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Original Research

Effects of Silymarin on milk production, liver enzymes, oxidative status and *HSP70* gene expression in postparturient Sanjabi ewes

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Abstract: In the sheep farming industry, breeders need suitable strategies in order to improve milk yields. Meanwhile, silymarin (a natural hepatoprotector substance) has beneficial effects on common oxidative stress at the beginning of lactation. This study was the first research to evaluate the effect of silymarin on milk production, liver enzymes, oxidative and *HSP70* responses in the postpartum period. Total 20 Sanjabi ewes were divided into two groups: control (group C: no addition) and treated (group T: received a diet supplemented with silymarin at 2000 mg/kg feed for 15 d after lambing). Data indicated that silymarin reduced postpartum body weight(BW) loss. At the same time, the feed intake (FI) rate increased. In addition, the peaks of milk yields could be achieved earlier compared with control ewes ($P< 0.05$). Treatment decreased milk compositions (fat and protein) on days 10 and 15. Furthermore, the reactive oxygen species (ROS) and malondialdehyde (MDA) contents in group T were significantly lower than group C ($P< 0.05$). Also, the activities of endogenous antioxidant enzymes (Glutathione peroxidase, Superoxide dismutase, and Catalase) were increased. Silymarin remarkable increased the serum alanine aminotransferase (ALT) values ($P< 0.05$). Meanwhile, an increasing trend in the total protein levels was recorded in group T as compared to group C ($P> 0.05$). The QRT-PCR analysis showed that silymarin supplemental reduced expression of *HSP70* gene in blood serum ($P< 0.05$). It means that changing the diet can affect the activity of heat shock proteins that consequently changes the quality of animal products. In conclusion, our observation relieved that silymarin treatment in the puerperium periodis potentially an effective strategy to improve milk quality via dual hep at oprotective and antioxidant functions.

Key words: *HSP70*; Liver; Oxidative status; Postpartum; Sanjabi ewes; Silymarin.

Introduction

The animal husbandry has faced major challenges for improvement of the features of milk, to enhance more value and nutritional quality to the products like cheeses and yogurts. Recently scientific research for milk production has been very progressive in dairy livestock. The production of sheep's milk and its by-products represent an important source of income for the sheep farming industry, with a 22% increase in production between 2003 and 2013(1).

Nutritional requirements of dairy sheep increased to coincide with onset of lactation, but the gradual decrease in dry matter intake (DMI) that occurs over the postpartum period (because these days are the most stressful for dairy animals), caused ewes cannot receive enough energy for supply of requirements, resulting in negative energy balance (NEB) (2). Thus, as a result of maladaptation to this NEB, liver lipidosis occurs (3). In addition, the body's antioxidant defense system is overcome and ideal blood antioxidant levels are so difficult to keep (4, 5).

In the sheep farming industry of Iran, herbal antioxidantshave become widely accepted as a standard breeding tool for modifying milk production in the peripartum and postpartum period, due to the savings in costs associated with animal husbandry(6). Dietary flavonolignans extracted (including isosilibinin, silibinin,silychristin, isosilychristin and silydianin) and a flavonoid (taxifolin) (7) from the milk thistle (silymarin, molecular formula: C25H22O10, molecular weight: 482.44 g/mol) is known as one of the most important nutrients in the small ruminants diet (8). The evidence accrued suggests that silymarin is an antioxidant nutrient which may prevent lipid peroxidation (9, 10), cell membrane stabilizing (11), hyperprolactinemic properties (12) and improved milk quality because of its hepatoprotective properties (13).

Medicinal herbs are rich in natural materials (14-18).

Recent studies reported that silymarin-paired with many organ systems in human and farm animals like the liver (19, 20). In addition, relative studies revealed that silymarin, a natural hepatoprotector substance, into the postpartum period diets alleviated the adverse impact of common oxidative stress at the beginning of lactation (21) by scavenging of reactive oxygen species (ROS). Inside the body, ROS produced by revelation to common oxidative stress is considered one of the most important reasons for the destruction of body tissue, for example, lipid peroxidation, DNA damage, enzyme inactivation and damage to other organs (22).

The 70-kDa heat shock proteins (Hsp70s) including a family of conserved ubiquitously expressed heat shock proteins. Proteins of a similar structure exist in almost

all organisms. The Hsp70s are an important part of the cell machinery for protein folding and help protect cells against stress (23, 24).

