid reactive substances (TBARS). These products are known to cross-link membrane components causing alterations in biomembrane permeability and lipid organization as well as cellular dysfunction and membrane damage.[13,14] Markers of oxidative stress were increased in cows with subclinical ketosis[15] and in hyperketonemic compared with normoketonemic people with type 1 diabetes.[12,16] Normal cells have the capacity to detoxify superoxide radicals using antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase, and catalase (CAT), which help maintain the intracellular concentration of reduced glutathione and NADPH necessary for the optimal function of the antioxidant defense system.[17]

The aim of this study was to evaluate the role of hyperketonemia in initiating the process of lipid peroxidation. Furthermore, the status of the enzymatic (GSH-Px, SOD, and CAT) and nonenzymatic (reduced glutathione) antioxidant defense system in ewes affected by pregnancy toxemia was examined.

Material and Methods

Animals and blood samples

The study included 10 pregnant ewes, aged 3.5–6 years, with pregnancy toxemia, 10 clinically healthy pregnant ewes, and 10 clinically healthy nonpregnant ewes of known age, gestational age, and parity (parity was not known for all healthy nonpregnant ewes) from 2 farms located near the Jordan University of Science and Technology. All protocols were approved by the animal care and use committee (JUST-ACUC). For ewes with pregnancy toxemia, the diagnosis was established based on clinical signs, mainly recumbency, stargazing, subnormal temperature, tremors, acetone breath, jaw clamping, blindness, and hyperesthesia to touch, and was confirmed by high serum concentration of β-hydroxybutyrate (BHB) (3.2–10.5 mmol/L; reference limit, $\leq$ 0.86 mmol/L[3]). Each ewe was fed a constant diet of 0.5 kg of concentrate consisting of protein, fiber, and fat, in addition to 2 kg of wheat straw twice daily. A mineral and vitamin premix (Minavit-Forte, VAPCO, Amman, Jordan) containing vitamin A, vitamin D3, vitamin E, potassium iodide, cobalt chloride, sodium selenite, copper sulfate, ferrous sulfate, magnesium sulfate, manganese sulfate, zinc sulfate, and dicalcium phosphate was added. Drinking water was provided ad labitum. Data on gestational ages and the number of fetuses were collected from farm records, and the attending veterinarian confirmed pregnancy using transabdominal ultrasonography on day 60 after breeding. The number of lambs born per ewe was determined after lambing.

Blood samples were collected from the jugular vein of all animals into 3 vacutainer tubes (BD, Franklin Lakes, NJ, USA) containing, in order, acid citrate dextrose for the measurement of plasma activities of antioxidant enzymes (GSH-Px, SOD, and CAT) and reduced glutathione (GSH) concentration; lithium heparin for measurement of plasma concentrations of TBARS; and no additive for measurement of serum concentrations of BHB, cortisol, and glucose in serum. Blood was immediately transported to the laboratory. For serum collection, blood samples were kept for 20 minutes at room temperature and then centrifuged at 3000*g* for 10 minutes at room temperature (24°C). Serum was harvested and stored frozen (−20°C) until analysis. All serum samples were analyzed within 1 month of collection. Hemolysates were prepared by lysing 500 µL packed RBCs by addition of 4 volumes of cold double-distilled water. The suspension was centrifuged twice to eliminate cell membranes and the concentration of GSH and the activities of GSH-Px, SOD, and CAT were measured in the supernatant (hemolysate).

Antioxidant enzymes and reduced glutathione assay

Activities of GSH-Px, SOD, and CAT were measured in the hemolysate as a functional assessment of the status of the trace minerals selenium, zinc, and copper. GSH-Px activity was assayed as described previously.[18] Briefly, GSH-Px catalyzes the oxidation of glutathione using tert-butyl hydroperoxide. Oxidized glutathione is converted to the reduced form in the presence of glutathione reductase and NADPH; NADPH is oxidized to NADP, and the reduction in absorbance of NADPH at 340 nm is measured. The absorbance change per minute and the molar extinction coefficient of NADPH are used to calculate GSH-Px activity, expressed as unit per gram of hemoglobin (U/g Hb). SOD activity was assayed using the xanthine/xanthine oxidase system for superoxide radical (O_2^-) generation.[19] This anion reduces nitroblue tetrazolium (NTB) to a red formasone compound. SOD activity is measured at 560 nm by detecting inhibition of this reaction. One unit of SOD activity is defined as the activity resulting in half-maximal inhibition of NTB reduction rate and is expressed as U/g Hb. CAT activity was measured by the method described by Aebi,[20] in which 1 U of CAT activity is defined as the amount of enzyme that produces

1 mmol H_2O_2 per minute at an initial concentration of 10 mmol/L at pH 7.4 and 37°C. Results are based on the calculated Hb concentration in the reaction mixture and expressed as U/g Hb. GSH concentration was detected in the hemolysate by titration with 0.1 mmol/L dithiobis in a 0.1 mol/L disodium phosphate buffer solution. The formation of the reduced product, thionitrobenzene, was measured by spectrophotometry at 412 nm (Spectrum 2000, Witeg Labortechink, Wertheim, Germany). GSH was expressed as g/L hemolysate.[21]

Lipid peroxidation products assay

Plasma TBARS were extracted with *n*-butanol according to the optimized method of Yagi[22] by adding 100 μL of plasma to a 0.37% thiobarbituric acid solution and measuring concentration by spectrophotometry at 535 nm.

