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NATIVE IMMUNITY AND OXIDATIVE TRAITS OF GROWING RABBITS

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Abstract: The evaluation of animal welfare through innate immunity (Serum Bactericidal Activity – SBA, Hemolytic Complement Assay – HCA, lysozyme) and the antioxidant status of the body (Reactive Oxygen Substances – ROS and Antioxidant Power of plasma, AP) offers a reliable prognostic and diagnostic tool. The aim of the present study was to investigate trends and correlations between some traits of innate immunity and the oxidative status of fattening rabbits at different ages. Blood samples from 120 New Zealand White fattening rabbits at 45, 55, 65, and 75 d of age were collected and analyzed. The results showed that SBA did not have a normal distribution because of numerous 0 values. Data distribution was normal when only SBA > 0 values were considered. Lysozyme (mean value 27.19 µg/mL) and HCA (mean value 50.84 $CH_{50\%}$) had stable trends at different ages and showed a tendency that was comparable to that obtained in other animal species. On the contrary, SBA (mean value 42.15%) showed an unexpected positive correlation with lysozyme (P<0.001) and a negative correlation with HCA (P<0.001). Oxygen free-radicals are involved in the pathogenesis of several diseases and oxidative stress alters immune competence. In this experiment, ROS and AP showed mean values of 0.60 mmol H_2O_2 and 421.67 µmol HClO, respectively. In this context positive correlation coefficients between oxidative status traits and immune traits (P<0.001) were found, although at a very low level; and surprisingly, only ROS and SBA did not show any significant correlation. In this study it emerged that, even in the absence of evident pathologies, the immune and oxidative traits of fattening rabbits could be affected by environmental stress (weaning, cage, neighbors).

Key words: rabbit, welfare, immunity parameters, oxidative status.

INTRODUCTION

Correct assessment of rabbit welfare should involve multiple indicators such as behavior, physiology, injury, disease and performance (Broom, 1997; Trocino and Xiccato, 2006). In recent years, it has become evident that there is a strong link between behavior, stress, and the neuroendocrine and immune systems (Straub *et al*., 2000; Marchetti *et al*., 2001; Mann, 2003). Therefore, unfavorable environmental conditions can lower homeostatic functions, such as immune response and, in particular, the innate immune system (Amadori *et al*., 1997). This portion of the immune system is affected by environmental stressors which have been shown to be significantly correlated to the health status of same animal species (Moscati *et al*., 2003). Monitoring innate immunity requires inexpensive analytical procedures, and is therefore applicable on a large-scale.

Furthermore, it has been widely reported that the antioxidant status is associated with the health of the animal and with the specific and non-specific response of the immune system (Hildeman, 2004).

The aim of the present study was to investigate trends and correlations between some traits of innate immunity and the oxidative status of fattening rabbits of different ages.

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MATERIAL AND METHODS

Animals and housing

The trial was carried out at the farm of the Department of Applied Biology (University of Perugia). Environmental temperature and relative humidity were controlled (ranges: 18-27°C and 60-75% RH, respectively). The building was artificially ventilated (0.3 m^3/sec). Four hundred and eighty New Zealand White kits (sex ratio 50:50), weaned at 30 d of age, were kept in double-cell flat-deck cages (two per cage) under a continuous photoperiod of 16 h light with an intensity of 40 lux.

Blood Sampling

Blood samples were collected from 120 animals at 45, 55, 65 and 75 d of age (n=480). Each rabbit was sampled only once due to the large blood volume taken from each rabbit.

Whole blood samples (about 6 mL per rabbit) were collected via the marginal ear vein and immediately sent to the laboratory of the Istituto Zooprofilattico Sperimentale dell'Umbria e Marche. At the laboratory, the samples were allowed to coagulate at room temperature for two hours, and then the collection tubes were rimmed and refrigerated at 4°C for a maximum of 24 h before analysis.

Plasma was produced by the same procedure, except that blood was collected directly into pre-cooled (on ice) plastic centrifuge tubes and cellular components were removed by centrifugation (3000 *g*, 20 min, 4°C) before coagulation.