Based on our knowledge until know no studies are available that examine the effect of silymarin on milk quality and oxidative status in the postpartum period ewes. Thus, our investigation tested the hypothesis that silymarin protects health, performance and milk quality of Sanjabi ewes in the postpartum period via enhancing the antioxidant state. The results provide a novel mechanistic approach with respect to common oxidative stress during the postpartum period.

Materials and Methods

Chemicals and ethical approval

All reagents were purchased from Merck (Darmstadt, Germany). The kits to evaluate ROS, various oxidative stress indices (including malondialdehyde (MDA), the activities of endogenous antioxidant enzymes (glutathione peroxidase (GSH), superoxide dismutase (SOD), and catalase (CAT)), and liver enzymes (aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP)) were purchased from Nanjing Jiancheng Bioengineering Institute (China) and Pars Azmun Co. (Tehran, Iran), receptively. The study protocol was carried out in compliance with the guidelines for care and handling of farm animals which was approved by an ethics committee at Razi University, Kermanshah, Iran (97-02-32-51985).

Managements of ewes

In the postpartum period, ewes were fed a lactation diet based on alfalfa hay and concentrate (corn, barley, soybean meal and wheat bran) diet provided three times daily with ad libitum intake according to the recommended of National Research Council (25).The components, ingredients and nutrient composition of the diet are presented in Table 1. Feed intake (FI) was offered to achieve five percent of refusals (measured daily before the morning feeding).

Animals and experimental procedure

Twenty healthy 2-5 years-old fat tail Sanjabi ewes (70±5.25 kg), were obtained from the Mehregan Farm located in Kermanshah Province in the west of Iran. Estrus synchronization was performed in the breeding season by using progesterone sponge in day 0 and administration of 400 IU of PMSG (Hipra Co., Spain) on day 12. Following the administration of PMSG, ewes were introduced to rams for mating. At lambing, the treatments were as follows (for 15 d after lambing): control (normal diet, n=10) and silymarin (2000 mg/kg DM, n=10). The silymarin (powder with a purity of 80.18%) prepared from the Zardband Pharmaceuticals Co., Tehran, Iran.

Laboratory analysis

Blood and milk samples were collected on days 0, 10 and 15 before morning feeding. Fresh milk samples were analyzed for the determination of the level of protein, fat, lactose and somatic cell count (SCC) using a MilkoScan FT 120 (FOSS Electric A/S, Hillerød, Denmark).

Blood samples (5 ml) were obtained via jugular veins (into vacuum tubes non-heparinized). The serum was separated by centrifugation (10 min at 20 °C and 4000×g), kept at –20 °C for later analyses. The levels of MDA and endogenous antioxidant enzymes were measured by ultraviolet spectrophotometer. The absorbance of the supernatant was measured at 532, 420, 550 and 405 nm, respectively. The concentration of total protein and liver enzymes in the serum were measured by using an atomic absorption spectrophotometer (Perkin-Elmer, AA-600, USA). Standard commercial kits were applied for analysis and the procedures were adopted as recommended with the kits' manufacturer.

Quantitative real-time PCR (QRT-PCR)

Serum total RNA was extracted using the TRIzol reagent. according to the manufacturer's instructions (Takara Bio, Japan). The 2% agarose gel electrophoresis was used to assess the integrity of total RNA and the A260/280 ratio evaluated by NanoDrop 2000 (Thermo

Table 1. Components, ingredients and nutrient composition of diets.

Ingredient	Dry alfalfa	Wheat	Corn	Soybean meal	Barely	Wheat barn	Total
DM feed (%)	55	15.3	12.6	8.1	5.4	3.6	100
ME (Mcal/Kg)	0.995	0.469	0.19	0.24	0.15	0.091	2.13
CP (% DM)	10.7	1.72	1.134	4.04	0.756	0.615	18.9
NDF (% DM)	31.9	1.48	3.52	1.2	1.51	1.83	41.44
CA (% DM)	3.85	0.306	0.239	0.58	0.216	0.248	5.43
Cu (mg/kg)	7.535	1.193	1.00	1.81	0.46	0.50	12.49
Zn (mg/kg)	12.155	4.59	1.75	4.61	2.39	4.60	30.09
Fe (mg/kg)	93.5	6.12	11.42	14.98	4.86	4.6	135.48

CP: crud protein; NDF: neutral detergent fiber, CA: crude ash.

