In Vitro Effect of Resveratrol Supplementation on Oxidative Balance and Intercellular Communication of Leydig Cells Subjected to Induced Oxidative Stress

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Many studies have revealed that oxidative stress is a primary factor in the pathogenesis of male reproductive system dysfunctions. The strong antioxidant and cytoprotective effects of resveratrol have previously been demonstrated, but its effect in the context of the male reproduction remains unconvincing. To observe the biological activity of resveratrol in protecting the male reproductive function, hydrogen peroxide-induced oxidative stress in Leydig cells was used as a cell model. The aim of the present study was to examine if resveratrol could induce changes in the gap junction intercellular communication (GJIC), nitric oxide production, total oxidant status (TOS) and total antioxidant capacity (TAC) in TM3 Leydig cells subjected to H2O2. The Leydig cells were exposed to a resveratrol treatment (5, 10, 20, 50 and 100 μM) in the presence or absence of H2O2 (300/600 μM) during a 24 h in vitro culture. The cell lysates to assess TOS and TAC, NO production were quantified in a culture medium using the Griess method, and the Scrape Loading/Dye Transfer (SL/DT) technique was used for the determination of GJIC in the exposed TM3 Leydig cells. Treatment with higher doses of resveratrol alone led to a significantly increased TOS (p<0.05 with 100 μM) and NO production (p<0.05 with 50 μM and 100 μM), but significantly reduced TAC (p<0.01 with 100 μM) and GJIC (p<0.05 with 100 μM), while the SL/DT evaluation in the cells exposed to resveratrol at concentrations 5 μM (p<0.05) and 10 μM (p<0.01) revealed a significant stimulation of GJIC. The most potent cytoprotective or stimulatory effect of resveratrol in the cells co-exposed to oxidative stress (300 μM H2O2) was observed at a concentration of 10 μM in the case of GJIC, which was manifested by a significant increase in the values (p<0.05) compared to the control group treated with H2O2 alone.

Key words: Antioxidant capacity, gap junction, male reproduction, nitric oxide production.

Oxidative stress (OS) is a result of an imbalance between the effect of an overproduced reactive oxygen species (ROS) and the ability of a biological system to promptly detoxify (through the available intracellular and extracellular antioxidant defences) the intermediates or to initiate repair processes in the biomolecules. Under normal conditions, prooxidants and antioxidants in the cells and tissues remain in balance (AGARWAL et al. 2014). However, in the context of male reproduction, OS is becoming more commonly associated with different dysfunctions in the male reproductive system (PASQUALOTTO et al. 2000). Different pathologies in the testes including infection, alcohol toxicity, inflammation, ischemia-reperfusion injury, cryptorchidism, endocrine disruption by environmental compounds, and impairments from UV radiation are associated with ROS overgeneration, while a prolonged exposure to ROS and the resultant cumulative oxidative damage is thought to be one of the main causes of aging (DIEMER et al. 2003).
A high amount of ROS can impair male fertility not only directly by developing OS in the germ cells, but also indirectly by affecting the Leydig cells and the hypothalamic axes for the release of male sex hormones (Hardy et al., 2005; Spiers et al., 2015). Potential oxidative alterations in the mitochondrial membrane, modifications of the DNA in Leydig cells and the impairment of testosterone release all may occur as a result of ROS overproduction (Chen et al., 2010). Also, LH sensitivity is decreased by reducing the LH receptors in Leydig cells, with a diminished ability of LH to induce the steroidogenic acute regulatory (StAR) protein which transports cholesterol from the outer mitochondrial membrane to the inner membrane (Diemer et al., 2003). ROS overgeneration and the condition of oxidative stress may play a role in age-related testicular degeneration, which is again associated with decreased reproductive functions (Koksal et al., 2000). The testicular Leydig cells’ susceptibility to ROS in vivo is defined by their close proximity to interstitial macrophages (Hales et al., 1999), but ROS are also produced continuously intra-cellularly as by-products in the reactions of microsomal and mitochondrial electron transport and other metabolic processes. In addition, the enzymes of steroidogenic cytochrome P450 in the Leydig cells also generate ROS as intermediates in the mechanism of their catalytic reactions (Quinn & Payne, 1985; Hornsby et al., 1987).

