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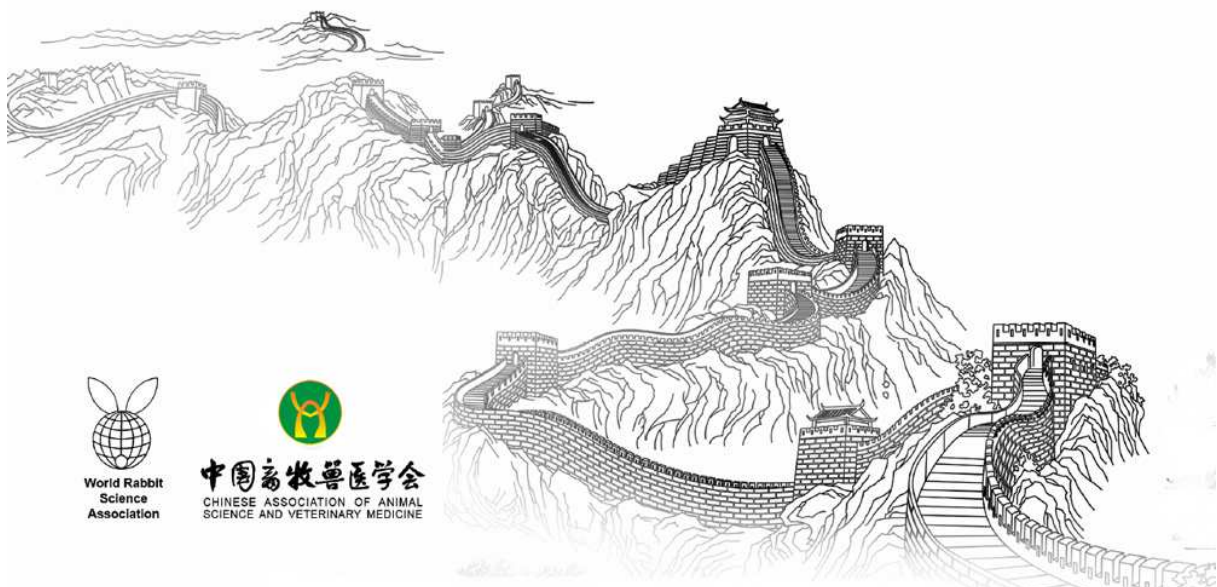
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CHANGES OF STRESS INDICATORS IN DIFFERENT MATRICES IN GROWING RABBITS BEFORE AND AFTER TRANSPORT

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ABSTRACT

The present study aimed at evaluating the change of glucocorticoids (cortisol and corticosterone), and oxidative and protein markers (malondialdehyde, MDA and heat shock protein 70, HSP70) as stress indicators in 31 growing rabbits before and after 1-h transport in collective cages from farm to slaughterhouse by a commercial truck. In order to identify tissues suitable for stress marker measurements and easy to be taken, different matrices were sampled: blood and hair for measuring glucocorticoid levels, and muscle and liver for MDA concentration and HSP70 expression. The stress status of rabbits was weakly affected since only plasma cortisol (4.58 to 6.29 ng/ml; $P < 0.05$) and liver HSP70 expression (100777 to 166132 pixel; $P < 0.001$) significantly increased after transport. The corticosterone and cortisol levels in hair were successfully measured, but did not change with transport. In fact, this matrix could be useful for long term stress status evaluation. In conclusion, under our conditions, the stress status of rabbits was not affected to a large extent by transport. Liver HSP70 expression resulted a suitable candidate tool for measuring stress in rabbits.

Key words: Animal welfare, Growing rabbits, Stress indicators, Transport.

INTRODUCTION

Relationships among animal welfare and product quality have been largely studied in several species for which a large impact of stress on meat quality is recognized (Schwartzkopf-Genswein et al., 2012). In growing rabbits, some information is available on the effects of transport, lairage or loading conditions on animal stress and meat quality (Cavani et al., 2009) and, surely, transport *per se* may be a stressing event as measured by means of primary and secondary stress indicators in blood (De la Fuente, 2007; Mazzone et al., 2010; Fazio et al., 2015). Among stress indicators, oxidative variables like malondialdehyde (MDA) and proteins like heat shock protein (HSP) may be measured. In fact, MDA level is widely used as a blood indicator of lipid peroxidation (Del Rio et al., 2005). The HSP are ubiquitous and highly conserved proteins in all organisms, which expression, in particular that of HSP70, increases under stressful conditions since they fold and unfold protein substrates during stress cell damage (Feder and Hofmann, 1999).