Table 2. Primers used for RT-PCR, sequence and product size.

Gene	Product size (bp)	Primer sequence
β-actin	300	Forward: 5'-TGCGTGACATCAAGGAGAAG -3' Reverse: 5'-TGCCAGGGTACATTGTGGTA -3'
HSP70	372	Forward: 5'-AGCGTAACACCACCATTCC -3' Reverse: 5'-TGGCTCCCACCCTATCTC -3'

Primer sets designed using free online software Primer3Plus (v. 0.4.0) http://primer3plus.cg

Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized using a Prime Script™ RT Master Mix kit. QRT-PCR was conducted using the QuantStudio 7 Flex qRT-PCR system (Stratagene, USA) and SYBR@ Premix Ex Taq™II kit. Specific primers were synthesized by Invitrogen, USA (Table 2). The house-keeping β-actin gene was for each sample as reference gene. Thermocycling conditions were as follows: after initial denaturation at 95 °C for 120s; 40 amplification cycles were performed at 95 °C for 15s, 60°C for the 30s; finally, 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. The $2^{-\sigma\sigma Ct}$ method was used to analyze the data.

Statistical analysis

Effects of silymarin were evaluated by one-way analysis of variance (ANOVA) using the command PROC GLM of SAS software (version 9.1), according to the model:

Yi = μ+Ti+eijk

Where Yi is the dependent variable; μ=the overall mean; Ti = effect of silymarin; and eijk = residual error.

The effects of treatment according to the diet were determined with averages calculated by command LSMEANS and Dunnett's new multiple ranges was used to determine the significant differences among the treatment groups at significant level of $P< 0.05$.

Results

Effects of silymarin treatment on the body weight (BW), FI and milk quality of ewes are presented in Table 3. Data indicated that silymarin reduced postpartum BW loss. At the same time, the FI rate increased. Also, the peaks of milk yields could be achieved earlier compared with unchallenged ewes ($P < 0.05$). Treatment decreased milk compositions (fat and protein) on days 10 and 15.

Table 4 shows oxidative biomarkers, total protein and liver enzymes in each group. The ROS and MDA

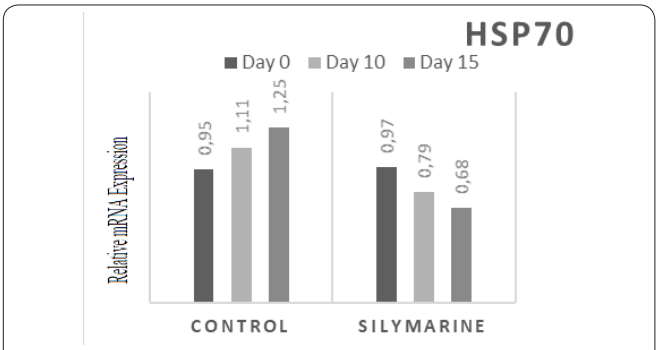

Figure 1. Changes in the expressions of *HSP70* in mRNA induced by silymarin ($P< 0.05$); mRNA expression levels of *HSP70* as detected by QRT-PCR. All data were expressed as relative values against their respective control groups. The values are presented as mean ±SEM (n=10). β-actin was used as an internal control.

Table 3. Effects of silymarin (2000 mg/kg/day) on performance and milk quality of Sanjabi ewes in the postpartum period.

Groups / Parameters	Day 0		Day 10		Day 15	
	Control	Silymarin	Control	Silymarin	Control	Silymarin
BW (kg)	74.6±7.1	73.9±6.66	71.8±5.7	70.9±7.2	73.1±6.1	72.4±6.8
FI (kg)	1.73±0.05	1.75±0.07	1.78±0.09	1.81±0.10	1.86±0.12	1.88±0.15
Milk (L)	0.81±0.02	0.79±0.03	1.1±0.05 ^{b}	1.4±1.11 ^{a}	1.01±0.08 ^{b}	1.38±0.98 ^{a}
Fat (g/100 g)	5.81±0.86	5.65±0.78	5.43±0.61	5.21±0.50	5.39±0.58	5.02±0.44
Protein (g/100 g)	5.21±0.41	5.14±0.39	5.45±0.46	5.32±0.40	5.41±0.33	5.29±0.46
Lactose (g/100 g)	4.89±0.61	4.81±0.52	4.61±0.42	4.66±0.48	4.93±0.52	4.90±0.45
SCC (x10^4/mL)	281±122	265±113	385±173	337±151	338±151	307±134

Values are given as means ± S.D (n=10). Superscripts (a-b) show significant differences in each row ($P< 0.05$). BW: body weight; FI: feed intake; SCC: somatic cell count.

