Effect of antigen stimulation on the oxidative stress parameters in sperm of outbred and inbred rabbits

Svetlin Tanchev\(^a\), Stanimir Dimitrov\(^b\), Galina Nikolova\(^b\), Yanka Karamalakova\(^b\), Donika Ivanova\(^b\), Deyana Hristova\(^a\), Svetlana Georgieva\(^b\), Antoaneta Zheleva\(^b\), Vladimir Petrov\(^b\) and Veselina Gadjeva\(^b\)

\(^a\)Department of Genetics, Breeding and Reproduction, Faculty of Agriculture, Trakia University, Stara Zagora, Bulgaria; \(^b\)Department of Chemistry and Biochemistry, Faculty of Medicine, Trakia University, Stara Zagora, Bulgaria; \(^c\)Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

**ABSTRACT**

The presence of an increased oxidative stress during antigen stimulation was established in the sperm of inbred rabbits by evaluation of the level of real-time free radical formation (ROS and Asc\(^\bullet\)), final products of oxidation of lipids (MDA) and the activities of antioxidant defence enzymes superoxide dismutase (SOD) and catalase (CAT). There was not a significant difference between levels of ROS, Asc\(^\bullet\) and MDA in inbred and outbred rabbits before immunization. The immune response, represented by levels of ROS and Asc\(^\bullet\) of inbred and outbred rabbits were statistically significantly higher compared to those before immunization \((p = 0.0001)\) and \((p = 0.00001)\). Moreover, the levels of ROS in the sperm of inbred rabbits were statistically significantly higher compared to outbred rabbits \((p < 0.05)\). Oxidative stress was accompanied by an adaptive increase of SOD and CAT activities during the immune response, compared to those before immunization. Furthermore, the increased SOD and CAT activities appeared to be sufficient to inactivate the oxidative stress. We measured reduced levels of ROS, Asc\(^\bullet\) and MDA 30 days after immunization. When immune response reduced, the observed SOD and CAT activities tended to return to the values before immunization. That might have been connected with the decreased oxidative burden. However, CAT activities remained about 1.5 times higher than that before immunization. In conclusion, our results indicate that the administered antigen stimulation induces oxidative stress in both groups inbred and outbred rabbits.

**Introduction**

The organisms are subjected to stress when a sudden increase in pro-oxidants overcomes their antioxidant defences that occur when the immune system is activated. Within a few hours following an infection, vertebrate organisms trigger an innate, nonspecific inflammatory response by immune cells, such as phagocytes (e.g. granulocytes, macrophages) which are recruited to the focal site and are essential for fighting invading pathogens, and thus for the survival of all multicellular organisms [1]. Phagocytes use highly reactive oxygen and nitrogen species (ROS and RNS) to kill engulfed pathogens and to regulate inflammation. Although ROS and RNS are effective antimicrobial agents, they may also generate local and systemic oxidative stress (OS) and cause oxidative damage to the host organism [2,3]. Vertebrate spermatozoa are highly susceptible to OS due to the high proportion of polyunsaturated fatty acids in their membrane, and their highly condensed DNA and reduced transcription machinery that limits DNA repair [4]. Oxidative damage to spermatozoa can reduce fertility in domestic animals and humans [5]. This has been demonstrated recently in wild bird species to affect sperm quality by the reduction of sperm motility and swimming velocity [6]. Remarkably, immune-induced systemic OS has been found to reduce male fertility in humans as well [5]. But whether immune-induced OS can result in oxidative damage to sperm and reduced sperm quality in non-human species still remains unexplored. Inbreeding increases homozygosity, and this, in turn, decreases fitness by either exposing deleterious recessive alleles or reducing the frequency of high fitness heterozygotes [7,8]. This reduction in fitness, and in trait values generally, is known as inbreeding depression and depends on the magnitude of directional dominance in a trait, which itself can be explained by selection [7]. Inbreeding also negatively impacts sperm motility and normal sperm morphology [9]. Furthermore, the production of abnormal sperm increases with increased homozygosity, suggesting that...
inbred individuals have lower quality sperm [10,11]. Quantitative genetics of inbreeding depression is generally well studied but its molecular bases have only recently begun to be explored. Metabolically active sites like the testes are likely to be especially prone to stress because of the production of reactive oxygen species (ROS) [12]. This could ultimately result in increased testis and sperm dysfunction, and therefore inbreeding may especially impact on ejaculate components because of this. However, while there is some evidence consistent with more ROS damage to sperm in inbred populations [13] this has never been specifically documented. The aim of this study was to test whether the antigenic stimulation would cause: (1) increase of radical formation in real-time; (2) increase of some y products of lipid peroxidation; (3) reduction of antioxidant capacity and thus increase the risk of oxidative damage to sperm in both hereditary and bred rabbits.

