STIMULATING AND PROTECTIVE EFFECTS OF RESVERATROL ON BOVINE SPERMATOZOA

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ABSTRACT

As spermatozoa are particularly sensitive towards oxidative damage, the search for a potential antioxidant substance to preserve and protect them under *in vitro* conditions has recently attracted the attention of the scientific community. The aim of this study was to assess the dose- and time-dependent in vitro effects of resveratrol, a natural phenol and phytoalexin with potential antiviral, anti-inflammatory and antioxidant properties on bovine spermatozoa during three different time periods (Time 0 h, 12 h and 24 h). Semen samples were collected from 15 adult breeding bulls, and diluted in physiological saline solution containing 0.5% DMSO together with 0, 1, 5, 10, 50, 100 and 200 µM of resveratrol. Spermatozoa motility was determined using the Sperm Vision^{TM} and CASA (Computer Assisted Semen Analyzer) system. Cell viability was measured using the metabolic activity MTT assay, the nitroblue-tetrazolium (NBT) test was used to assess the intracellular superoxide formation. Spermatozoa motility difference between the control and group A was the only one significant ($P<0.001$) at 0 h, however, significantly ($P<0.001$) increased motility parameters were observed in group D after 12 h and in groups C, D, E and F after 24 h when compared to the control. The MTT assay indicated that none of the resveratrol concentrations had a negative or cytotoxic effect on the spermatozoa mitochondrial activity and furthermore showed a significantly ($P<0.001$) improved cell viability in groups B, C, D, E and F at 24 h. The NBT test showed that the addition of 10 µM resveratrol had an instant positive effect on the spermatozoa protection against free radical production. This protection remained present with a significant impact at 12 h ($P<0.001$) as well as 24 h ($P<0.001$). Furthermore, resveratrol concentrations from 50 to 5 µM exhibited significant ($P<0.05$; $P<0.001$) protective effects on the spermatozoa free radical formation. The results indicate that the addition of resveratrol, especially in concentrations of 50 µM to 5 µM to the culture medium could be beneficial for the overall stimulation of spermatozoa activity and protection against possible *in vitro* oxidative stress development.

Key words: resveratrol, spermatozoa, bulls, motility, CASA, MTT assay, NBT test

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INTRODUCTION

Uncontrolled overproduction of reactive oxygen species (ROS) and the resulting oxidative stress (OS) development has become a serious problem in matters related to male fertility (Agarwal et al., 2003). ROS attacks usually result in a decrease in sperm motility, presumably by a rapid loss of ATP, causing axonemal damage (de Lamirande and Gagnon, 1992), a decrease in sperm viability and an increase in middle piece morphology defects, with deleterious effects on sperm capacitation and acrosome reaction, ultimately leading to infertility.

Studies have shown that antioxidants protect spermatozoa from ROS produced by leukocytes, prevent DNA fragmentation, improve semen quality, reduce cryodamage to spermatozoa, block premature sperm maturation and provide an overall stimulation to the sperm cells (Agarwal et al., 2007). However, the majority of them are still uncontrolled, focus on healthy individuals or have indirect end-points of success. Several other studies are noted due to the quality of their study design, and demonstrate compelling evidence regarding efficacy of antioxidants towards improving semen parameters (Kefer et al., 2009).

Resveratrol (trans-3,5,4'-trihydroxystilbene; RES) is a naturally occurring polyphenol synthesized by a variety of plant species in response to injury, UV irradiation and fungal attack. It is present in grapes, berries, peanuts, as well as in red wine (Savouret and Quesne, 2002). Besides known cardio protective effects, RES exhibits anticancer properties: it suppress cell proliferation, has a growth inhibitory effect, potentiate apoptotic effects of cytokines, chemotherapeutic agents and ionizing radiation as reviewed by Aggarwal et al. (2004). In addition of being an antioxidant and a vasorelaxing agent, it modulates lipoprotein metabolism, inhibits platelet aggregation, and exerts a therapeutic activity. Given the structural similarities of RES to diethylstilbestrol (DES) and estradiol, and its activity as a modulator of the estrogen-response systems, it has been classified as a phytoestrogen (Levenson et al., 2003; Aggarwal et al., 2004).

Regarding male fertility, recent *in vivo* studies in animal models demonstrated that RES administration enhances sperm production in rats by stimulating the hypothalamic–pituitary–gonadal axis without inducing adverse effects (Juan et al., 2003). RES may have a positive effect by triggering penile erection and by enhancing blood testosterone levels, testicular sperm count and epididymal sperm motility, as demonstrated in rabbits (Shin et al., 2008). A protective effect of RES against oxidative damage but not against the loss of motility induced by the cryopreservation of human semen has recently been observed as well (Garcez et al., 2010).

