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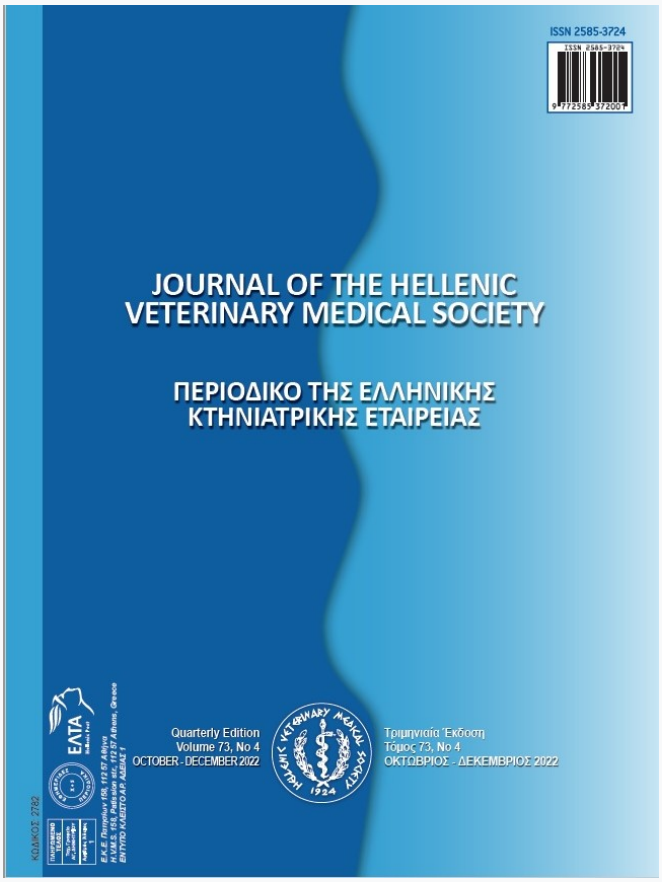

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Research article
Ερευνητικό άρθρο

NRF2 is a potential biomarker for the evaluation of weaning-related oxidative stress in goat kids

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ABSTRACT: Weaning stress is crucial threatening factor in animal breeding. The aim of this study was to investigate the effects of weaning to oxidative stress and stress status of goat kids at the molecular levels. In the study, blood samples were collected at the weaning process (7 days before weaning- Pre-W, Day of weaning- Day-W and 7 days after weaning- Post-W)from 24 healthy Damascus goats. Cortisol, MDA (Malondialdehyde), COX-2 (Cyclooxygenase-2), and NRF2 (Nuclear factor erythroid 2-related factor 2) proteins levels were investigated in plasma, while *COX-2* and *NRF2* genes expression levels were determined from leukocytes. Compared to Pre-W, *COX-2* gene was upregulated almost 2-fold in Day-W. On the other hand, *NRF2* gene expression levels were tended to upregulation in Day-W, and it was upregulated approximately 2-fold in Post-W. While MDA levels were gradually increased in the weaning process, the levels of COX-2 and NRF2 proteins showed similar changes with gene expression trends. Positive correlations were also found between cortisol and COX-2 and NRF-2 protein levels in plasma. It was thought that significant outputs gained in terms of MDA levels in plasma of kids at the weaning process. For the first time, determination of COX-2 and NRF2 levels both mRNA and protein levels in the weaning process of goat kids were satisfactorily evaluated in this study. Particularly, NRF2 was found to have the potential to be a fundamental biomarker to assess the oxidative status of weaning kids.

Keywords: COX-2; NRF2; weaning stress; oxidative stress; Damascus goat

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INTRODUCTION

Milk contains essential nutrients necessary for both the continuation and development of vital activities in the process from birth to weaning in mammals. Although there are various changes between the species, the milk sucking period, which begins with colostrum after birth, ends with the transition to roughage and concentrated feeds (Jasper and Weary, 2002). Weaning in goat breeding is one of the important factors potentially affecting the growth performance and health of kids (Izuddin et al., 2019).