Table 4. Effects of silymarin (2000 mg/kg/day) on serum levels of oxidative biomarkers and liver enzymes of Sanjabi ewes in the postpartum period.

Groups / Parameters	Day 0		Day 10		Day 15	
	Control	Silymarin	Control	Silymarin	Control	Silymarin
ROS (U DCF[1]/ml)	7.12±1.81	7.16±1.72	11.03±2.03 ^{a}	6.04±1.55 ^{b}	8.81±1.46 ^{a}	5.23±1.09 ^{b}
MDA (nmol/ml protein)	2.30±0.23	2.41±0.25	2.81±0.41 ^{a}	1.48±0.18 ^{b}	3.12±0.55 ^{a}	1.12±0.92 ^{b}
GSH (μmol/ml)	7.72±1.77	7.61±1.64	4.12±0.88	6.01±0.63	8.25±2.03	10.9±2.62
SOD (μmol/ml)	3.21±0.52	3.18±0.48	2.81±0.44	3.35±0.61	2.95±0.58	3.55±0.74
CAT (U/ml)	5.23±0.31	5.30±0.32	4.61±0.41	5.38±0.71	4.55±0.49	5.41±0.51
TP (mg/dL)	4.95±0.75	5.11±0.76	5.03±0.81	5.13±0.79	5.51±0.92	5.82±0.97
AST (U/L)	101.5±44.3	98.9±45.1	95.4±37.2	105.7±48.4	93.9±40.1	108.8±49.5
ALT (U/L)	23.8±5.1	22.9±4.8	21.7±4.5 ^{b}	31.1±6.2 ^{a}	20.3±4.1 ^{b}	33.8±6.1 ^{a}
ALP(U/L)	321±116.6	327±121.5	319±113.8	331±120.2	316±109.1	335±124.2

Values are given as means±S.D(n=10). Superscripts (a-b) show significant differences in each row ($P< 0.05$). ROS: reactive oxygen species; MDA: malondialdehyde; GSH: glutathione peroxidase; SOD: superoxide dismutase; CAT: catalase; TP: total protein; AST: aspartate aminotransferase; ALT: alanine aminotransferase, ALP: alkaline phosphatase. Superscripts (a-b) show significant differences in each column ($P<0.05$).

contents in the group silymarin were significantly lower than that of the group control ($P< 0.05$). At the same time, the activities of endogenous antioxidant enzymes (GSH, SOD, and CAT) were increased. Silymarin remarkable increased the serum ALT values ($P< 0.05$). Meanwhile, an increasing trend in the total protein, AST and ALP levels were recorded in the silymarin group as compared to those in the control group ($P> 0.05$).

The mRNA expression level of *HSP70* was shown in Figure 1. The QRT-PCR analysis showed that silymarin supplemental reduced *HSP70* gene expression in serum ($P < 0.05$).

Discussion

In the farming industry, animal health science needs to be highlighted with the major challenges in managing the postpartum period requirements, and common therapeutic methods usually do not lead to satisfactory results. Thus, this experiment was carried out to study the effects of silymarin (as an effective antioxidant) on health, performance and milk quality of the postpartum period.

To the best of our knowledge, this is the first comprehensive research exploring the mechanistic basis for the functional effects of silymarin during the postpartum period in the sheep model. In the present study, the difference in BW, FI, oxidative biomarkers, liver enzymes and expression of gene related to heat shock protein 70(HPS70) between control and treated animals has likely attributed to differences in daily content of the diet and the general condition of the ewes remained satisfactory.

Milk production and compositions are of the main economic significance for dairy farmers. Our study proved that dietary inclusion of silymarin could improve milk quality during the postpartum period of Sanjabi ewes. In addition, to these animals, the peaks of milk yield were reached earlier, leading to optimal milk production across the entire experiment, although silymarin was directed for a short period (15 d after lambing). Previous studies have reported that silymarin administration in the prepartum period enhances milk production and compositions(i.e. fat, protein and lactose)in dairy cows, thus it reduces the NEB in transition to metabolic adaptation at the start of lactation and thus improved the milk yields (21, 26).The results of current research contribute to the study of the development of dairy sheep, both in terms of productive and herbal extract, as natural products do not produce harmful residues in food and tend not to make problems such as antibacterial resistance (27). Collectively, our findings indicated that silymarin exposure could minimize the common oxidative stress problems associated with the postpartum period allows the ewes to express their potential for peak milk production at the beginning of lactation.