Gap junction intercellular communication (GJIC) is an important type of cell-to-cell communication that maintains the balance in different tissues. Gap junctions are composed of transmembrane proteins called connexins (Cxs) (Chen et al., 2018) and play an important role in the exchange of ions and molecules up to a molecular mass of 1 kDa, including exchanges of Na, K, Ca, cyclic AMP and ATP between neighbouring cells (Herve et al., 2007). The most abundant and ubiquitously expressed gap junction protein, Connexin 43, is necessary for healthy testicular development and spermatogenesis, and its expression is correlated with various reproductive anomalies or disorders in males including male subfertility or infertility (Cyr, 2011; Yawer et al., 2020). In adult males, Connexin 43 is predominantly localised at the region of the blood-testis barrier and among Leydig cells within the interstitial tissue. In several studies on humans, rodents, and non-rodent species such as stallions, European bison and pigs, alterations in Cx43 expression in the testes have been documented and associated with impaired reproductive functions (Chojnacka et al., 2016). TM3 cells express the Cx43 couple through intercellular gap junctions in a manner similar to that of freshly dissociated Leydig cells from the mouse testis, while GJIC may be a modulator of hormone secretion (Goldenberg et al., 2003). It has been suggested that the Cx43 gap junctions in Leydig cells may coordinate the androgenic secretory activities of these cells associated with specific stages of the seminiferous epithelium.

Studies on the TM3 Leydig cell line have demonstrated that increased testosterone secretion in response to LH is associated with reduced intercellular communication, suggesting that gap junction mediated coupling could be the modulator of hormone secretion in the Leydig cells and consequently of spermatogenesis (Pointis et al., 2010). Thus, testicular GJIC and connexin dysregulation, especially during the critical early stages of development, in many cases may participate in testicular etiopathology (Herve et al., 2007). The connexin genes regulate many of the cellular responses to oxidative stress and inflammation, while there is a direct link between an increased concentration of reactive oxygen species and impaired GJIC through the gap junctions, with extensive studies showing that there are many bioactive compounds that can repair or improve GJIC (Halliwell & Gutteridge, 2015).

Currently, there is an increasing interest in the development of natural phytopharmaceutical agents, as their usage is considered more beneficial than synthetic chemotherapeutic or chemopreventive compounds (Baur & Sinclair, 2006). It is well known that the antioxidants in biological systems can scavenge and dispose of ROS, suppress their production and act to oppose ROS actions. Many studies have revealed that antioxidant supplementation is strongly associated with an improvement of the reproductive capacity in males of different species, with a higher semen quality and lower incidence of DNA damage in the sperm (Schmid et al., 2012). Studies related to antioxidant supplementation have also shown an increase in fertilisation rates by reducing the ROS levels, oxidative stress and lipid peroxidation (Eskenazi et al., 2005; Zareba et al., 2013).