Materials and methods
The experiment included 10 male New Zealand White rabbits, 8 months of age, weighing 3.8–4.0 kg. The rabbits were healthy and housed in controlled conditions (12 h light/dark cycles), the temperature of 18–23 °C and humidity of 40%–70%, with free access to tap water and standard laboratory chow. Experiments were carried out in accordance with European directive 86/609/EEC of 24.11.1986 for the protection of animals used for scientific and experimental purposes. The rabbits were divided into two groups. The first group comprised five outbred rabbits (control). The second group included five inbred rabbits, a progeny of full siblings (inbreeding coefficient Fx = 0.375).

Semen collection and immunization
Semen was collected using a teaser rabbit doe and the artificial vagina (MEGAPOR — Spain) once weekly between 12 May 2014 and 18 June 2014. From each rabbit, three ejaculates were obtained prior to the immunization and another two – after the immunization. After the collection of semen, samples were immediately transported to the laboratory for examination. As a stress factor, a live attenuated lyophilized vaccine Myxoren (Bioveta, Ivanovice na Hane, Czech Republic) was used, containing in each dose Poxvirus myxomatosae attenuatus minimum 103.3 TCID50. Until use, the flasks with the bio-product were stored in a refrigerator at 4–8 °C. Immediately before application, the vaccine was reconstituted with the solvent provided by the manufacturer with each flask. The vaccination of animals was done by subcutaneous injection of 1 mL/dose with individual disposable syringe and needle.

Electron paramagnetic resonance (EPR) real-time studies
All EPR measurements were performed at room temperature on an X-band EMXmicro, spectrometer Bruker, Germany, equipped with standard resonator. All EPR experiments were carried out in triplicate and repeated thrice. Spectral processing was performed using Bruker WIN-EPR and Simfonia software.

EPR ex vivo evaluation the levels of ROS products in the studied sperm samples
The levels of ROS were determined according to Shi et al. [14] with modifications. To study the formation of ROS in real-time in sperm, was used EPR spectroscopy in combination with spin -trap. The spin trapping agent Phenyl Butyl Nitron (PBN) upon reaction with unstable radicals forms a relatively stable spin adduct. In brief, to volume of 100 μL of sperm were added 900μL 50mM PBN dissolved in DMSO, centrifugated at 792.96 g for 10 min at 4 °C, and recorded EPR spectrum of the supernatant. The levels of ROS products were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units. EPR settings were as follows: centre field 3503.73 G, microwave power 20.00 mW, modulation amplitude 5.00 G, sweep width 50.00 G, gain 1 × 10⁵, time constant 81.92 ms, sweep time 125.95 s, five scans per sample.

EPR ex vivo evaluation the levels of ascorbate radicals in the studied sperm samples
The levels of Asc were studied according to Bailey et al. [15] with some modifications. Briefly, the sperm was prepared in DMSO in a ratio of 1:3. After centrifugation at 792.96 g for 10 min at 4 °C, the supernatants were collected and immediately transferred into a quartz tube and placed in EPR cavity. The levels of Asc were calculated as double integrated plots of EPR spectra and the result was expressed in arbitrary units. EPR settings were as follows: centre field 3505.00 G, microwave power 20.00 mW, modulation amplitude 1.00 G, sweep width 15.00 G, gain 1 × 10⁵, time constant 40.96 ms, sweep time 60.42 s, 10 scans per sample.

