Impact of Oxidative Stress and Supplementation with Vitamins E and C on Testes Morphology in Rats

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Abstract. The aim of the study was to verify whether an increased supply of vitamins E and C prevents the detrimental effects of ozone on the testes. The experiment was performed on 5-month-old rats exposed to ozone (0.5 ppm) for 50 days (5 h daily). Simultaneously, the animals were injected with the vitamins in 5-day intervals and at different doses (0.5, 1.5, 4.5, 5 and 15 mg of vitamin E; 0.5, 3, 9, and 50 mg of vitamin C; or both vitamins together, respectively). Gonad sections were PAS stained. In the ozonized males, depletion of germ cells occurred. In the vitamin E groups, the testes were comparable to the controls, excluding the 0.5-mg-dose vitamin E group in which perivascular fibrosis and intertubular hyalinization were observed. In the vitamin C groups, intertubular hyalinization, partial arrested spermatogenesis, and desquamation of the seminiferous epithelium appeared proportionally to the vitamin dose. Additionally, premature spermiation was found at a vitamin C dose of 50-mg. In the rats injected with both vitamins, hyalinization and fibrosis appeared in addition to partial arrest of spermatogenesis and vacuolar degeneration. In conclusion, vitamin E protects against the detrimental effects of ozone in rat testes irrespective of the dose applied. This was not observed for vitamin C. Moreover, administration of higher doses of vitamin C intensified the damage to the testes caused by ozone.

Key words: Ozone, Rat, Testes, Vitamin C, Vitamin E

biochemical properties of the membrane, further impairing cell function [3–5].

The short half-life and limited diffusion possibilities of ROS enable these molecules to play an important role in regulation of many cell functions (e.g. activation of transcription, proliferation, apoptosis, etc.). They also affect the quality of sperm, thus enabling, for example, acrosomal reaction or capacitation. An increased content of ROS, both of exo- and endogenous origin, causes damage to DNA, inhibits the motility of spermatozoa, and disables the fusion of spermatozoa with oocytes through changes in the plasma membrane [6, 7].

The antioxidative system in organisms functions to ensure a balance between the oxidative and antioxidative processes. This system includes, among others, α-tocopherol (vitamin E) and ascorbic acid (vitamin C)—compounds with antioxidative characteristics. Vitamin E, acknowledged as a stabilizer of protein-lipid membranes, prevents peroxidation of fatty acids and acts as a “scavenger” of free radicals. On the other hand, vitamin C is a donor of electrons for the redox systems of interplasma membranes, and enables regeneration of fatty acids and as a “scavenger” of free radicals. On the other hand, vitamin C is a donor of electrons for the redox systems of interplasma membranes, and enables regeneration of vitamin E [8]. High doses of ascorbic acid with an increased supply of peroxides (which occurs upon oxidative stress) intensify, however, the oxidative processes in tissues by activating the Fenton reaction. The pro-oxidative activity of vitamin C intensifies with an increased dose of the vitamin [9, 10]. Lipid ozonation products (LPO) tend to accumulate, and as result of disintegration, form highly toxic aldehydes. One of the main end products of this process is malonic dialdehyde, which is formed upon activation of this reaction by the iron-ascorbate system [7]. There is a possibility that excessive doses of vitamin C may damage DNA upon oxidation of purine and pyrimidine bases [11]. Experiments in vitro showed that vitamin E also reveals pro-oxidative properties that have not been confirmed so far in in vivo studies [12].

The available literature lacks data on the impact of ozone on gonad function in males. Our own study has indicated that an increased ozone content in the air lowered both the blood testosterone level and activity of 17β-hydroxysteroid dehydrogenase in the rat testes [13]. Considering all these findings and the above-described mechanism of the activity of vitamins commonly acknowledged as antioxidants, these studies were undertaken to verify whether, and to what extent, an increased supply of vitamins E and C prevents the negative effects of ozone in the rat testes.