Trans-resveratrol (3,5,4'-trihydroxystilbene) is a natural polyphenol that normally occurs in grapes and other plants. This compound has attracted intense interest due to its ability to provide protection against cardiovascular disease, ischemic injury and different inflammatory processes, as well as to extend the lifespan and an ability to inhibit the growth of tumours (Baur & Sinclair, 2006). Resveratrol has broad spectrum of bioactive properties, with its anticancer, antiaging and antioxidative effects being the most commonly studied (Svecnikov et al., 2009). The mechanism of the resveratrol cytoprotective properties is still not fully understood, but it has been observed to affect the activation of some proteins and genes. It involves an increase of AMP-activated kinase, Sirtuin 1 and Peroxisome proliferator-activated receptor gamma coactivator-1 alpha activity (Baur & Sinclair, 2006; Lagouge et al., 2006; St-Pierre et al., 2006). Several studies have demonstrated the ability of resveratrol to activate the pathways of survival, such as mitogen-activated protein kinases and the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (Aggarwal et al., 2004; Kundu et al., 2004; Holme & Peruaiz, 2007). However, as numerous experiments to date have shown,
the biological outcome of resveratrol depends on the concentration and form of the resveratrol that is used, and also on the type of exposed cell (Kairisalo et al. 2011). Although resveratrol has a demonstrable ability to improve fertility in a mouse model of reproductive capacity (Ungvari et al. 2009; Liu et al. 2013), it has been characterised as a phytoestrogen because of its property to bind to the androgen and oestrogen receptors and to develop mixed agonist and antagonist effects on them (Gehm et al. 1997; Bowers et al. 2000). Because of the oestrogen-like effect of resveratrol, several studies have indicated that it may negatively affect hormone-dependent cells (Basly et al. 2000). Our previous study emphasised that resveratrol is able to prevent a decline of the Leydig cell viability parameters and their functional activity, including androgens biosynthesis, in conditions of H2O2-induced oxidative stress. Our data suggested a protective effect of resveratrol at lower concentrations, while high concentrations led to cytotoxicity, which indicates a biphasic, hormetic response in biological systems (Greifova et al. 2020). Based on the inconsistent results of different studies, there is a need to further examine the activity of resveratrol on Leydig cells under normal conditions, as well as under the conditions of induced oxidative stress, with respect to its potential antioxidant and cytoprotective activity in the context of male fertility.

Materials and Methods

Cell culture, treatment and sample preparation

A TM3 Leydig cell line (ATCC, Manassas, VA, USA) derived from mouse Leydig cells was used as the cell model in this study. The cells were cultivated in an Dulbecco’s modified Eagle’s medium/F-12 (DMEM/F-12; Sigma-Aldrich, St. Louis, USA) supplemented with 5% horse serum (A&E Scientific, PA, USA), 2.5% foetal bovine serum (FBS; Merck, Berlin, Germany), 2.5 mM L-glutamine (Sigma-Aldrich, St. Louis, USA) and 1% penicillin/streptomycin (Invitrogen, CA, USA), and were maintained in a humidified incubator with 5% CO2 at a temperature of 37°C in a standard T75 cell culture flask (Corning, NY, USA) until reaching a 80-90% confluent monolayer. Before exposition, the cells (1x10^6 cells/well) were seeded into 96-well plates and were pre-cultivated for 24 h without the tested compounds. To determine the effect of resveratrol alone, as well as resveratrol in the condition of H2O2-induced oxidative stress, the cells were cultured for 24 h in a medium containing 300 or 600 μM H2O2 (30% (w/w) in H2O; Sigma-Aldrich, St. Louis, USA) diluted to the desired concentrations with a culture medium, with the presence (experimental groups) or absence (H2O2 control group) of resveratrol (99%, Sigma-Aldrich, St. Louis, USA) diluted to the tested concentrations (5, 10, 25, 50 and 100 μM). The ranges of the H2O2 and resveratrol concentrations were selected according to the results of our pilot range-finding experiments.

The sample preparation involved collecting the exposure medium from the culture plates after 24 hours of exposure to determine the NO production. The cells themselves that adhered in the wells of the culture plates after the exposure were used in the intercellular communication assay. A n analysis of the total antioxidant capacity and the total oxidant status of the cells was preceded by the preparation of cell lysates. Cells that adhered to the culture plates were washed with DPBS, followed by the application of a mammalian cell lysis buffer (Mammalian Cell Lysis Buffer, GoldBio, St. Louis, USA) supplemented with a protease inhibitor (Protease Inhibitor Cocktail, Sigma-Aldrich, St. Louis, USA) to reach a final concentration of 0.1% in the lysis solution. Lysates including the cell debris were then centrifuged (20,000 x g, 30 min) and pure lysates were used in the further assays. Each experiment was performed four times independently, with cells from different passages.