Analytical determinations

Lysozyme. Serum lysozyme was measured with a lysoplate assay (Osserman and Lawlor, 1966), carried out in a moist incubator at 37°C for 18 min. The method is based on the lyses of Micrococcus lysodeikticus in 1% agarose. The diameter of the lysed zones was measured with a ruler and compared with the lysed zones of a standard lysozyme preparation (Sigma, Milan, Italy, M 3770). The value is expressed as µg/mL.

Serum Bactericidal Activity (SBA). The SBA was performed according to a method previously validated for cattle (Amadori *et al*., 1997). The test is based on the challenge of serum with non-pathogenic *E. coli*. Its concentration is expressed as a percentage.

Haemolytic Complement Assay (HCA). The haemolytic complement assay (Barta and Barta, 1993) was carried out in microtitre plates. The complement titre is the reciprocal of the serum dilution causing 50% lyses of red blood cells of ram. Its concentration is expressed as $CH_{50\%}$.

Oxidative status. Reactive Oxygen Substances (ROS) of the plasma were evaluated with a commercial kit (Diacron, Grosseto, Italy) and are expressed as mmol H_2O_2.

The Antioxidant Power of plasma (AP) was measured with a commercial kit (Diacron, Grosseto, Italy) that evaluates the ability of plasma to oppose the massive oxidative action of a hypochlorous acid (HClO) solution. AP levels of the sample are expressed as µmol of neutralized HClO.

Statistical analysis

The normality of distribution was evaluated through skewness and kurtosis. As SBA was not distributed normally because a certain number of samples were equal to 0, such samples were not considered and the normality of distribution was evaluated. The data was analyzed using the GLM procedures of STATA (StataCorp, 2005) evaluating the effect of rabbit age. To appreciate the time-dependent trend of such variables, the estimated values obtained using quadratic regression (REG) are presented as figures.

Table 1: Skewness/kurtosis tests for normality of analyzed traits.

		Probability χ^2		
	n	Skewness	Kurtosis	Total
Lysozyme	480	0.04	0.05	0.05
Serum Bactericidal Activity > 0	480	0.28	0.01	0.20
Serum Bactericidal Activity > 0	408	0.07	0.01	0.01
Haemolytic Complement Assay	480	0.04	0.08	0.05
Antioxidant Power	480	0.06	0.02	0.05
Reactive Oxygen Substances	480	0.23	0.02	0.05

Comparisons betweens means were made using the Student 't' test at the 0.05 significance level, while non-parametric variables were analyzed with χ^2. Pearson correlation coefficients between variables were also performed using CORR procedures of STATA.

RESULTS AND DISCUSSION

The analysis of location and variability of the data set by skewness and kurtosis are reported in Table 1. All traits presented a normal distribution; only SBA had non-normal curves which appeared close to the normal distribution curve when the values equal to zero were excluded. This last result is more clearly shown in Figures 1a and 1b. SBA showed a bimodal distribution due to the presence of values (about 15%) equal to zero (Figure 1a); when such values were omitted the data distribution became normal (Figure 1b).

The overall mean data of the analyzed variables is reported in Table 2.

Lysozyme (mean value = 27.19 µg/mL; SD = 13.17) is a strong antibacterial enzyme (against Gram positive) that has a synergic action with immune humoral response, and factors of the serum complement. Carroll and Martinez (1979) demonstrated that lysozyme is the major anti-Gram positive bactericidal agent present in rabbit plasma serum and is presumably found in the soluble portion of whole rabbit blood. Lysozyme titration is essentially related to the function of the macrophage system and basically indicates the presence of inflammation. The values observed in this study were higher than those reported by Carroll and Martinez (1979) in healthy rabbits (0.85 µg/mL) and by Tessler and Weinberg (1975) in

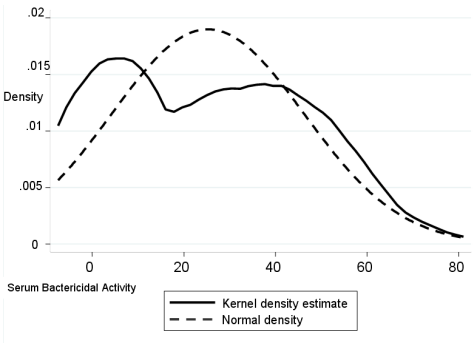

Figure 1a: Probability density function of the normal distribution of Serum Bactericidal Activity (all the samples).