BHB, cortisol, and glucose assays

Serum concentration of BHB was determined by a kinetic enzymatic method using a commercially available kit (Ranbut D-3-hydroxybutyrate, Randox, Crumlin Co., Antrim, UK).[3] The assay is based on the reversible reaction between 3-hydroxybutyrate and NAD^+ catalyzed by 3-hydroxybutyrate dehydrogenase, and the change in NADH concentration is measured by changes in the absorbance at 340 nm. Serum glucose concentration was measured by spectrophotometry using a commercially available kit (Glucose GOD-PAP, Linear Chemicals, Barcelona, Spain) following the manufacturer's instructions. Serum cortisol concentration was measured using a commercially available ELISA kit (Biocheck, Foster City, CA, USA). All analyses were performed in duplicate to control for interassay and intra-assay discrepancies.

Statistical analysis

Results were expressed as median and range. Differences between data were calculated using the nonparametric Kruskal–Wallis test. *P*-values $\leq .05$ were considered significant. Intra-assay coefficient of variation for the assays was calculated by dividing the mean of the SD of duplicates by the mean of the duplicates. Statistical analysis was performed using the SPSS program v 13 (SPSS Inc., Chicago, IL, USA).

Results

The ewes in the 3 groups did not differ by age, parity, or gestational age (Table 1, $P > .05$). Serum concentrations of BHB, cortisol, and TBARS were significantly higher in ewes with pregnancy toxemia when compared with concentrations in healthy pregnant and nonpregnant groups (Table 2, $P \leq .05$). A significant difference was also found when healthy pregnant and nonpregnant groups were compared. In contrast, serum concentrations of glucose and GSH were significantly lower in the group of ewes with pregnancy toxemia when compared with concentrations in both pregnant and nonpregnant healthy groups ($P \leq .05$). However, there were not significant differences in glucose or GSH concentrations between healthy pregnant and nonpregnant ewes. The activities of GSH-Px, SOD, and CAT were significantly decreased in affected ewes when compared with activities in the 2 healthy groups (Table 2, $P \leq .05$). However, significant differences in the activities of the antioxidant enzymes between the healthy pregnant and healthy nonpregnant ewes were not found. The concentration of serum cortisol was significantly ($P \leq .05$) higher in ewes with pregnancy toxemia when compared with the concentration in healthy pregnant and nonpregnant ewes. Furthermore, cortisol concentration in healthy pregnant animals was 4-fold higher when compared with that in healthy nonpregnant ewes. In ewes with pregnancy toxemia, a strong significant positive correlation was found between concentrations of plasma TBARS and serum BHB ($r = .80$; $P = .002$), GSH ($r = .69$; $P = .01$), and cortisol ($r = .76$; $P = .05$). A significant negative correlation was found between BHB concentration and GSH concentration ($r = -.50$; $P = .04$), glucose concentration ($r = -.43$; $P = .01$), and CAT activity ($r = -.38$; $P = .05$).

Table 1. Characteristics of ewes with pregnancy toxemia (PT) and healthy pregnant (HP) and healthy nonpregnant (NP) ewes.

	PT	HP	NP
Number of ewes	10	10	10
Age (years)*	4.5 ± 0.85 (3–6)	4.6 ± 0.70 (3–7)*	4.75 ± 0.82 (3–6)*
Breed	Awassi	Awassi	Awassi
Gestational age (weeks)*	17.8 ± 1.03 (16–19)	17.5 ± 0.85 (16–19)	Not applicable
Parity*	4.1 ± 1.52 (2–6)	3.9 ± 0.74 (3–5)	Not available

*Mean ± SD (range).

Table 2. Serum concentrations of β-hydroxybutyrate, glucose, cortisol, lipid peroxidation products (TBARS), and reduced glutathione and activities of antioxidant enzymes in ewes with pregnancy toxemia and in healthy pregnant and nonpregnant ewes.