Materials and Methods

The experiment was performed on 120 clinically healthy 5-month-old Wistar Hannover male rats weighing 370 ± 10 g. The animals originated from the Division of Pathophysiology, University of Warmia and Mazury in Olsztyn, Poland. They were kept in a room under natural lighting, at a temperature of 21–22°C, and with gravitational ventilation, and were fed ad libitum Murigran standard pellets for rodents (Motycz n/Lublin, Poland) with free access to water.

All animals were randomly divided into 15 groups (8 rats each). The abbreviations for the control animal groups are as follows:

Group CON—control animals; and

Group PS—control animals receiving intramuscular (im) injections of 0.2 ml of physiological saline for 50 days, in 5-day intervals.

All the rats from the remaining groups were exposed to 0.5 ± 0.2 ppm of ozone daily for 5 h (0830–1330) for 50 days. Moreover, these animals, except for Group O3, were given im injections of vitamin E (Vitaminum E, Pharmaceutical Company “Polfa”, Warsaw, Poland) and/or vitamin C (Vitaminum C, PLIVA, Kraków, Poland) in 5-day intervals. The abbreviations and doses (mg/rat) for these groups are as follows:

Group 0.5E–0.5 mg of vit. E; Group 1.5E–1.5 mg of vit. E; Group 4.5E–4.5 mg of vit. E; Group 15E–15 mg of vit. E; Group 0.5C–0.5 mg of vit. C; Group 3C–3 mg of vit. C; Group 9C–9 mg of vit. C; Group 50C–50 mg of vit. C; Group 0.5E + 0.5C–0.5 mg of vit. E and 0.5 mg of vit. C; Group 1.5E+3C–1.5 mg of vit. E and 3 mg of vit. C; Group 4.5E+9C–4.5 mg of vit. E and 9 mg of vit. C; Group 15E+50C–15 mg of vit. E and 50 mg of vit. C; and Group O3—only exposed to ozone, without vitamin injections.

Ozone was generated from compressed air in an IMPOZ-4 ozonizer (Institute of Precise Mechanics, Warsaw, Poland) and transferred through a polyethylene conduit to an exposure chamber (a room chemically-sealed with a chemically neutral and biologically friendly polyethylene foil) with a capacity of 42.6 m³. In the chamber, ozone was
spontaneously mixed with air, and its concentration was monitored using an iodometric method [14]. Taking the data on oxygen consumption by rats [15] into consideration the air in the chamber was exchanged at the midpoint during exposure to avoid excessive accumulation of CO₂ and the ozone concentration was returned to the initial level. All the rats, the control and experimental rats, were kept in cages (4 rats per cage). During exposure to ozone, the animals had free access to water, while the pellets were removed due to the oxidizing effect of ozone. Except during the 5 h of exposure to ozone, all rats were kept under the same conditions with reference to air composition, temperature, and manner of feeding.

After 50 days, the testes were collected from all the animals under halothane anesthesia (Narcotan, Leciva, Czech Republic). Samples of the gonads were fixed in Bouin’s fluid and embedded in paraffin blocks. The microtome sections (8 µm) obtained were routinely stained with periodic acid Schiff (PAS).

The experimental procedures were approved by the Local Ethics Committee, according to the Polish Guide for the Care and Use of Animals (1997).

**Results**

In the histopathological preparations of both control animal groups (CON and PS), the course of spermatogenesis was regular in all males (Fig. 1a).

All animals exposed to ozone without vitamin injections (O₃) were characterised by a depletion of germ cells, i.e. round spermatids and spermatocytes, as well as the occurrence of single giant spermatid cells and focal desquamation of the epithelium up to denuding of the basement membrane. Reduced seminiferous epithelial layers were found in numerous tubules (Fig. 1b).