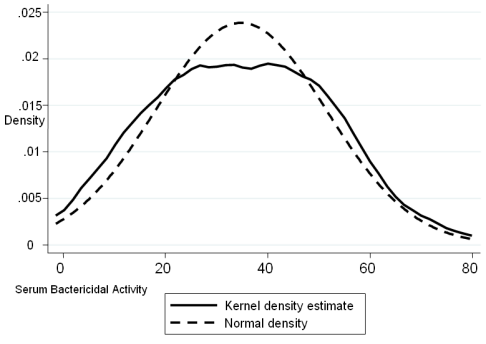

Figure 1b: Probability density function of the normal distribution of Serum Bactericidal Activity (samples with values >0).

Table 2: Mean values and standard deviation of innate immunity and oxidative status parameters (n=408).

Variable		Mean	SD	Min	Max
Lysozyme	µg/mL	27.19	13.17	0.51	118.06
Serum Bactericidal Activity	%	42.15	18.23	9.00	90.77
Haemolytic Complement Assay	CH_{50}/150 µL	50.84	16.9	0.16	182.40
Antioxidant Power	µmol HCLO neutralised	421.7	24.4	78.0	939.3
Reactive Oxygen Substances	mmol H_2O_2	0.60	0.09	0.42	0.92

healthy albino (4.1 µg/mL) experimentally induced uveitis male rabbits (6.0 µg/mL and 4.2 µg/mL not immunized and immunized, respectively). These last mentioned authors concluded that if the infection is not severe, the serum lysozyme value does not increase. Bonnafous and Raynaud (1980) observed lysozyme values of 10.7 µg/mL in the plasma of male rabbits. They reported that lysozyme is strongly present in the salivary secretions of rabbits; and absent in the digestive material of the stomach, large intestines, and intestinal secretions taken from fistulae located in different areas of the intestine.

The high values observed in the present trial could be due to the intensive housing conditions that favor environmental stress with the consequence of continuous inflammatory response. It is known that an increase in serum lysozyme levels is often associated with inflammatory processes of the intestinal apparatus (Nugent *et al*., 1976; Klass and Neale, 1978). This hypothesis was confirmed in a recent study (Mugnai *et al*., 2008) in which weaned young rabbits reared in extensive housing conditions showed a decreasing trend of serum lysozyme with age – with the post-weaning phase being the most critical period for morbidity and mortality (Padilha *et al*., 1995; Maertens and Štruklec, 2006).

In general, values observed in other animal species were always lower then those found in rabbits. In beef cattle, Ponti *et al*. (1989) found lysozyme serum values ranging from 0.10 to 1.87 1 µg/mL, while Moscati *et al*. (2005) reported values from 4.8 to 11.0 µg/mL. Also in pig, sheep, and chickens, the serum lysozyme levels are lower than in rabbits (Moscati *et al*., 2003; Sensi *et al*, 2006; Sotirov *et al*., 1997; Sotirof *et al*., 2007).

SBA is a major parameter of innate immunity. Indeed, the bactericidal activity of mammalian serum has been studied as nonspecific host defense mechanisms and may play an important role in the initial stages of microbial attack. Beginning with the early observations of Fodor (1887) and Von Behring (1888), which demonstrated the existence of heat-stable bactericides in blood and serum, numerous attempts have been made to isolate and characterize the factors involved. Pettersson (1926) investigated the microbicidal spectrum of a class of thermostable substances found in various sera and demonstrated their principal activity against Gram positive bacteria. He applied the generic term β-lysin to heat-stable substances, in contrast to α-lysin (antibody and complement) heat-labile (Buchner, 1998). The serum of rabbits contains high levels of β-lysin activity, whereas the serum of humans, dogs, cows, and pigs consistently exhibits low levels of bactericidal action (Mackie and Finkelstein, 1932; Myrvik and Weiser, 1955). According to this assumption, we found a mean value of SBA of 42.15% (SD = 18.23), while in cattle, some authors found higher values of serum SBA. In Holstein Frisian and cross-bred calves Amadori *et al*. (1997) found values ranging from 80% to 40%, while Moscati *et al*. (2005) reported from 87% to 99% in Chianina calves.