This study was designed to evaluate the dose- and time-dependent effects of resveratrol on bovine spermatozoa activity, viability and free radical formation during a 24 hour *in vitro* cultivation.

MATERIAL AND METHODS

Bovine semen samples were obtained from 15 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The samples had to accomplish the basic criteria given for the corresponding breed. The samples were obtained on a regular collection schedule using an artificial vagina. After collecting the samples were stored in the laboratory at room temperature (22–25°C). Each sample was diluted in physiological saline solution (PS; sodium chloride 0.9 % w/v; Bieffe Medital, Italia) containing 0.5% Dimethyl Sulfoxide (DMSO; Sigma-Aldrich, St. Louis, USA), with various concentrations of RES (trans-resveratrol; Sigma-Aldrich, St. Louis, USA; A – 200; B – 100; C – 50; D – 10; E – 5; F – 1 μM/L) using a dilution ratio of 1:40. The samples were cultivated at room temperature (22–25°C). We compared the control (K) group (medium without RES) with the experimental groups (exposed to different concentrations of RES).

Motility analysis was carried out using the CASA (Computer Assisted Semen Analyzer) system equiped with the SpermVision^{TM} program (MiniTub, Tiefenbach, Germany) and the Olympus BX 51 microscope (Olympus, Japan) at cultivation Times 0 h, 12 h and 24 h. Each sample was placed into the Makler Counting Chamber (depth 10 μm, Sefi-Medical Instruments, Israel) and the percentage of motile spermatozoa (motility>5μm/s; MOT) was evaluated. 1000–1500 cells were assessed in each analysis (Massányi et al., 2008).

Viability of the cells exposed to RES *in vitro* was evaluated by the metabolic activity (MTT) assay (Mosmann, 1983; Knazicka et al., 2012). This colorimetric assay measures the conversion of 3-(4,5-dimetylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT; Sigma, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. Formazan can then be measured spectrophotometerically at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data were expressed in percentage of control (i.e. optical density of formazan from cells not exposed to RES). Results from the analysis were collected during two repeated experiments at each concentration.

The nitroblue-tetrazolium (NBT) test was used to assess the intracellular formation of superoxide radical (Esfandiari et al., 2003). This assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the membrane permeable, water-soluble, yellow-colored, nitroblue tetrazolium chloride (2,2′-bis(4-Nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)ditetrazolium chloride; Sigma, St. Louis, USA) and superoxide radical. Formazan can be measured spectrophotometerically at a measuring wavelength of 620 nm against 570 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data were expressed in percentage of control (i.e. optical density of formazan from cells not exposed to RES). Results from the analysis were collected during two repeated experiments at each concentration.

Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical

characteristics (mean, standard error) were evaluated at first. One-way ANOVA with Dunnett's post test was used for statistical evaluations. The level of significance was set at *** (P<0.001); ** (P<0.01); * (P<0.05).

RESULTS AND DISCUSSION

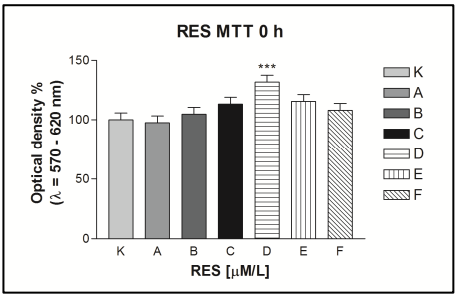
Evaluation of the percentage of motile spermatozoa showed decreased values in all groups cultured for 24 hours (Table 1). Initial (time 0 h) spermatozoa motility was significantly (P<0.001) low in group A cultured with 200 µM RES (54.84±1.01%), when compared to the control group K, cultured without any RES addition (68.07±2.23%). After 12 h, significantly (P<0.001) lower spermatozoa motility values were detected in group A (18.53±2.88%) together with group B (100 µM; 39.52±2.16%), but a significantly higher spermatozoa motility was observed in group D (10 µM; 67.91±3.49%) in comparison with the control (53.09±1.62%). The highest inhibitory effect of the spermatozoa motility after 24 hours was detected in group A (2.25±0.51%), which was significant when compared to the K group (40.69±3.07%). A significant (P<0.001) motility stimulation was found in groups C (50 µM RES; 52.99±2.84%), D (57.17±1.79%), E (5 µM RES; 56.19±2.58%) and F (1 µM RES; 52.79±3.49%).