Among matrices, hair, feces and muscle/meat could be easier than blood to be sampled and may avoid the influence of sampling procedure on stress status. Cortisol and corticosterone concentrations have been successfully measured in hair and hard feces (Buijs et al., 2011; Comin et al., 2012; Prola et al., 2013; Trocino et al., 2014) of growing rabbits and rabbit does. Besides, MDA and HSP70 have been measured in muscle and organs of different species kept for farming purposes to evaluate the effect of transport or rearing systems (Poltronieri et al., 2007; Negrato et al., 2013; Xing et al., 2015).

The objective of the present study was to measure primary (cortisol and corticosterone) and secondary (MDA, HSP70) stress biomarkers in plasma, hair, muscle and liver of growing rabbits before and after transport from

farm to slaughterhouse in order to evaluate the effect of transport on their levels and to investigate the relationships among them, besides comparing tissue matrixes.

MATERIALS AND METHODS

Animals and samples collection

Rabbits were reared at the experimental facilities of the University of Padova from 34 d until 74 d of age into collective open-top wire-net pens (1.28 x 0.78 m, i.e. 1.00 m²) at a stocking density of 16 animals/m² and during the months of January-March. In the stable, temperatures varied between 18°C and 24°C and the rabbits were submitted to a natural photoperiod. The day before commercial slaughter, 15 rabbits were used for sampling stress biomarkers (group BEFORE transport). The following day, all the rabbits present in the experimental farm were loaded in transport plastic cages (0.50 x 1.0 x 0.30 m; 6 rabbits per cage) after about 6 hours of fasting and transported for about one hour to a commercial slaughterhouse by a commercial truck authorized for animal transport. External environmental conditions were 4-6°C for temperature and 80% relative humidity. Slaughtering took place after approximately 45 min of lairage; animals were stunned by electro-anaesthesia followed by jugulation according to the current practice of the slaughterhouse; hot commercial carcasses were refrigerated during 2.5 h at 3-4°C. A total of 16 rabbits was used for sampling physiological stress indicators at the commercial slaughterhouse (group AFTER transport). Before slaughter, rabbits of the two groups (BEFORE and AFTER transport) were used to sample blood by the ear vein and hair by gentle pulling from the back. After carcass refrigeration, 5 g were sampled from hind leg muscle and liver of each carcass. After collection, blood was immediately centrifuged and plasma and tissue samples were stored at -80°C until analyses. All experimental procedures and animal care were performed in accordance with Italian legislation on the protection of animals used for experimental and other scientific purposes (LD-26/2014).

Analyses of physiological indicators of stress

The glucocorticoid levels in plasma and hair were measured by microtitre radioimmunoassays (RIAs) using species-specific antibodies (Biogenesis, Poole, England, UK; Analytical Antibodies Bologna, Italy) as detailed by Simontacchi et al. (2009) and validated for rabbits. Glucocorticoids were extracted from the hair as described by Trocino et al. (2014) and from the plasma according to Bertotto et al. (2010). To validate RIAs in plasma and hair, parallelism, recovery and intra and inter-assay precision tests were performed: optimal parallelism between standard and diluted extract curves and reproducibility (CVs <0.10%) were obtained; extraction efficiency reached over 70%. The MDA concentration in muscle was assayed by measuring thiobarbituric acid-reactive substances (T-BARS) as described in Pascoli et al. (2011). The HSP70 expressions in muscle and liver were measured by Western Blot followed by densitometric analysis as detailed by Negrato et al. (2013) after protein concentration evaluation by the BCA Protein Assay Kit (Thermo Pierce, IL, USA).

Statistical Analysis

Individual data of stress biomarker levels in the different matrices were analysed by ANOVA with transport group as the main factor by using the PROC GLM of SAS (Statistical Analysis System, Inc., Cary, NC, USA). Differences between the means with $P \leq 0.05$ were accepted as statistically significant differences. Thereafter, the same data were submitted to the PROC CORR of SAS.