On the other hand, in animals exposed to ozone and given injections of vitamin E at different doses, expect at a dose of 0.5 mg, the morphological characteristics of testes were comparable to those in the control groups (Fig. 1c). In the 6 rats receiving 0.5 mg of vitamin E, perivascular fibrosis and hyalinization of intertubular tissue were observed (Fig. 1d). In the rats exposed to ozone and injected with vitamin E, spermatogenesis proceeded normally.

In the ozonized animals receiving vitamin C, the seminiferous epithelium was the most injured. Intensification of these lesions increased in proportion to the vitamin dose applied. In the rats receiving 0.5, 3, and 9 mg of vitamin C, the following changes were observed (number of males in relation to dose, respectively): hyalinization of intertubular connective tissue (1, 3, 5), partially arrested spermatogenesis (3, 5, 7), occurrence of few round spermatids and spermatocytes by desquamation of the epithelium up to the basal compartment (1, 5, 6), and totally denuded basement membrane in some places (0, 1, 2) (Fig. 1e). Spermiation proceeded regularly, and was at stage VIII, according to the definitions of LeBlond and Clermont [16]. Moreover, when a 9-mg-dose of vitamin C was administered, focal detachment of the seminiferous epithelium was observed in 2 rats (Fig. 1f). In the animals injected with 50 mg of vitamin C, apart from the above-mentioned changes that occurred after increasing lower doses of vitamin C, perivascular fibrosis was found in 4 rats (Fig. 2a) and peritubular fibrosis in 2 rats (Fig. 2b). In the 2 rats, with peritubular fibrosis spermiation was premature, even up to stage V (Fig. 2b).

In the animals exposed to ozone and injected with vitamins E and C together, lesions of seminiferous epithelium were similar to those reported in the groups of animals receiving 0.5, 3, and 9 mg of vitamin C. Moreover, wrinkling (in 3 rats), thickness, and hyalinization of the basement membrane of the tubules (in 7 rats) was observed in addition to perivascular (in 8 rats), intertubular (in 2 rats), and peritubular fibrosis (in 3 rats) (Figs. 2c, d). The lesions intensified with an increased dose of vitamins. In the animals of the 15E+50C group, a tri-layered seminiferous epithelia occured in 5 rats (Fig. 2e), partial arrest of spermatogenesis occured in 6 rats, and vacuolar degeneration and giant cell formation occurred in 2 rats (Fig. 2f). In the E+C groups, spermiation proceeded normally, i.e. at stage VIII.

**Discussion**

The present study, for the first time, showed that exposure of rats to ozone resulted in intensification of histopathological changes and loss of a considerable number of germ cells. These alterations indicate degenerative processes of the
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The decrease in the number of seminiferous epithelial layers could be the result of spermatogonium B mitosis inhibition, which denotes elongation of the G1 phase of the cell growth cycle. It has been shown that elongation of the G1 phase is caused by inhibition of cycline 1 synthesis evoked by excessive XBP1 gene expression. This gene’s promoter contains several stress-regulated elements, one of which responds positively to oxidative stress [19]. Elongation of the

Fig. 1. Histopathological changes in the testes of the rats-PAS staining. Bar: 30 µm. a. The CON group—regular course of spermatogenesis (× 200). b. Reduced seminiferous epithelium layers (arrows) in all tubules in the O3 group (× 200). c. Regular course of spermatogenesis in 1.5E group (× 200). d. Perivascular fibrosis (arrows) and hyalinization (asterisks) of intertubular tissue in the 0.5E group (× 200). e. Depletion of germ cells, intraluminal cellular debris (arrowheads) formation in all tubule lumens and tubules denuded to the basal compartment (arrows) in the 9C group (× 200). f. Focal detachment (arrows) of the seminiferous epithelium in the 9C group (× 200).
G1 phase provides time for synthesis of elements that may impair oxidative stress and repair potential DNA damage. It should proceed prior to replication, before the resulting changes are subject to conversion to irreversible mutations, which will be further multiplied. Elongation of the G1 phase and inhibition of the S phase are also necessary because cells—if necessary—may be directed
towards apoptosis, as it is well known that spermatozoa with damaged DNA are still capable of fertilization, and this be the cause of prolonged effects of oxidative stress, including reproductive disturbances, genetic diseases and neoplasms [20].