Estimation of lipid peroxidation products
The total amount of lipid peroxidation products in the sperm of inbred and outbred rabbits was estimated using the thiobarbituric acid (TBA) method, which measures the malondialdehyde (MDA) reactive products [16].
In brief, in volume 1.0 mL sperm sample from each animal, 1.0 mL of normal saline and 1.0 mL of 25% trichloroacetic acid (TCA) were mixed and centrifuged at 396.48 g for 20 min. One millilitre of protein-free supernatant was taken, mixed with 0.25 mL of 1% TBA and boiled for 1 h at 95 °C. After cooling, the intensity of the pink colour of the obtained fraction product was read at 532 nm.

**Measurement of antioxidant enzymes in sperm**

Superoxide dismutase (SOD) activity was estimated as described by Sun et al., with minor modifications [17]. The xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion reduces nitroblue tetrazolium (NBT) to formazan, which is monitored at 560 nm. SOD in the sample removes the superoxide anion and inhibits the reduction. The level of this reduction is used as a measure of SOD activity. The final concentrations of xanthine, xanthine oxidase and NBT in the samples were 50 mmol/L, 10 U/mL and 0.125 mmol/L. One unit of SOD activity is defined as the amount of enzyme causing 50% inhibition of the reduction of NBT to formazan observed. CAT activity was assessed in the sperm samples by the method described by Beers and Sizer [18]. Briefly, hydrogen peroxide (30 μmol/L) was used as a substrate and the decrease in H2O2 concentration at 22 °C in phosphate buffer (50 mmol/L, pH 7.0) was followed spectroscopically at 240 nm for 1 min. One unit of CAT activity is defined as the amount of enzyme that degrades 1 μmol/L H2O2 per minute. Results are presented as units per millilitre.

The results are reported as means ± SE. Statistical analysis was performed with Student’s t-test and multiple regression analysis. P < 0.05 was considered statistically significant.

**Results and discussion**

Inbreeding in animals can increase their susceptibility to pathogens, but direct evidence from wild populations is scarce [19]. In the current study, we explored to our knowledge for the first time the influence of antigen stimulation on: (1) both real-time biomarkers of OS, namely ROS products and Asc. radicals using EPR spectroscopy technique, (2) levels of MDA stable products of lipid peroxidation and (3) activities of the antioxidant enzymes SOD and CAT in sperm of inbred rabbits and no relative heterogeneous rabbits.

Certain stages of the course of lipid peroxidation generate a variety of unstable radical species that can be measured in real-time [20,21]. Short-lived unstable radicals, formed during peroxidation, can be only confirmed by EPR spin trapping technique [22,23]. For the assessment of the oxidative status of the sperm of inbred and outbred rabbits, we used PBN as a spin trapping agent. Although, PBN does not exhibit specificity towards different unstable radicals, is widely used in *in vitro* and *in vivo* spin trapping EPR spectroscopy due to the stability of its spin adducts. In all studied sperm samples EPR spectrum of the PBN spin adduct was registered, consisting of a triplet of doublets (not shown). Since, the values of the hyperfine splitting constants aN and aH of the registered PBN adducts were consistent with those of alkoxy radical adducts of PBN previously reported [24]. The radicals trapped were assigned to secondary oxygen-centred alkoxy radicals resulted from the attack of primary oxygen-centred radicals towards membrane phospholipids.

Results from the study of ROS products in sperm are shown in Figure 1. As seen, before immunization there was no significant difference between ROS levels measured in inbred and outbred rabbits (P > 0.05). After immunization, in the sperm of both inbred and outbred group of rabbits statistically significant higher levels of ROS products were found compared to the same groups before immunization (mean 10.58 ± 3.75 vs. 2.47 ± 2.17, for outbred rabbits and mean 13.53 ± 3.18 vs. 2.55 ± 2.08, for inbred rabbits, respectively, P < 0.001, t-test). The obtained result indicates that the antigen stimulation induces significant OS, and furthermore oxidative processes are available in the sperm of the tested animals at the time of experiment. Moreover, after antigen stimulation the levels of ROS products in the sperm of inbred rabbits were slightly higher compared to outbred group which means, inbred rabbits were more sensitive to OS. We report about 20% increase in ROS production in inbred group of rabbits compared to the same group before antigen stimulation. Furthermore, 30 days after