After weaning, carbohydrate, protein and lipid content of the kids changes depending on the ration. In addition, it has been reported that remarkable changes occur in the microbial load of the offspring (Baldwin et al., 2004; Magistrelli et al., 2013; Wüst et al., 2000). Stress, may be defined as the biological response that occurs when a threat to homeostasis is perceived, takes place in offspring as a result of weaning (Kim et al., 2011). In addition to psychological and physiological changes, weaning stress can reduce growth performance and make kids susceptible to disease factors by causing oxidative stress with weakened immune system (Liao et al., 2019; Redondo et al., 2010).These dramatic changes in kids may cause significant economic losses in breeding (Ugur et al., 2007; Liao et al., 2019).

Stress in the organism can be determined by behavioral observations and various parameters. Plasma cortisol levels are used as a stress biomarker for determining the weaning stress (Pol et al., 2002; Bertoni et al., 2005; Magistrelli et al., 2013). However, it has been reported that cortisol levels may vary depending on factors such as season, circadian rhythm and season, as well as weaning stress (Wüst et al., 2000; Magistrelli et al., 2013).On the other hand, it has been suggested in some studies that different molecules that could be markers such as albumin, acute phase proteins and liver function tests should be considered in the control of stress status, rather than only the determination of plasma cortisol levels (Magistrelli et al., 2013; Zobel et al., 2020). However, these parameters have limited potential to be specific due to environmental factors (Magistrelli et al., 2013).

Stress in the organism is an indicator of homeostasis that may impaired due to oxidative stress. The reactive oxygen species (ROS) and free radical production have led to oxidative stress (Acaroz et al., 2019; Izuddin et al., 2020). Higher ROS production in the blood activates antioxidant mechanism of the organism (Galarza et al., 2021). MDA,final products of lipid peroxidation, is major biomarker of oxidative stress (Yakan et al., 2021). The level of MDA is served as a reliable biomarker of lipid peroxidation (LPO) and usually served as a marker of LPO (Acaroz et al., 2018). In addition tothe levels of cortisol and MDA in plasma, COX-2 and NRF2 levels which are the strongly related factors with antioxidant potential of the organism have been investigated at the mRNA and protein levels in Pre-W, Day-W and Post-W in Damascus kids in this study.

MATERIALS AND METHODS

Animals, study design and samples collection

The study was carried out with the permission of Hatay Provincial Directorate of Agriculture and Forestry (Notification date: 12.02.2021) and the approval of Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (Decision no: 2021/02-08). The animal material of the study consisted of 24 healthy Damascus kids in a private farm in Hatay province of Turkey (36° 18' 65.6" North and 36° 18' 63.9" East) located in the Eastern Mediterranean region at an altitude of about 100 m above sea level. The study was conducted in May 2021 and a minimum of 2 m^2 area was arranged for each animal.

The kids were given *ad libitum* dry alfalfa and 100 gr concentrate feed in the Pre-W. Also, water for consumption was always available. The concentrate feed had 88.91% dry matter, 5.96% crude ash, 2.58% crude fat and 16.51% crude protein.

Blood samples were collected under sterile conditions into tubes containing EDTA with a volume of 10 mL from left jugular vein at Pre-W, Day-W and Post-W. The samples were quickly (within about 30 min) transferred to the Genetics laboratory (Veterinary Faculty, Genetics Department) in the cold chain and centrifuged at 3000 xg for 10 minutes at +4°C. Plasma samples obtained after centrifugation were portioned for the determination of cortisol, MDA, COX-2 and NRF2 proteins levels and kept at -80°C until the relevant analysis.

The leukocyte layer formed after centrifugation was collected to new tubes for RNA isolation, and treated with Red Blood Lysis Buffer (Poh et al., 2016). Then the leukocyte pellets were homogenized in approximately 1 ml of TRIzol Reagent (Thermo Fisher Scientific, USA, Cat No: 15596018) in accordance with the manufacturer's recommendations. Ho-

mogenized samples in TRIzol Reagent were stored at -80°C until molecular analysis.