	Median (Range)		
Analyte	Pregnancy Toxemia	Healthy Pregnant	Healthy Nonpregnant
β-Hydroxybutyrate (mmol/L)	7.05 (3.2–10.5)*	0.89 (038–1.32)**	0.29 (0.10–0.53)**
Glucose (mmol/L)	1.08 (0.72–1.83)*	4.19 (3.22–5.11)	4.72 (3.72–5.44)
Cortisol (nmol/L)	2.25 (1.05–4.03)*	0.42 (0.26–0.78)**	0.09 (0.02–0.31)**
TBARS (μmol/L)	4.5 (3.4–5.2)*	1.93 (0.87–2.7)**	1.21 (0.87–1.860**
GSH-Px (U/g Hb)	42 (32–52)*	69 (49–86)	75.5 (64–82)
SOD (U/g Hb)	1071 (850–1410)*	1595 (1387–2045)	1716 (1340–1965)
Catalase (U/g Hb)	89 (68–112)	111 (89–144)	132 (96–145)
GSH (nmol/L)	1.81 (1.56–2.28)*	2.48 (1.89–3.25)	2.83 (2.21–3.38)

For all analytes, intra-assay coefficients of variation were $< 5\%$.
*$P \leq .05$ for comparison of pregnancy toxemia and other 2 groups;
**$P \leq .05$ for comparison of healthy pregnant and nonpregnant groups.
TBARS, thiobarbituric acid reactive substances; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; Hb, hemoglobin.

Discussion

The increase in plasma concentration of TBARS found in this study suggests there is significant increase in lipid peroxidation in ewes with pregnancy toxemia compared with healthy pregnant and healthy nonpregnant animals. Increased lipid peroxidation may be caused by disturbed redox balance[14] as was evident from the significant reduction in the levels of nonenzymatic and enzymatic antioxidants in the group of affected ewes with higher concentrations of ketone bodies in their blood. The decrease in antioxidant enzyme activity may be associated with an imbalance between lipid peroxidation and antioxidant capacity in pregnant ewes. In contrast to these findings, an increase in activities of antioxidant enzymes in cows with subclinical ketosis has been reported.[15]

The negative correlation of GSH-Px and CAT activities with BHB concentration in ewes with pregnancy toxemia suggests a reduction in the protective activities of these antioxidant enzymes against lipid peroxidation, which is evident in the increase of the concentration of plasma TBARS in affected animals, similar to results in lactating cows with subclinical ketosis[15] and in diabetic people.[23] In contrast, SOD activity correlated positively, but not significantly, with BHB concentration. Moreover, the positive correlation of GSH-Px, SOD, and CAT activities with reduced glutathione concentration in affected ewes indicates dependence of these enzymes on glutathione levels under conditions of both increased and decreased oxidative stress, in agreement with a study pregnant women.[24] The significant increase in the concentration of TBARS and its strong positive correlation with BHB in toxemic ewes with low-energy intake concur with results from a study of periparturient dairy cows.[25] In contrast, in studies evaluating human blood samples and brains of developing rats, ketone bodies were not found to mediate generation of oxidative stress.[26,27] The negative correlation between BHB and GSH suggests that increased ketone body concentrations in blood of affected ewes influence cellular GSH levels, similar to what has been demonstrated in people.[12] The level of cellular lipid peroxidation and GSH depletion was significantly greater in the toxemic ewes with increased concentrations of ketone bodies, suggesting that hyperketonemia is a risk factor for increased lipid peroxidation and GSH depletion in these ewes with pregnancy toxemia, as is found in cows and people.[25,28]

Although increased serum cortisol concentrations were found in all pregnant ewes, the increase in ewes with pregnancy toxemia was significantly greater than in healthy pregnant ewes, as reported previously.[29] Positive correlation between BHB and cortisol concentrations and between serum cortisol and TBARS in pregnancy suggests that cortisol and BHB may play a role in induction of lipid peroxidation and oxidative stress in ewes with higher concentrations of ketones. Similar results are reported in cows with subclinical ketosis[15] and in buffaloes affected with dystocia.[30]

Oxidative stress has been reported in several studies of healthy pregnancy in animals and people[12,31–33] and is caused by increased free radical production resulting from increased metabolic activity during pregnancy,[34] negative energy balance and ketone body formation,[35] reduction of antioxidant reserve during pregnancy, and physiologic adaptation of pregnant animals to lactation.[15,34,36] Other mechanisms, including exhaustion of the antioxidant defense system,

metabolic acidosis, and acute or chronic inflammatory processes, may also cause oxidative stress in animals.[37,38] We have demonstrated that oxidative stress and lipid peroxidation are involved in the development and complications of pregnancy toxemia. In addition, an association between hyperketonemia and lipid peroxidation was shown, suggesting that ketonemia is a risk factor for lipid peroxidation and oxidative stress in ewes affected with pregnancy toxemia. These findings should be considered in the therapeutic approach to pregnancy toxemia. Antioxidants may be useful additions to protocols for prevention and treatment of pregnancy toxemia.

Acknowledgment

This study was supported by the Jordan University of Science and Technology.

Disclosure: The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

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