The capacity of the serum to inhibit bacterial growth is assessed by the presence of complement factors which modulate the concentrations of natural antibodies against other ubiquitous environmental bacterial agents, mainly Enterobacteriacee (Gram negative). It gives some indications regarding the defense mechanisms of the animal which activate the complement system. The HCA test is very useful in assessing

Table 3: Pearson correlation coefficients analysis between immune and oxidative status traits.

	Lysozyme	Serum Bactericidal Activity	Haemolytic Complement Assay	Antioxidant Power
Serum Bactericidal Activity > 0	0.29**			
Haemolytic Complement Assay	−0.13*	−0.23**		
Antioxidant Power	0.61**	0.23**	−0.38**	
Reactive Oxygen Substances	0.38**	0.10	0.26**	0.21**

* $P<0.05$; ** $P<0.01$.

the risk of an onset of infectious disease, or measuring the severity of already existing pathologies. Indeed, complement consumption has been reported to occur in many bovine parasitic infections (Ortiz-Ortiz *et al*. 1978; Boulard and Bencharif, 1984). In humans, complement activity has been extensively studied and, in particular, Atkinson and Frank (2006) showed that innate immunity suffices for most organisms. The complement system is a major player in this innate immunity. It can also be used in pigs as a parameter for monitoring general health status, including stress assessment (Burger *et al*., 1998). Regarding rabbits, the literature offers only a few studies for comparison. In the present trial, the HCA mean value was 50.84 $CH_{50\%}$ (SD = 16.9).

Oxidative stress, resulting from an increased production of free-radicals and ROS, and/or a decrease in AP, damages biological macromolecules and disrupts normal metabolism and physiology (Tse *et al*., 2004). In the present experiment, ROS and AP mean values were 0.60 mmol H_2O_2 (SE = 0.09) and 421.67 µmol neutralized HClO (SD = 24.39), respectively.

Oxygen free-radicals generated during biological processes are involved in the pathogenesis of several diseases and various reports have indicated that oxidative stress alters immune competence (Koner *et al*., 1996). High levels of ROS significantly reduce the primary and secondary antibody responses; furthermore, cell-mediated immunity, tuberculin sensitivity, and leukocyte migration inhibition have been shown to decrease in oxidatively stressed rabbits (Merendino *et al*., 1998).

An analysis of the relationship between traits is of more interest than an analysis of single traits – even if the observed correlation coefficients are very low (Table 3). Lysozyme and SBA (values >0) presented a ($P<0.01$) positive correlation, confirming their immune function as early defense barriers. Lysozyme and HCA were negatively correlated ($P<0.05$) and this is probably due to the presence of sub-inflammatory processes which enhance the release of lysozyme by neutrophiles and macrophages, and reduce the free complement which is mainly found in immuno-complexes.

In a previous study, Castellini *et al*. (2003), found that n-3 fatty acid dietary supplementation affects the lysozyme levels in rabbits vaccinated against hemorrhagic viral disease, thanks to the immune modulator function of this fatty acid (Calder, 2001; Anderson, 2002.)

The same explanation could be hypothesized for the relationship between lysozyme and ROS ($P<0.01$) released by leucocytes during inflammatory processes. Under such conditions the positive correlation between lysozyme and AP ($P<0.001$) could be due to the body's need to compensate for the higher ROS level.

Previous studies on oxidative stress in rabbits related to locomotory activity (Dal Bosco *et al*., 2002) indicate a positive correlation between the ROS values and the antioxidant response of the animal. The lowest antioxidant capacity (387 µmol HClO/mL) and ROS levels (26 mg hydrogen peroxide/100 mL) were observed in cage-reared animals, while the highest value (506 µmol HClO/mL and 33 mg hydrogen peroxide/100 mL, respectively) were found in pen-reared rabbits.

On the other hand, the SBA trend was anomalous and differed from that described in other intensively reared animal species (Amadori *et al*., 1997; Moscati *et al*., 2003). The positive correlation with lysozyme ($P<0.001$), and the negative response with HCA ($P<0.05$) would imply that rabbits have some difficulty in balancing environmental stimuli, which results in an increase in non-specific body defenses.