In our experiment, administration of vitamin E had a protective effect on the seminiferous epithelium, as demonstrated by the lowering of degeneration and restitution of the regular course of spermatogenesis. The effective prevention against injury to the seminiferous epithelium in ozonized rats may be connected with the stabilizing activity of vitamin E on cellular membranes, which protects the joint between the Sertoli and germ cells [12].

We found, that the processes of fibrosis and hyalinization what occurred in the ozonized rats receiving 0.5 mg of vitamin E, in those receiving vitamin C, and in those receiving vitamins E and C together, were intensified (especially fibrosis) with an increased dose of vitamin C. The observed thickness of the tubule membranes may indicate disturbances in the cooperation of Sertoli cells and peritubular myoid cells. Such occurrences often accompany regulative and defensive response, and form a barrier to toxic substances of external origin. Perivascular, intertubular, and peritubular fibrosis demonstrate a defence reaction that proceeds as in other tissues. One of the elements of the defense reaction is the proliferation of fibroblasts in a damaged location [21]. In our study, hyalinization of intertubular tissue intensified with an increase in vitamin dose in the rats of the E+C groups. Increased production of glycosaminoglycans (GAGs) and proteoglycans (PGs) has been also considered a defence reaction against the damaging activity of free radicals. The protective activity of GAGs and PGs was shown in different experimental models where oxidation was induced by the presence of additional donors of electrons—ions of transition metals (e.g. Cu and Fe) [22]. Our results suggest that ascorbates, being a source of free electrons may evoke the oxidative stress and may provoke similar defensive reactions as seen with iron and copper ions.

In the present study, inhibition or arrest of meiosis was observed in the ozonized animals given injections of both vitamins E and C. This was also demonstrated by a characteristic pyknosis of the chromatin in the nuclei of pachytene spermatocytes [23]. As in the arrest of the G1 phase, these observations indicate that oxidative stress may selectively cause repression of the genes controlling the cell growth cycle. In some types of mammalian cells, an increase in H2O2 was found to evoke periodic inhibition of growth and elongation of the cell growth cycle connected with a lowered ability for replications and divisions. A higher H2O2 concentration led to changes in the type of apoptosis, and eventually led to the death of the cell [24]. Similarly, DNA damage induced by peroxidation and an increased concentration of free radicals accelerated apoptosis of germ cells [24]. Other studies showed that loss of glutathione in human cells provokes arrest of the G2/M phase and inhibits the G1 and S phases by a p53-independent mechanism [26].

Accumulation of LPO damages cellular membranes, not only in situ. The described premature spermiation was caused by destabilization of Sertoli cell membranes, which evokes impairment of their joints with germ cells, loss of spermatocytes and spermatids, and early release of spermatozoa. Sertoli cells and peritubular cells possessing a complete set of active antioxidative enzymes and germ cells are characterised by high activity of the superoxide dismutase (SOD) and low activity of the enzymes responsible for glutathione transition. Due to the lack of protective enzymes, these cells cannot repair the damage caused by ROS, and thus they are especially prone to superoxide- and LPO-induced damage [27].

The fact that the animals receiving both vitamins E and C, unlike those receiving vitamin E alone, exhibited a large amount of seminiferous epithelia damage may indicate that at least part of the changes are connected with the activity of vitamin C. Studies on artificial fertilization of cattle with the addition of ascorbic acid to vitamin E medium demonstrated significantly decreased efficiency of in vitro fertilization [8].

The obtained results indicate that vitamin E exhibits a clear protective action on the rat testes exposed to ozone, probably through stabilization of Sertoli cell membranes. On the other hand vitamin C did not exert the same effect as vitamin E, and furthermore, intensified the damage to the testes evoked by ozone when administrated in higher doses.
References


