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Original Article
Özgün Araştırma

Changes in oxidative stress parameters in ear-tagged calves in acute period

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Abstract: This study aimed to investigate the effects of ear-tagging on blood malondialdehyde (MDA), reduced glutathione (rGSH), Vitamin C (Vit C) levels, and glutathione peroxidase (GPx) activity in the acute period. Ear-tagged (with polyurethane ear-tag) animals consisted of nine healthy Holstein calves (2-3 months old). Blood samples were taken just before ear-tagging (baseline), at the 20th min, on the 3rd day, and 8th day after the ear-tagging procedure. Six Holstein calves were involved the sham group. In ear-tagged animals, plasma MDA levels increased on the 3rd day compared to baseline values ($p<0.01$) and returned to baseline values on the 8th day, whereas no change was found in the sham group. In ear-tagged animals, plasma rGSH levels increased at the 20th min, 3rd day, and 8th day ($p<0.001$) in comparison to baseline values, which in the sham group, there was no change. In ear-tagged animals, erythrocyte rGSH levels on the 3rd and 8th days were higher compared to baseline and 20th min level ($p<0.01$). In the sham group, the 3rd and 8th day erythrocyte rGSH levels increased compared to baseline values ($p<0.01$). In ear-tagged animals, the 20th min, 3rd day, and 8th day erythrocyte GPx activities were lower than the baseline values ($p<0.001$), whereas in the sham group, no change was observed. While Vit C levels increased progressively in the sham group ($p<0.05$), this increase was not observed in the ear-tagged group. In conclusion, ear-tagging was found to cause oxidative stress and increase antioxidant requirement in calves.

Keywords: Calf, ear-tagging, oxidative stress, polyurethane ear-tag

Kulak küpesi takılan buzağılarda akut dönemde oksidatif stres değişimleri

Özet: Bu çalışmanın amacı kulak küpelemenin akut dönemde kan malondialdehid (MDA), redükte glutatyon (rGSH), vitamin C (Vit C) düzeyleri ile glutatyon peroksidaz (GPx) aktivitesi üzerine etkilerinin araştırılmasıdır. Çalışmanın deney grubunu, kulak küpeleme (poliüretan kulak küpesi ile) yapılan dokuz adet buzağı (2-3 aylık yaşta) oluşturdu. Kan örnekleri kulak küpeleme işleminden hemen önce (temel seviye), kulak küpeleme sonrası 20. dakika, 3. gün ve 8. gün'de alındı. Ayrıca, belirtilen zamanlarda sadece kanları alınan altı adet Holstein ırkı buzağı da sham grubu olarak kullanıldı. Kulak küpeli buzağılarda 3. gün MDA seviyeleri temel seviyelere göre yükselirken ($p<0,01$), 8. günde tekrar temel seviyelerine indi. Sham grubunda ise MDA düzeyleri yönünden bir değişiklik gözlenmedi. Kulak küpeleme grubunda plazma rGSH seviyeleri 20. dakika, 3. gün ve 8. günde temel düzeylere göre arttı ($p<0.001$); ancak, sham gurubunda bir değişim gözlenmedi. Kulak küpeli buzağılarda eritrosit rGSH seviyeleri 3. ve 8. günlerde, 20. dakika ve temel seviyelere göre yüksek olarak belirlendi ($p<0.01$). Sham grubunda ise 3. ve 8. gün eritrosit rGSH seviyeleri temel seviyelere nazaran yüksek olarak belirlendi ($p<0.01$). Kulak küpeleme grubunda eritrosit GPx aktiviteleri 20. dakika, 3. gün ve 8. gün'lerde temel seviyelere kıyasla düşük ($p<0.001$) olmasına rağmen, sham grubunda bir değişim göstermedi. Sham grubunda Vit C seviyeleri giderek artmasına rağmen ($p<0.05$), kulak küpeli hayvanlarda bu artış görülmedi. Sonuç olarak kulak küpelemenin buzağılarda oksidatif strese neden olarak antioksidan ihtiyacı arttırdığı düşünüldü.