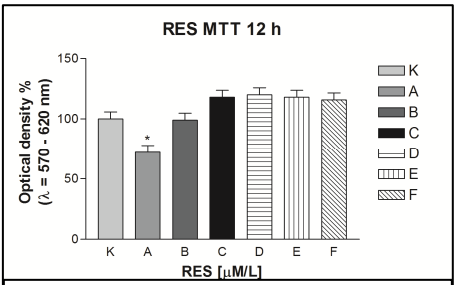
Tab. 1 Spermatozoa motility (MOT; %) in the presence of resveratrol in PS during different time periods (Mean±SEM; n=15)

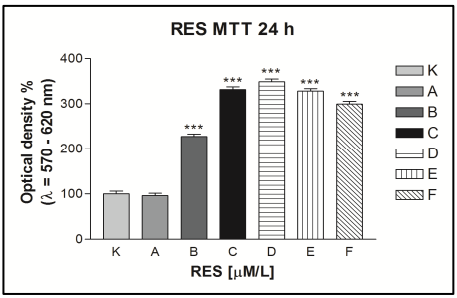
K	A	B	C	D	E	F
0 h						
68.07±2.23	54.84±1.01*** *	64.80±1.73	72.75±2.39	72.64±3.73	73.46±1.59	65.23±4.57
12 h						
53.09±1.62	18.53±2.88*** *	39.52±2.16*** *	59.38±2.22	67.91±3.49*** *	57.14±1.45	57.09±3.15
24 h						
40.69±3.07	2.25±0.51***	24.82±2.73*** *	52.99±2.84*** *	57.17±1.79*** *	56.19±2.58*** *	52.79±3.49*** *

*** (P<0.001); ** (P<0.01); * (P<0.05)

The MTT assay revealed a similar cell viability in all of the experimental groups with a significant (P<0.001) increase in the D group (131.6±4.04%) when compared to the control (100±2.48%; Figure 1). After 12 h the spermatozoa viability increased in all experimental groups. The lowest spermatozoa viability was observed in the A group (P<0.05; 71.95±4.49%; Figure 2). Spermatozoa viability increased significantly (P<0.001) in all of the experimental groups after 24 h, with the exeption of the A group, which was however similar to the cell viability of the control group (95.76±5.47% and 100±3.82%, respectively; Figure 3).

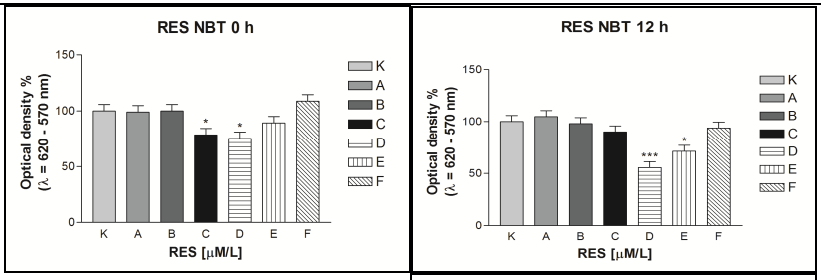






*Figs. 1, 2, 3. Effect of various doses of RES on the viability of spermatozoa at 0, 12 and 24 h. Each bar represents the mean (±SEM) optical density as the percentage of controls (n=15), which represent 100%. The data were obtained from two independent experiments. The control group received PS without RES administration. Groups: A – 200 μM/L; B - 100 μM/L; C – 50 μM/L; D - 10 μM/L; E – 5 μM/L; F – 1 μM/L RES. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05.*

The NBT test revealed that 50 and 10 μM of RES had an instant and significant (P<0.05) protective effect against superoxide production in the sperm cells (Figure 4). The positive effect of the D group remained persistent and significant (P<0.001) over the course 12 h and was subsequently joined by the E group (5 μM; P<0.05; Figure 5). After 24 h, experimental groups with the addition of 100 to 5 μM of RES exibited a long-term and significant (P<0.05; P<0.001) antioxidant protection of the sperm cells and prevention of the escalating intracellular superoxide production, with a special positive effect in case of the groups D (37.02±3.02%) and E (41.37±3.70%) when compared to the control group (100±1.01%). The highest concentration of RES proved to be significantly (P<0.001) stimulating towards superoxide formation and hereby causing a higher risk of oxidative stress development (135.20±2.05%; Figure 6).