RNA isolation and cDNA synthesis

Total RNA isolation was performed to samples according to the modified trizol method (Rio et al., 2010). The samples were passed through chloroform-isopropyl alcohol and ethyl alcohol steps and the obtained total RNAs were diluted with 30-100 µL of nuclease free water (NFW) according to the pellet size.

RNA quality control was then performed by nucleic acid meter (SMA-1000 Spectrophotometer, Merinton) and agarose gel electrophoresis. After checking the purity (A260/280 ratio), concentration and integrity (control of 28S and 18S rRNA subunits in 1% agarose gel electrophoresis), cDNA synthesis was performed.

DNA digestion was performed to the samples before cDNA synthesis for possible genomic DNA elimination via genomic DNA digestion kit (DNase I, RNase free Thermo Scientific, USA, Cat no: EN0521). Then, cDNA was synthesize dusing RevertAid First Strand cDNA synthesis kit (Thermo Fischer Scientific, USA, Cat no: K1621) and samples were completed to 200 µL with NFW.The protocol in thermal cycler (BioRad T100, USA) was as follows: 10 min at 25°C, 120 min at 37°C and 5 min at 85°C, respectively.

Real-Time PCR application

COX-2,*NRF2* and *ACTB* genes amplifications were performed in Real-Time PCR (Rotorgene Q, Qiagen, USA). *ACTB* was used as housekeeping gene. SYBR Green Dye containing kit (Power SYBR Green PCR Master, Thermo Scientific, USA, Cat no: 4367659) was used for amplification. 10 µL volume sample was used for the reaction and each sample was studied in duplicate. The reaction protocol was as follows in Real-Time PCR: 10 min at 95 °C, followed by 15 s at 95°C, 60 s at 60°C, and 40 cycles. Primers of genes used for the amplification was presented in Table 1.

Table 1. Forward and Reverse primer sequences of studied genes

Genes	Forward and Reverse Sequences	Product Length	Reference
COX-2	F: 5'-GTAGGCCAGGAGGTCTTTGG-3'	142 bp	Yakan et al., (2021)
	R: 5'-GCCTGCTTGTCTGGAACAAC-3'		
NRF2	F: 5'-CCAACTACTCCCAGGTAGCCC-3'	227 bp	Deng et al., (2017)
	R: 5'-AGCAGTGGCAACCTGAACG-3'		
ACTB	F: 5'-CTTCCAGCCGTCCTTCCT-3'	105 bp	Modesto et al., (2013)
	R: 5'-TGTTGGCATACAGGTCCTTTC-3'		

Determination of MDA levels and ELISA application

The levels of MDA from plasma samples were determined spectrophotometrically according to the method of Esterbauer and Cheesman (1990) reported. On the other hand, cortisol, COX-2 and NRF2 levels of samples were determined by ELISA reader (AMR-100, Allsheng, CHINA)at 450 nm via commercial kits (E0021Go, E1375Go, E1376Go, Bioassay Technology Laboratory, respectively).

Statistical analysis

Before performing the statistical analysis, all data were examined for normality and homogeneity of variances. Descriptive statistics were calculated and presented as "Mean ± Standard Error of Mean" and figure. To determine the correlation between plasma cortisol, MDA, COX-2 and NRF2 levels of samples, Pearson correlation coefficient was used. The effect of day of sampling on plasma cortisol, MDA, COX-2 and NRF2 levels were analyzed with linear mixed models. The following model with repeated measures design was: $Y_i = \mu + D_i + e$ Where, Y_i, dependent variable; µ, overall mean; D_i, effect of day of sampling (i = Pre-W, Day-W and Post-W) and *e*, residual error. Animals included in the study were assessed as a random effect, while the day of sampling was assessed as a fixed effect. When a significant difference was revealed, any significant terms were compared by Simple effect analysis with Bonferroni adjustment. Expression levels of *COX-2* and *NRF2* genes were calculated with $2^{-\Delta\Delta Ct}$ method and given as fold change (Livak and Schimittgen, 2001). $P<0.05$ was considered as significant in all analyses. Stata 12/MP4 was used in analysis of the data.