In Figure 2 it can be seen that the SBA trend differs with age, especially during the first days after weaning. Whereas the lysozyme and HCA trends were stable, the level of SBA, even in the absence of visible pathologies, indicates an effort to continuously adapt to environmental stress (weaning, change of cage, neighbors, etc). The SBA in apparently healthy kits is unbalanced and this increases with age. A recent study (Mugnai *et al*., 2008) showed that, in a similar way to the lysozyme level, the HCA of caged fattening New Zealand rabbits decreased from weaning to 90 d of age (from 68.60 to 25.80 $CH_{50\%}$).

Ponti *et al*. (1989) and Sensi *et al*. (2006) showed an age-related effect in the serum lysozyme of calves and pig. The social and physical environment of animals is implicated in the etiology of infective diseases. To thoroughly understand animal health, the interaction between stress and disease must be studied. Little data has been reported about the influence of environmental stressors on the native immunological parameters of rabbits.

Such parameters are strongly affected by the environment and management practices as already shown in pigs (Moscati *et al*., 2003) and dairy cows (Amadori *et al*., 1997) where an alteration of the body defenses can affect the health of the animal as well as its performance. These parameters can help indicate if the impact of environmental stressors leads to abnormalities in the homeostatic function through the evaluation of restoration time.

The values reported in this study can be considered as preliminary reference values for intensively reared rabbits. Further studies are needed to better understand the SBA trend, and in particular, the physiological explanation of why SBA equals zero should be better analyzed.

To better understanding the dynamics and interaction between such traits, healthy animals should be compared with animals experimentally infected or suffering sub-acute infections – especially during

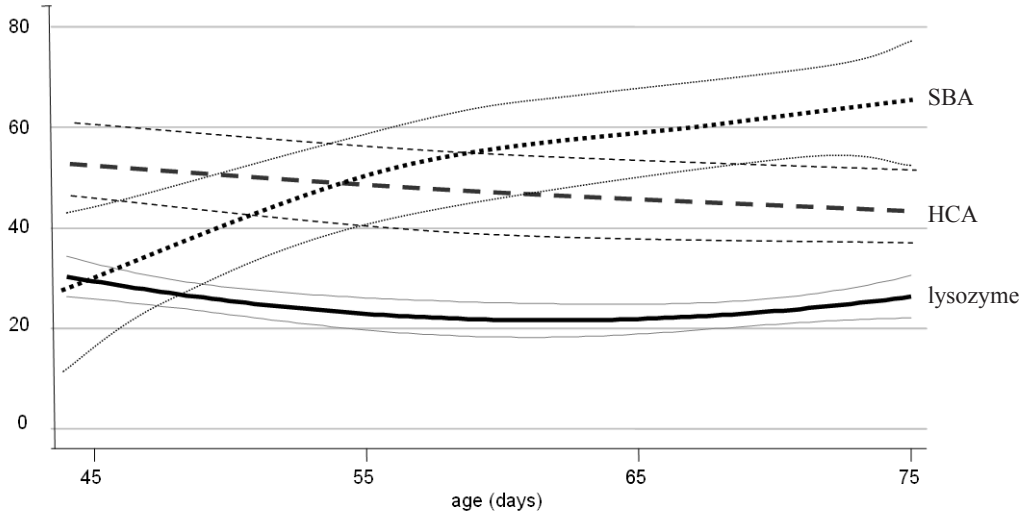

Figure 2: Effect of the age of rabbits on the trends of Serum Bactericidal Activity (SBS; %), Haemolytic Complement Assay (HCA; CH_{50}/150 µl) and lysozyme (µg/ml) (prediction and 95 confidence interval) (n=408).

the first phase of infection/inflammation. More detailed research into acute phase proteins could clarify homeostasis of innate immune status, its actions, and presence being strongly correlated to inflammatory response and stress (Murata *et al*., 2004).

Future research could be aimed at evaluating the economics of animal welfare, and by detecting the first phases of immune homeostatic alteration it may be possible to reduce excessive drug use in intensive rabbit production.

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