*Figs. 4, 5, 6. Effect of various doses of RES on the superoxide production at 0, 12 and 24 h. Each bar represents the mean (±SEM) optical density as the percentage of controls (n=15), which represent 100 %. The data were obtained from two independent experiments. The control group received PS without RES administration. Groups: A – 200 μM/L; B - 100 μM/L; C – 50 μM/L; D - 10 μM/L; E – 5 μM/L; F – 1 μM/L RES. The level of significance was set at *** P<0.001; ** P<0.01; * P<0.05.*

Resveratrol has emerged as an agent with very complex biological activity. Due to its remarkably broad range of effects, especially with respect to cardiovascular, anticancer, antiinflamatory protection, and longevity, RES has attracted numerous researchers and widespread consumer interest (Calabrese, 2010).

According to numerous *in vivo* studies, RES, if supplemented, is well absorbed, rapidly metabolized, mainly into sulfo and glucuronides conjugates which are eliminated in urine. RES seems to be well tolerated and no marked toxicity was reported (Cottart et al., 2010).

However, substantial *in vitro* studies show, that RES has a dose-dependent diverse activity on the cultivated cells. This compound displays an important dichotomy: low doses improve cell survival, as in cardio-and neuro-protection, yet high doses increase cell death, as in cancer treatment (Brown et al., 2009).

The results of Szende et al. (2000) show a dose-dependent effect of RES on cultivated normal endothelial cell. The cell number in culture decreased drastically at 10 and maroly 100 mg/mL concentration of RES for 24 to 48 h. One mg/mL of RES exerted a slight antiproliferative effect. However, a significant, well-measurable proliferation promoting effect on cells in culture for 24 to 48 h was detected if the amount of RES applied was very small. Mitotic index was practically zero at high doses of RES and at the small dose (0.1 mg/mL) the mitotic index exceeded the control value for 24 to 48 h. The apoptotic index decreased with the decrease of the concentration of RES and this change was near to zero at the 0.1 mg/mL dose. The authors suggest that RES may be a natural concentration-dependent formaldehyde-capturing molecule. It seems that RES facilitated the elimination (collection) of uncontrolled formaldehyde from tissues. The first step in this

elimination could lead to the formation of reaction products (hydroxymethyl derivatives), which may promote apoptotic activity.

Holian and Walter (2001) evaluated the viability and proliferation of cultured normal human keratinocytes exposed to RES. They found that RES, even at submicromolar concentrations, inhibited the proliferation of these cells in vitro and, at higher concentrations, was cytotoxic to them.

Zou et al. (1999) investigated the effects of RES on proliferation and cell-cycle control of cultured smooth muscle cells. RES reduced their proliferation in a dose-dependent manner, with concentrations of 50-100 μM RES resulting in 70-90% reduction of the cell proliferation. In this case, the authors assumed, that the antimitogenic effects of RES were not mediated by the induction of apoptosis, but appeared to relate to a G1/S phase block in cell cycle.

Regarding the male reproductive system, Forgacs et al. (2005) examined the effect of various RES concentrations on the viability and activity of the 48 h mouse Leydig cells culture. RES cytotoxicity appeared only at the 200 μM concentration. The basal testosterone level showed a mild (2.5 times), but significant increase in the presence of 12.5-50 μM of RES. Interestingly, RES caused a biphasic effect on the human chorionic gonadotropin (hCG) simulated testosterone production. Their results showed that RES had a small additive effect at lower concentrations (3 μM) whereas at higher concentrations (>25 μM) it turned to be an inhibitor of the testosterone production. Similar effects were observed by Juan et al. (2005) in his *in vivo* experiments, where RES increased the serum testosterone. Based on the presumptions of the authors, one possible way could be through the LH (luteinizing hormone) receptor. Since RES is an estrogen-like molecule, and its effect on estrogen receptor has been proven, there is another possibility that RES modify the hCG-LH response through estrogen receptors (Levenson et al., 2003; Aggarwal et al., 2004). The authors state, that RES has an additive effect (at low concentration) to hCG stimulated testosterone production and suggest that RES had an alternative route to the LH-receptor mediated way, which could be the mentioned receptor-cross-talking or an LH-receptor independent increasing of cAMP-level.