RESULTS

While non-significant increase was detected in plasma cortisol levels were detected in the Day-W and Post-W days, MDA levels gradually increased in the study period ($P<0.05$) (Figure 1).

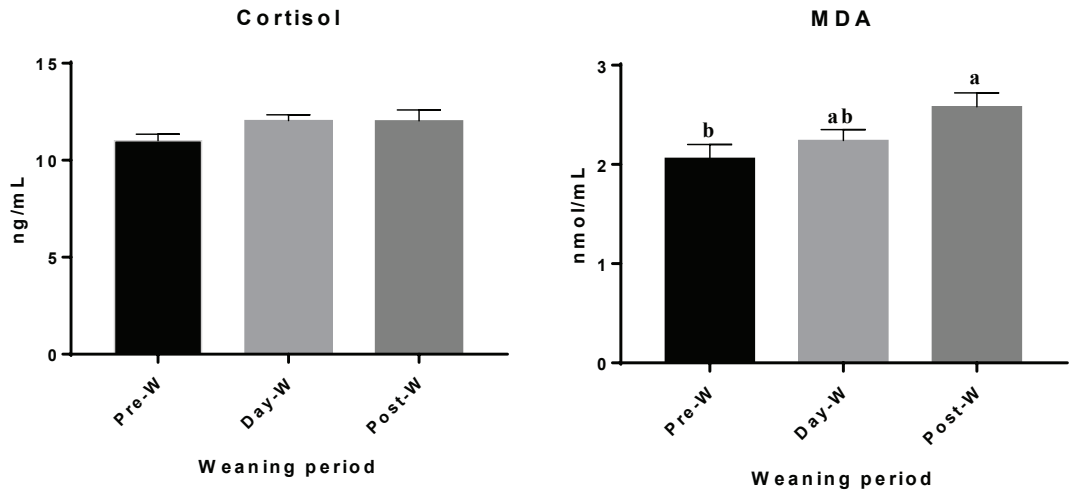

Figure 1. Cortisol and MDA levels in the Pre-W, Day-W and Post-W days in plasma, (Mean±SEM) a,b: Different letters show differences between sampled days

COX-2 gene was expressed almost 2-fold more in Day-W compared to Pre-W. On the other hand, the expression levels of *COX-2* were similar in Pre-W and Post-W. In addition, *NRF2* gene expression levels were tended to upregulation in Day-W and Post-W. Particularly, it was approximately 2-fold upregulated in Post-W ($P<0.05$) (Figure 2).

Protein levels of encoded by *COX-2* and *NRF2* genes in plasma were also found different in the studied period. Similar to gene expression results, while COX-2 protein levels increased in Day-W ($P<0.05$), it was similar in Pre-W and Post-W. In addition, NRF2 levels gradually increased in Day-W and Post-W ($P<0.01$) (Figure 3).

Positive and significant correlations were found between plasma COX-2 and NRF2 protein levels ($P<0.05$). Plasma cortisol levels were also positively correlated with COX-2 and NRF2 ($P<0.05$; $P<0.001$, respectively). On the other hand, negative correlation was found between plasma cortisol and MDA levels ($P<0.05$) (Table 2).