Anahtar kelimeler: Buzağı, kulak küpeleme, oksidatif stres, poliüretan kulak küpesi

Introduction

According to European legislation, identification and registration of livestock such as cattle, sheep, goats, and pigs are obligatory to observe animal diseases and behavior in order to improve veterinary practices. This implementation will enable earlier detection of diseases, control fertility, and yield changes resulting in improved animal welfare and efficacy (Ammendurp and Fussel 2001; Neethirajan 2017; Bailey et al. 2018; Vazquez-Diosdado et al. 2019). There are three different methods used around the globe for identification, registration

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and, traceability of livestock animals: 1-stamping, ear marking (notching, tattooing, and tagging), 2-electronic identification (subcutaneous injection, inside ear-tag, bolus, accelerometer), 3-Biometric methods (DNA and retinal image) (Caja et al. 2004; Thomson et al. 2016; Krieger et al. 2017, 2018). Due to its low cost in comparison to other methods, ear-tagging is currently the most widely used method around the world for individual identification of animals (Petherick 2005; Aydın 2007; Lomax et al. 2017). Also, accelerometers and radio frequency identifications are attached via ear-tags (Doğan et al. 2016; Krieger et al. 2018). Ear-tags can be present in many different shapes (rosette, button, hoop, etc.), sizes and colors, and can be made from various materials (metal, plastic) (Aydın 2007). After determining that the metal ear clips that have been used in the past could cause some lesions (Kitagaki and Hirota 2007), flexible plastic ear-tags made of polyurethane have been shown to be less harmful (Meingassner 1991; Kitagaki and Shibuya 2004) are used more commonly, today. Although studies have reported less harm with the use of plastic ear-tags compared to metal tags, plastic ear-tags have also been shown to cause some changes including ear lesions (Johnston and Edwards 1996; Edwards and Johnston 1999), ear pain (Leslie et al. 2010; Lomax et al. 2017), tachycardia (Stewart et al. 2013), and ear wounds and necrosis (Salina et al. 2016). Animal welfare targets a life free from undesired emotions such as pain, suffering, and stress (Bryant 1972; Dantzer 2001), whereas lack of stress response is regarded as an indicator of an animal's well being (Barnett and Hemsworth 1990; Broom 1998). Stress can be expressed as a biological response in the form of anatomical, physiological, and behavioral changes to internal or external stimuli that threaten the homeostasis of the organism. The response against stress may cause various effects in the organism such as changes in the immune system, problems with digestion, and cessation of reproductive and developmental functions (Kelley 1980). Stress may result in the disturbance of oxidant-antioxidant balance in favor of free radicals (Freeman and Crapo 1982). Free radicals show their effects by causing oxidation of proteins, lipids, carbohydrates, and DNA, leading to pathological changes in cell membranes, cellular organelles, and DNA. As a result, cellular dysfunctions or death occur, or resulting mutations can lead totumourigenesis (Akkuş 1995). An antioxidant is defined as a substance that significantly delays or inhibits the oxidation of a substrate. The physiological role of antioxidants is to prevent damage to cellular components that occur as a result of chemical reactions involving free radicals (Young and Woodside 2001). The imbalance between oxidants and antioxidants results in the formation of reactive oxygen species and leads to oxidative damage. This condition is defined as oxidative stress (Kurutaş et al. 2004; Yeum et al. 2004). In animals, major antioxidant defense mechanisms include the enzymes glutathione peroxidase, catalase and superoxide dismutase, and glutathione. Malondialdehyde levels provide information about lipid peroxide levels (Akkuş 1995). Glutathione is a low-molecular weight tripeptide synthesized from the amino acids cysteine, glutamic acid, and glycine, and it is the most important soluble antioxidant in the body (Kohen and Nyska 2002). The most effective antioxidant enzyme in the protection mechanism against lipid peroxidation in erythrocytes is GPx (Kehrer 1993; Akkuş 1995). Decreased GPx activity leads to increased hydrogen peroxide and severe cell damage (Halliwell and Gutteridge 1989). Together with α-tocopherol and Vit C (ascorbic acid), GPx suppresses the free radicals attacking biological membranes and tissue lipids. Vitamin C is a good antioxidant because of its strong reducing activity (Banudevi 2006).