The aim of our study was to analyze the effects of resveratrol on the bovine spermatozoa viability and free radical production *in vitro*. Our results agree with those presented by Collodel et al. (2010) who evaluated the effects of RES on human spermatozoa and rat germinal cells. According to the authors, RES at 100 μM exerted cytotoxic activity against both cell models and it acted in a dose-dependent manner. The LD50 for both models was 50 μM, whereas spermatocytes were more sensitive to the harmful effect of RES with a LD50 was between 50 and 30 μM. In addition to the viability, the authors examined the effects or RES on the motility of swim-up selected sperm. At RES of 100 μM, the motility was absent in all analyzed samples. Progressive motility reached high values at 6 and 15 μM of RES.

In our case, RES concentrations of 200 and 100 μM primarily did not have lethal effects on the spermatozoa viability, however, its effects suppressed the spermatozoa activity parameters. This

observation was apparent especially when analyzing the NBT parameters. 200 µM of RES induced a significant decrease of the motility together with a significant increase of the superoxide formation. 100 µM of RES had similar effects, even though less radical. Although the exact mechanism of RES toxicity has not been defined yet, elevated concentrations of RES were shown to inhibit the F1 complex of the F0/F1-ATPase proton pump of the mitochondrial inner membrane, responsible for the synthesis of ATP from ADP in the oxidative phosphorylation pathway (Zheng and Ramirez, 2000). Since mitochondria are recognized organelles for aerobic production of energy and bear a central role in cellular metabolism, especially in cells and tissues with high metabolic activity (Lagouge et al., 2006), it is not surprising that their disfunction leads to a decrease of spermatozoa viability. Also, the ability of RES to interfere with the machinery of mitochondrial electron transport could be partially responsible for the depolarization of the mitochondrial membranes and a subsequent cell apoptosis (Shaman et al., 2006). The most probable apoptotic path activated in this case could be the type II pathway associated with the activation of caspase-9 independently of Fas signaling (Dorrie et al., 2001) or via a novel mitochondrial mechanism controlled by Bcl-2 (Tinhofer et al., 2001).

Interesting results were obtained, when the RES concentration was 200 µM. All of the viability markers of the bovine spermatozoa decreased significantly immediately after the semen samples were diluted in the medium. In this case, it is obvious that the RES toxicity can not be explained by a molecular mechanism, since the time for interactions between RES and the cellular structures was too short. However, we did observe that the spermatozoa cultivated in the medium containing 200 µM of RES, had coiled tails, which, together with a severely impaired viability, are signs of the spermal osmotic shock (Khan and Ijaz, 2008). Based on this observations we may assume that a RES concentration of 200 µM, apart from being toxic, changes the osmotic pressure of the medium, which subsequently leads to an altered plasma membrane integrity and a decreased viability of spermatozoa.

Apart from the cytotoxic effects of high RES concentrations, our results show a significant stimulation of all the spermatozoa viability parameters when the concentrations of RES were between 50 and 5µM). Based on these results we conclude that lower concentrations of RES have beneficial effects on the overall spermatozoa viability and antioxidant status. This conclusion is supported by a large body of evidence from *in vitro* and *in vivo* studies indicating that RES may be favorable to many health aspects. One of the biological activities of RES is its antioxidant potential, since RES is able to reach peroxidized rigid membranes and increase membrane fluidity in order to interact more efficiently with radicals in the altered lipid bilayer (Brittes et al., 2010). Therefore, RES exhibits a protective effect against lipid peroxidation in cell membranes and DNA damage caused by ROS (Frèmont, 2000). Also, Lagouge et al. (2006) proved that the effects of small concentrations of RES were associated in with an induction of genes for oxidative phosphorylation and mitochondrial biogenesis, therefore stimulating mitochondrial functions of the cell, from what we may conclude that small concentrations of RES, apart from being antioxidant, could mobilize the spermatozoa energetic metabolism and therefore improve spermatozoa viability.

CONCLUSIONS

Our results, even though being preliminary, support evidence of the dose-dependent activity *in vitro* and the scavenger potential of low concentrations RES against oxidative stress induced in bovine spermatozoa. The development of new spermatozoa culture media that can better protect sperm from the ROS damage and improve their energy requirements is absolutely required. RES, in small concentrations, could be used as a ROS scavenging and a metabolic promoting supplement, especially in techniques such as IVF-ICSI or cryopreservation of semen (Tremellen, 2008; Garcez et al., 2010). These results obviously cannot foresee an *in vivo* outcome, since the effect of RES supplementation on male infertility has not yet been explored. To translate this findings into clinical reality, studies on the potential toxicity, pharmacokinetics and bioavailability of RES in the organism, followed by clinical trials, are definitely needed.

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