**Figure 1.** Levels of ROS products expressed in arbitrary units in sperm of inbred and outbred rabbits. The results are expressed as mean ± s.d.: *P < 0.05 compared to the group of outbred rabbits; ***P < 0.001 (A, B) compared to the corresponding group before immunization.
immunization, the ROS levels in both groups were almost the same (mean 1.23 ± 0.65 and 1.21 ± 0.42, \(P > 0.05\)) and reached values close to those measured before immunization. This suggests a complete reduction of the ongoing oxidative processes in outbred and inbred rabbits. Further confirmation that oxidative processes take place in real-time is the statistically higher level of ascorbate radicals, measured in the sperm of both tested groups undergoing immunization as compared to the same groups before immunization (Figure 2).

After immunization, in the sperm of both inbred and outbred group statistically significant higher levels of Asc* radicals were found compared to the same groups before immunization (mean 0.83 × 10^5 ± 0.29 × 10^5 vs. 0.11 × 10^5 ± 0.095 × 10^5, for outbred rabbits and mean 0.874 × 10^5 ± 0.23 × 10^5 vs. 0.119 × 10^5 ± 0.071 × 10^5, for inbred rabbits, correspondingly, \(P < 0.001\), t-test).

During the immune response, higher levels of ascorbate radicals, although not statistically significant, were measured in the sperm of inbred rabbits compared to outbred rabbits (\(P > 0.05\)). Ascorbate anion AH is an endogenous soluble antioxidant present in biological systems, and its oxidation produces a relatively stable Asc* radical. Pathologically, generated ROS are able to oxidize endogenic ascorbic acid to its stable radical structure and the last can be detected by direct EPR spectroscopy, the only method that does not interfere with the biochemical processes [25]. For the first time, Buettner and Jurkiewicz proposed the intensity of the EPR spectrum of Asc* radical to be used as an indicator of pro-oxidative changes in biological systems [26]. Since then, as a biomarker of the OS Asc* radical has been detected in a variety of biological samples [27,28]. In the present study, the elevated levels of Asc* radicals established in both antigen-stimulated groups were in accordance with the elevated levels of ROS products measured in the same groups (see Figures 1 and 2). Since, Ascorbate radicals and ROS products are OS biomarkers and moreover, they are radical structures registered by EPR spectroscopy in real-time, is evident that oxidative processes still take place in both antigen-stimulated groups of rabbits throughout the study. Thirty days after antigen stimulation in both groups of rabbits, a statistically significant reduction not only in the levels of ROS products (Figure 1) but also in Asc* levels (Figure 2) was observed.

Conclusions that the levels of ascorbate radicals in both tested groups were close to those measured before their treatment, shows that after 30 days the oxidative processes induced during antigenic stimulation have been overcome, as in inbred and in outbred rabbits.

The results of MDA-reactive products estimated in the sperm of inbred and outbred rabbits before and after immunization are given in Figure 3. MDA levels measured in the antigen-stimulated inbred and outbred group were insignificantly higher comparing to those found in the same group before immunization (mean 3.62 ± 0.31 \(\mu\)mol/L and 3.49 ± 0.37 \(\mu\)mol/L for inbred group; 3.69 ± 0.33 \(\mu\)mol/L and 3.61 ± 0.44 \(\mu\)mol/L for outbred group, respectively, \(P > 0.05\)). There was not a significant difference between levels of MDA in inbred and outbred rabbits during the immune response (\(P > 0.05\)). Moreover, 30 days after immunization, the plasma levels of MDA, for both inbred and outbred rabbits, decreased and reached values close to those before immunization (mean 3.34 ± 0.26 \(\mu\)mol/L and 3.46 ± 0.18 \(\mu\)mol/L, \(P > 0.05\)).