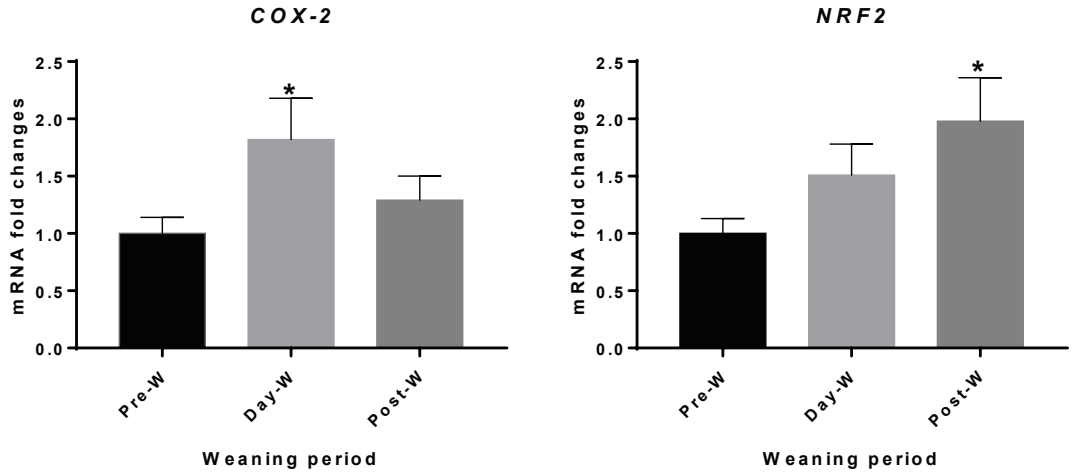

Figure 2. Expression Levels of *COX-2* and *NRF2* Genes, (Mean±SEM), *: $P<0.05$

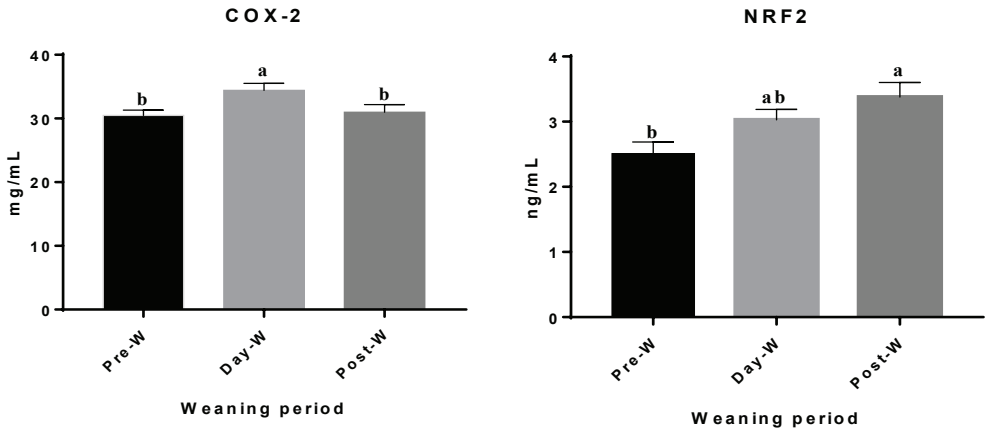

Figure 3. Plasma Levels of COX-2 and NRF2 Proteins (Mean±SEM), a,b: Different letters show differences between sampled days

Table 2. Correlations between plasma cortisol, COX-2, NRF2, and MDA levels

	Cortisol	COX-2	NRF2	MDA
Cortisol (ng/mL)	-			
COX-2 (mg/mL)	0.269*	-		
NRF2 (ng/mL)	0.563***	0.239*	-	
MDA (nmol/mL)	-0.283*	-0.117	-0.095	-

*: P<0.05; ***: P<0.001

DISCUSSION

Lactation is a physiological feature in mammals that begins with colostrum after birth and continues for varying periods of time depending on the species and environmental conditions such as breeding strategies. In goat breeding, the survival and healthy development of the kids, along with other factors, significantly depends on the weaning process. Although it is applied with varying duration and periods in farm animals, weaning is a crucial stress factor (Magistrelli et al., 2013).