Ear-tags used in the identification of livestock animals are foreign to the natural life of the animals, and therefore they are thought to induce stress, affecting well being of the animals. In our review of the literature, we observed that while some stress factors have been investigated for their stress-inducing potential (Avcı et al. 2008; Fidan et al. 2009; Çetin et al. 2011), no previous study investigated oxidative stress development due to ear-tagging in calves. In the present study, it was aimed to examine possible effects of flexible ear-tags made of polyurethane material on oxidative stress in 2-3 months old calves.

Material and Method

Materials

In the study, experimental and sham groups consisted of nine healthy Holstein calves (four males + five females) and six healthy Holstein calves (two males + four females) from a private dairy cattle farm, respectively. The calves were aged about 2-3 months old, and were not ear-tagged previously. Animals were kept in the same care and feeding conditions. The study protocol was approved by the Local Ethical Committee of Experimental Animal Ethics of Hatay Mustafa Kemal University and was performed entirely according to ethical rules (Approval no: 2014-04/1).

In the experimental group (Ear-tagged with polyurethane ear-tags, Figure 1), blood samples were properly drawn from the jugular vein of the calves into tubes containing EDTA, just before ear-tagging (baseline values). Afterward, blood samples were collected from the same animals at 20 minutes (20th min), 3rd day, and 8th day after the ear-tagging procedure. For the sham group, in order to examine the effect of stress caused by the blood drawing procedure, blood samples were drawn with the same intervals specified above from the calves which did not undergo the ear-tagging procedure. Blood samples were properly centrifuged (3000 rpm x 10 minutes x +4°C) in order to obtain plasma samples. After separating the plasma portion of the samples, the remaining cellular portion was washed three times with PBS solution. Consequently, one unit of erythrocyte suspension was mixed with five units of distilled water in order to obtain erythrocyte hemolysate. Plasma and erythrocyte hemolysate samples were then stored at -20°C until the biochemical analyses.

Methods

Reduced GSH activity in the erythrocyte hemolysate was measured using the method described by Ellman (1959) as a dithionitrobenzoic acid recycling assay. Erythrocyte hemolysate GPx activity was determined by Beutler's (1975) method, which is based on detection of the change in absorbance at 340 nm using hydrogen peroxide in the presence of NADPH. Erythrocyte hemoglobin levels were determined using Drabkin's (Sigma) solution. Plasma MDA levels were determined by the method described by Ohkawa et al. (1979), which is based on spectrophotometric detection of the formation of the pink-colored complex from MDA and tribarbutyric acid at 532 nm. Analysis of plasma Vit C was performed using Haag's (1985) method based on the formation of 2,4-dinitrophenylhydrazone following reaction of dehydroascorbic acid and diketogulonic acid with 2,4-dinitrophenylhydrazine (DNPH), and spectrophotometric detection of this product was at 520 nm.

Figure 1. Ear-tags used in the study.

Statistical Analysis

All the values are expressed as mean ± SE. The results of the groups were analyzed by ANOVA with posthoc Duncan multiple range tests (SPSS for Windows, release 12.0). In all cases, the probability of error of less than 0.05 was chosen as the criterion of statistical significance.

Results

Plasma and erythrocyte oxidative stress parameters in Ear-Tag and control groups are given in Table 1 and 2.

Table 1. Plasma and erythrocyte oxidative stress parameters in ear-tagged group (Mean±SE), (n=9).