Results from studying SOD activity before and after antigen stimulation of the tested groups are given in Figure 4. Before immunization, SOD activity measured in the sperm of inbred rabbits was statistically significantly lower than that in the sperm of outbred rabbits (\(P < 0.05\)).
Results of CAT activity estimated in the sperm of inbred and outbred rabbits are given in Figure 5. There was a significant difference between CAT in inbred group of rabbits and outbred group before immunization (mean 437.43 ± 234.26 U/mL vs. 695.90 ± 238.75 U/mL, \( P < 0.05 \)). During immune response CAT activity in both tested groups was increased, but not significantly compared to the corresponding group before immunization (mean 882.86 ± 436.72 U/mL and 695.90 ± 238.75 U/mL, \( P > 0.05 \)), for outbred group; mean 629.03 ± 233.75 U/mL and mean 437.43 ± 234.26 U/mL, \( P > 0.05 \), for inbred group). Also, there was no significant difference between CAT activities in inbred and outbred rabbits during the immune response (\( P > 0.05 \)). It should be noted that CAT activity in outbred rabbits, 30 days after immunization, decreased and reached levels close to those before immunization (mean 726.06 ± 295.98 U/mL and 695.90 ± 238.75 U/mL, \( P > 0.05 \)), while CAT activity of the inbred rabbits remained significantly higher than that measured before antigen stimulation (mean 753.43 ± 194.43 U/mL vs. 437.43 ± 234.26 U/mL, \( P < 0.01 \)).

Our results showed that prior to antigen stimulation the MDA levels in both studied groups were similar and lower than those measured after immunization of the corresponding group. However, SOD and erythrocyte CAT activities were significantly lower in inbred rabbits compared to outbred, probably due to primarily decreased capacity of the antioxidant enzyme system in inbred rabbits.

The presence of oxidative processes during the antigen stimulation in the tested groups of rabbits has been demonstrated by an elevation of MDA levels and a sharp rise in the levels of both ‘real-time’ biomarkers of OS such as Asc. and ROS production in comparison with the levels of those parameters measured before immunization. It is known that during the mounting of the immune response important sources of increased ROS production might be the monocytes [29,30] as well as lymphocytes. According to our results, the increase of MDA levels during antigen stimulation in both groups was accompanied by significant increase in the activity of antioxidant enzymes SOD and CAT in order to compensate the enhanced OS. Reduction observed in the ROS and Asc. radical levels after 30 days in both groups was in accordance with decreased levels of MDA end products of lipid peroxidation. The strong reduction in ROS levels means that OS has been overcome and this might attribute to attenuation of the immune response. We believe also that the maintenance of a higher level of CAT activity throughout the antigenic stimulation, as well as 30 days after its implementation, compared with this activity measured before immunization is one of the reasons for the observed effective ROS neutralization in both tested groups.

**Figure 4.** Superoxide dismutase (SOD) activity in sperm of inbred and outbred rabbits. The results are expressed as mean ± s.d.; \( P < 0.05 \) (*) compared to inbred rabbits before immunization; \( P < 0.01 \) (**) compared to the inbred group before immunization; \( P < 0.001 \) (***) compared to the outbred group before immunization and (***)\( A \) compared to the outbred group before immunization and (***)\( B \) compared to the outbred group before immunization.

**Figure 5.** Catalase (CAT) activity in sperm of inbred and outbred rabbits. The results are expressed as mean ± s.d.; \( P < 0.05 \) (*) compared to outbred rabbits before immunization; \( P < 0.01 \) (**) compared to inbred rabbits before immunization.
Our findings indicate a protective role for antioxidant enzymes SOD and CAT in sperm of rabbits against OS induced by applied antigen stimulation.

Since CAT activity measured in inbred rabbits 30 days after antigen stimulation is about 1.5 times higher comparing to the same before immunization, one might suggest that they are more adaptive to the OS than outbred rabbits. It is known that CAT is non-essential for some cell types under normal conditions, so that in the semen of rabbits could play an important role in the development of adaptive response to OS.

Conclusions

In conclusion, our results indicate that the administered antigen stimulation induces OS in both groups inbred and outbred rabbits. We also consider that although before antigenic stimulation outbred rabbits exhibited significantly higher SOD and CAT enzyme activities comparing to the inbred ones, after the immunization the inbred rabbits managed to compensate in a certain extent this deficiency which means they were more adaptive to the induced OS.

Disclosure statement

No potential conflict of interest was reported by the authors.

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