In the silymarin group increased the FI, thus during the postpartum period they did not compensate their need to energy through lipolysis. It can be concluded that silymarin decreases lipolysis through decreased creation of free radicals and the formation of ROS and increased FI. On the other hand, MDA (the end product of lipid peroxidation) is an index for the level of ROS-induced biological damage (28). Results also showed that silymarin decreased serum MDA concentration of ewes in the postpartum period. In agreement with the results of the current study, previous studies have reported that silymarin could mitigate free radicals production and formation of ROS(9, 11, 22), thus it decreases lipid peroxidation and subsequently MDA levels in blood and tissues. As has been previously reported, extensive oxidation increases the production of unstable compounds known as free radicals (29), which would damage the biological components in the body and lead to lipid peroxidation, protein carboxylation and DNA strand damages, finally causing various clinical consequences (30).Meanwhile, in the treated group, the decreased serum ROS and MDA concentrations confirm the antioxidant activity of silymarin treatment. This phenomenon can be explained by the antioxidant theory. The antioxidant theory states that increased antioxidant proportions decrease lipid peroxidation. Our study proved that silymarin could compensate for antioxidant deficiencies and prevent lipid oxidation under common oxidative stress at the beginning of lactation. It is possible that the silymarin reduces lipid peroxidation by decreasing corticosterone concentration because corticosterone increases lipid peroxidation. Hence, it is recommended that future trials should focus more on exact relations among herbal antioxidant therapy and glucocorticoid secretion in animal models (31, 32).

In the control ewes group, common oxidative stress at the beginning of lactation could attenuate the host system by altering the levels of endogenous antioxidant enzymes in the serum. On the contrary, based on our laboratory results dietary inclusion of silymarin at the dosage of 2000 mg/kg/day, increased the serum concentration of antioxidant machinery including GSH, SOD, and CAT. Basically, the herbal antioxidants like silymarin have beneficial actions on oxidative stress biomarkers. This idea was confirmed by other researchers who indicated that silymarin prevented oxidative changes produced by ROS production and inhibited oxidation of lipoproteins (6, 33).The liver plays a central role in metabolism, modified concentrations for many serum biochemical components are anticipated by supplying antioxidant properties (11).

The oxidative stress at the beginning of lactation is a common cause of liver injury. In this experiment, silymarin increased considerably the activity of liver enzymes (especially ALT), however, the previous study has demonstrated that silymarin supplementation can increase liver enzymes activity(8, 20), which was consistent our results. Because of their hepatoprotective and antioxidant functions, silymarin can sustenance the metabolism of animals during the prepartum period, because these days are the most stressful for dairy cows (13).Conversely, other studies have shown that the types and levels of silymarin did improve the levels of liver enzymes in livestock (34).

The analysis of QRT-PCR showed that silymarin differently decreased the expression of the *HSP70* gene in the serum of Sanjabi ewes in the postpartum period. Over time, the total protein level was increased in the animals of the treatment group. The capability of homeostasis can diminish extracellular damage (35) by changing gene expression in the presence of common stress and recurring to basal conditions (36, 37).The stu-

dies have been reported that some herbal antioxidants like silymarin are a secondary defense system (10). The results approve the role of silymarin in these metabolic pathways; suggesting that silymarin is not only an important antioxidant but also a regulator of *HSP70* gene expression.

The effect of the environment on gene expression has been previously reported by several researchers (38-41).

Collectively, our findings indicated that the enhanced *HSP70* can show to increase the health in animals because it inhibits oxidation in the postpartum period. It means that nutrition can have an important role in changing the heat shock genes expression which influences the quality of the products derived from ruminants through nutrition.

In summary, the data presented here demonstrated that silymarin administration is potentially an effective strategy for modifying the detrimental effects of common oxidative stress at the beginning of lactation of sheep via dual hepatoprotective and antioxidant functions, however, a full understanding of the mechanism by which these herbal extract function is still lacking.

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