Ear-Tagged group	MDA nmol/ml	rGSH µM plasma	rGSH µmol/ g hg	GPx U/g hg	Vit C mg/dl
Baseline	1.41 ± 0.06 ^{bc}	33,25±3,40^{a}	2.60 ± 0.53 ^{a}	71,94 ± 1.93 ^{a}	0.45 ± 0.04 ^{ab}
20th min	1.64 ± 0.08 ^{ab}	43,76 ± 3.26 ^{b}	2.75 ± 0.29 ^{a}	36,75 ± 3.08 ^{b}	0.32 ± 0.04 ^{a}
3rd day	1.81 ± 0.12 ^{a}	48,57 ± 2.22 ^{bc}	4.85 ± 1.07 ^{b}	39.68 ± 4.96 ^{b}	0.37 ± 0.04 ^{ab}
8th day	1.36 ± 0.10 ^{c}	52.12 ± 1.52 ^{c}	6.94 ± 0.66 ^{b}	37.11 ± 5.11 ^{b}	0.48 ± 0.08 ^{b}
p	<0.01	<0.001	<0.01	<0.001	<0.05

a, b, c, d-values with different letters in the same column are statistically significant (p < 0.05).

Table 2. Plasma and erythrocyte oxidative stress parameters in the sham (non-ear-tagged) group (Mean±SE), (n=6).

Sham Group	MDA nmol/ml	rGSH µM plasma	rGSH µmol/ g hg	GPx U/g hg	Vit C mg/dl
Baseline	0.38± 0.04	34.90± 4.73 ^{ab}	0.56± 0.23 ^{a}	60.30± 8.44	0.53± 0.07 ^{a}
20th min	0.38 ± 0.06	30.73±1.88 ^{a}	0.74 ± 0.32 ^{ab}	62.18 ± 3.35	0.68 ± 0.12 ^{ab}
3rd day	0.45 ± 0.11	41,15 ± 2.04 ^{b}	1.85 ± 0.48 ^{b}	69,85 ± 2.20	0.80 ± 0,20 ^{ab}
8th day	0.59 ± 0.02	36,98 ± 3.37 ^{ab}	3.32 ± 0.44 ^{c}	62,64 ± 1.44	1.10 ± 0.26 ^{b}
p	-	<0.05	<0.01	-	<0.05

a, b, c, d-values with different letters in the same column are statistically significant (p < 0.05).

In ear-tagged animals, plasma MDA levels were found to be increased on the 3rd day compared to the baseline values ($p < 0.01$), whereas MDA levels returned to the baseline values on the 8th day. On the other hand, non-ear-tagged animals (sham group) did not show any change in serum MDA levels.

In ear-tagged animals, plasma rGSH levels were found to be increased at 20th min, 3rd day, and 8th day ($p < 0.001$) in comparison to baseline values. In non-ear-tagged animals, plasma rGSH levels at 20th min, 3rd day, and 8th day were not significantly different compared to the baseline values. However, 3rd day plasma rGSH levels were higher than 20th min levels ($p < 0.05$). In ear-tagged animals, erythrocyte rGSH levels on the 3rd and the 8th days were higher compared to baseline and 20th minute levels ($p < 0.01$). In non-ear-tagged animals, 3rd day and 8th day erythrocyte rGSH levels were increased compared to baseline values ($p < 0.01$).

In ear-tagged animals, 20th min, 3rd day, and 8th day erythrocyte GPx levels were lower than the baseline values ($p < 0.001$), whereas in non-ear-tagged animals, erythrocyte GPx levels measured at the same time intervals did not change significantly.

Plasma Vit C levels did not show a significant change in ear-tagged animals. In non-ear-tagged animals, 20th min, 3rd day, and 8th day plasma Vit C levels were higher than the baseline; however, the difference was only significant on the 8th day. ($p<0.05$).