RESULTS AND DISCUSSION

Under our transport conditions (1-h transport during winter) and with rabbits reared in collective systems, plasma cortisol concentration significantly increased after transport (4.58 to 6.91 ng/ml; $P < 0.05$), whereas corticosterone concentration was not affected (Table 1). In fact, large increases in plasma cortisol in rabbits at slaughter had been previously measured in rabbits exposed to heat stress before slaughter (9.33 to 28.8 µg/dl, $P < 0.05$; De la Fuente et al., 2007) or transported and slaughtered in summer (34.7 ng/ml in summer vs. 19.5 ng/ml in winter, respectively; De la Fuente et al., 2004). Differently from our results, other Authors (Mazzone et al., 2010) had found serum corticosterone concentration to double in growing rabbits as a consequence of transport (6.23 to 14.88 ng/ml; $P = 0.001$). In hair, as expected, glucocorticoid levels remained stable before

and after transport (Table 1). In fact, they slowly accumulate in this matrix which could be rather useful to measure any stressful condition during the rearing period (Trocino et al., 2014). As what regards secondary stress indicators, only HSP70 expression in liver significantly increased in rabbits after transport (100777 to 166132 pixel; $P < 0.001$) (Table 1). To our knowledge, the only data available on HSP expression in rabbits reported an increase of HSP70 expression in the encephalic tissue of animals submitted to hypoxic conditions (Wang et al., 2006). As what concerns other species kept for meat production, the HPS70 muscle or liver expression increased in poultry and pig after transport (Zhang et al., 2012; Xing et al., 2015)

Table 1: Stress biomarkers in growing rabbits before and after transport to the slaughterhouse

	Transport group		Prob.	MSE
	Before	After		
Rabbits, no.	15	16		
Plasma corticosterone (ng/ml)	9.22	8.89	0.87	5.51
Hair corticosterone (ng/g)	7.78	6.91	0.19	1.81
Plasma cortisol (ng/ml)	4.58	6.29	0.04	2.22
Hair cortisol (ng/g)	0.75	1.11	0.13	0.64
Muscle MDA (μ M/g)	29.8	28.2	0.87	23.9
Muscle HSP70 (pixel)	64841	67019	0.81	21107
Liver HSP70 (pixel)	100777	166132	<0.001	27822

Few significant correlations were found among biomarkers and only within the same matrix: in details, corticosterone concentration was moderately correlated with cortisol level in plasma ($r=0.52$; $P < 0.01$) and in hair ($r=0.40$; $P < 0.05$) (Table 2). Besides, plasma cortisol concentration tended to be correlated with liver HSP70 expression ($r=0.36$; $P=0.09$). To our knowledge, no correlations among these biomarkers have been previously reported in rabbits or in other species kept for meat production. Only in fish (common carp, rainbow trout and European seabass), cortisol levels in different matrices (plasma, mucus, gut content, muscle and fin) resulted highly correlated in animals submitted to transport (Bertotto et al., 2010).

Table 2: Correlation coefficients among stress biomarkers (P -value between parenthesis).

	Plasma corticosterone (ng/ml)	Hair corticosterone (ng/g)	Plasma cortisol (ng/ml)	Hair cortisol (ng/g)	Muscle MDA (μ M/g)	Muscle HSP 70 (pixel)	Liver HSP 70 (pixel)
Plasma corticosterone (ng/ml)	-						
Hair corticosterone (ng/g)	-0.05 (0.79)	-					
Plasma cortisol (ng/ml)	0.52 (<0.01)	0.01 (0.99)	-				
Hair cortisol (ng/g)	-0.14 (0.44)	0.40 (<0.05)	-0.05 (0.80)	-			
Muscle MDA (μ M/g)	-0.23 (0.26)	0.05 (0.83)	-	0.05 (0.82)	-		
Muscle HSP70 (pixel)	0.03 (0.89)	0.06 (0.79)	0.02(0.92)	0.17 (0.43)	0.32 (0.20)	-	
Liver HSP70 (pixel)	0.05 (0.83)	-0.19 (0.38)	0.36 (0.09)	0.33 (0.12)	-0.22 (0.37)	-0.17 (0.44)	-

CONCLUSIONS

Given the results, the present transport conditions (1-h transport, low density in the transport cage, cool temperature) were likely too mild to affect the animal stress status of rabbits reared in collective systems to a large extent. In fact, blood cortisol level and liver HSP70 expression showed significant increases after transport, whereas plasma corticosterone and oxidative stress indicators did not change. Out of the tested matrices, hair proved to be unsuitable for measuring acute stress, and, out of measured biomarkers, liver HSP70 expression appeared to be a suitable candidate for measuring acute stress. More severe conditions of transport, more oxidative stress indicators and other matrices should be used to confirm our findings.

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