Plasma cortisol levels have been reported to increase in early weaning period in goat kids (Redondo et al., 2010; Katoh et al., 2005). On the other hand, the animals used in this study were weaned approximately 3.5 months later after the births and plasma cortisol levels have been detected similar during the weaning process. In addition to the period from birth to weaning, it has been thought that the season may be effective in the formation of cortisol activity. There is a significant relationship between the hypothalamus-pituitary-adrenal axis and plasma cortisol levels (Wüst et al. 2000; Redondo et al., 2010). It has been reported that the activation of this axis may change depending on the melatonin activity in kids (Redondo et al., 2010). While it has been mentioned in some studies that plasma cortisol levels increased because of weaning stress (Redondo et al., 2010; Kim et al., 2011), Magistrelli et al. (2013) have been reported that cortisol levels might have the variation because of circadian rhythm and physiological status of animals and might led bias.

Oxidative stress is one of the leading factors causing various disease and disorders in mammals. It has been reported that limited information has known about the MDA levels in the area of animal breeding and veterinary science (Yonny et al., 2016; Liguori et al., 2018; Sies 2018). Determination of MDA levels in goat kids plasma at the weaning process can be a potential contribution to assess the evaluation of the health status. MDA, a crucial indicator of oxidative stress, have gradually increased in weaning period in this study. It has been reported that MDA levels in plasma might increase in weaning process depending oxidative stress (Tao et al., 2016; Yin et al., 2014). Even though MDA level of Day-W have been found similar both Pre-W and Post-W days, it has increased in Post-W. Similar to our study, it has been reported that MDA levels of plasma had tended to increase in piglets after weaning (Tao et al., 2016). Also, Ognik

et al. (2017) has been stated that oxidative stress increases short after weaning in lambs.

The oxidative state of the organism, like many other parameters, is under the control of molecular regulation.COX-2, is a gene controlled by more than 20 transcription factors and whose activity is increased due to conditions such as oxidative stress and inflammation (Luo et al., 2011).It has been reported that COX-2 in leukocytes increases in the level of mRNA and protein in the case of oxidative stress (Barbieri et al., 2003).*NRF2* activity, on the other hand, increases the transcription levels of many antioxidant genes. When the increased activity of this transcription factor, which plays a primary role in the response to oxidative stress that may occur, is evaluated together with COX-2, it is understood that weaning causes the oxidative stress in kids (Yakan et al., 2021).

NRF2 is involved in the reduction of oxidative stress by binding to the promoter regions of antioxidant response elements in case of oxidative stress. It is largely responsible for the regulation of oxidative stress-related genes such as CAT, SOD, GSTα1, NQO1 and HO-1 in inflammation cases characterized by the increase in free radicals (Jin et al., 2016; Deng et al., 2017).It has been reported that inflammation and oxidative damage can be reduced as a result of upregulation of genes involved in the antioxidant system by triggering NRF2, which has a dominant effect on antioxidant activity in monocyte and macrophage cells in case of oxidative stress in goats (Deng et al., 2017). The varying activity of this gene due to oxidative stress has been studied in many tissues and organs in a large number of animal species such as cattle, rats, sheep and goats (Gessner et al., 2013; Kerasioti et al., 2016; Memon et al., 2019). However, in the studies conducted, the activity of this gene was evaluated mainly in cases that resulted in pathological changes. To the best of our knowledge, NRF2has been investigated for the first time at both mRNA and protein levels in weaning process in goat kids.

In addition to gene expression results and protein levels of COX-2 and NRF2, significant and positive correlations have been detected between plasma cortisol levels with plasma COX-2 and NRF2 protein levels in the study. On the other hand, these two studied genes have positively correlated as expected. Interestingly, negative correlation has been found between MDA and cortisol levels in plasma. The factors that effect on the variation of the cortisol has been thought to be responsible of this result (Magistrelli et al., 2013).

CONCLUSIONS

In conclusion, it has been thought that significant outputs have been gained in terms of MDA levels in plasma of kids at the weaning process. For the first time, determination of COX-2 and NRF2 levels both mRNA and protein levels in the weaning process of goat kids are satisfactorily evaluated in this study. Particularly, NRF2 has been found to have the potential to be a fundamental biomarker to assess the oxidative status of weaning kids.

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CONFLICT OF INTEREST

The article authors declare that they have no conflict of interest.

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