Discussion

In the present study, we investigated the effects of ear-tagging on oxidant/antioxidant equilibrium in calves by measuring plasma MDA, rGSH, Vit C, and erythrocyte rGSH and Gpx. Measurement of thiobarbituric acid reactants such as MDA, conjugated dienes, and lipid hydroperoxides is commonly used in the determination of cellular damage caused by oxidative stress (Carr et al. 1995). In the present study, while plasma MDA levels were increased on the 3rd day of ear-tagging compared to the baseline values, this increase was not observed in non-ear-tagged animals. To our knowledge, the effects of ear-tagging on MDA levels were not reported. As transport stress has been shown to increase serum MDA levels in cattle (Chirase et al. 2004), we may conclude that the ear-tagging procedure causes stress during the first days of the procedure, because on the 8th day MDA levels returned to the baseline values.

Glutathione peroxidase is a highly effective antioxidant enzyme against oxidative stress in erythrocytes. It functions in reduction of the hydrogen peroxide and organic hydroperoxides by oxidizing the rGSH in the cell. Reduced GPx activity leads to increased hydrogen peroxide and severe cell damage (Halliwell and Gutteridge,1989; Dundar and Aslan 2000; Çetin et al. 2011). In the present study, while erythrocyte GPx activity did not change in the sham group, it was found to be significantly reduced at 20th min, 3rd day, and 8th day in the ear-tagged animals when compared to the baseline values ($p<0.001$). Also, the findings of the present study indicated that following ear-tagging, rGSH levels (both in plasma and erythrocyte) were increased. Reduced GSH is transformed into oxidized GSH during scavenging of free radicals by GPx (Akkuş 1995). In ear-tagged animals, reduced GPx activity in the erythrocytes may have caused decreased rGSH utilization, and thereby, resulted in increased intracellular rGSH levels. Erythrocyte lysis has been shown as a source of antioxidants found in the plasma. Decreased erythrocyte GPx activity and increased MDA level have been reported to be responsible for the increased plasma GPx activity (Ines et al. 2006; Öztürk et al. 2008). In the present study, plasma rGSH levels are thought to be increased as a result of reduced erythrocyte GPx activity and increased MDA levels in parallel with erythrocyte lysis. In the sham group, there was also an increase in erythrocyte rGSH levels, however, unaltered erythrocyte GPx, plasma MDA, and plasma rGSH levels suggest that the oxidative stress was less intense in the non-ear-tagged animals.

Vitamin C is synthesized in the liver in ruminants. In calves, plasma Vit C levels vary according to developmental stages (Bouda et al. 1980). In the present study, Vit C levels increased progressively in the sham group (statistically significant difference was observed on the 8th day). This increase may be attributed to the Vit C content of feed given to the animals during the study. On the other hand, serum Vit C increase was not observed in the ear-tagged group which maybe because of the consumption of Vit C due to the increased oxidative stress in the ear-tagged animals. Plasma Vit C levels have been shown to decrease following oxidative stress in the body (Polidori et al. 2001; Weiss 2001), and Vit C supplementation as an antioxidant in the diet has favorable effects on stress parameters in sheep that were subjected to transport stress (Avcı et al., 2008).

Today, as a result of the developments in the field of biotechnology, non-invasive animal identi-

fication methods (DNA and retinal image) are being developed, but due to the high cost of these techniques, ear-tagging still maintains its popularity. Analgesic use during the ear-tagging procedure has been shown to reduce pain in the animals (Lomax et al. 2017); therefore, it may also reduce the stress associated with ear-tagging as well. A recent study on the identification of sheep using leg tagging method (Abacia and Palacin 2014) has shown that leg tags could be used conveniently in different stages of development in sheep.

In conclusion, ear-tagging was found to cause oxidative stress and increase antioxidant requirement in calves. For that reason, we believe it would be beneficial to provide supplementation of Vit C and other agents known to have beneficial effects on stress before and/or during ear-tagging, and also to use analgesic and antiinflammatory agents during ear-tagging.

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