Determination of the total oxidant status (TOS) of the cells

Our samples for this experiment were cell lysates made after 24 hours of culture with the exposure substances. We followed the individual steps of the protocol according to Erel (2005). In the first step, we prepared the reaction solutions. Reaction Solution 1 consisted of 150 μM xalenol orange (Xylenol Orange disodium salt, Sigma-Aldrich, St. Louis, USA), 140 mM NaCl (Sodium chloride, Sigma-Aldrich, St. Louis, USA) and 1.35 M glycerol (Sigma-Aldrich, St. Louis, USA) in 25 mM of H2SO4 (Sulfuric acid, Sigma-Aldrich, St. Louis, USA). Reaction Solution 2 contained 5 mM ammonium ferrous sulphate hexahydrate (Centralchem, Bratislava, SR) and 10 mM o-Dianisidine dihydrochloride (Sigma-Aldrich, St. Louis, USA) in 25 mM of H2SO4 (Sulfuric acid, Sigma-Aldrich, St. Louis, USA). The experimental samples and standards were pipetted in triplicate into 96-well plates. After adding Reaction Solution 1, we immediately measured the first absorbance (560 nm). Subsequently, Reaction Solution 2 was added and after 3-4 minutes, the second absorbance of the samples was measured at a wavelength of 560 nm using a Glomax Multi Detection System plate reader (Promega, Madison, Wisconsin, USA). The test was calibrated with hydrogen peroxide and the results were expressed in micromoles of H2O2 equivalent per litre.

Determination of the total antioxidant capacity (TAC) of the cells

The method for evaluating the total antioxidant capacity was based on the oxidation of the colourless reduced molecule 2,2’-azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS) to the characteristically coloured, blue-green
radical cation ABTS\(^+\) using hydrogen peroxide in an acidic reaction medium (pH 3.6). Our samples for this experiment were cell lysates prepared after 24 hours of culture with the tested substances and we followed the steps of the protocol according to EREL (2004). In the first step, we prepared the reaction solutions. Reaction Solution 1 was a 0.4 M acetate buffer (0.4 M glacial acetic acid, Centralchem, Bratislava, SR) +0.4 M sodium acetate solution (Anhydrous sodium acetate, Centralchem, Bratislava, SR) with a pH of 5.8. Reaction Solution 2 was prepared as a 2 mM solution of H\(_2\)O\(_2\) (30% H\(_2\)O\(_2\), Sigma-Aldrich, St. Louis, USA) in a 30 mM acetate buffer (pH 3.6), in which ABTS (2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diaminonium salt, Sigma-Aldrich, St. Louis, USA) was dissolved to reach a final concentration of 10 mM. After a one-hour incubation at room temperature, the characteristic blue-green colouring of ABTS\(^+\) developed. The samples and standard solutions were pipetted in triplicate into 96-well plates with Reagent 1, followed by a measurement of the first absorbance (660 nm). Subsequently, Reaction Solution 2 was added, and after 5 minutes of incubation, the second absorbance of the samples was measured spectrophotometrically at a wavelength of 660 nm using a Glomax Multi + Detection System plate reader (Promega, Madison, Wisconsin, USA). The assay was calibrated with Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma-Aldrich, St. Louis, USA). The resulting values were expressed as milimoles of Trolox equivalent per litre.

Determination of the nitric oxide (NO) production

Due to the very short half-life of NO, the direct measurement of NO in biological systems is complicated, as NO is rapidly metabolised to nitrites and nitrates in the presence of oxygen. Therefore, the indirect method for measuring nitrites and nitrates (NO\(_x\)) in the samples is most often used to determine NO production. The amount of NO produced by the exposed cells after 24 hours of culture was determined according to LYLE et al. (2009). The test was based on the quantification of nitrites (NO\(_2\)) in the supernatant (in our case in the exposure medium, which was tested immediately after 24 hours of cultivation) using the Griess reagent.

The Leydig cells were seeded into 6-well plates, followed by culturing with the tested substances for 24 h. After 24 hours of preculturing, the culture medium was changed to the MEM medium (Minimum Essential Medium Eagle, Sigma-Aldrich, St. Louis, USA) without phenol red and supplements to minimise the presence of compounds which could lead to a misinterpretation of the results. After a filter the exposure, the medium was collected and immediately centrifuged (10,000 x g/15 min) and pipetted in triplicate into a microtiter plate in a volume of 100 \(\mu\)l per well. A n equal volume (100 \(\mu\)l) of the Griess reagent (1% sulphanilamide (Sigma-Aldrich, St. Louis, USA), 0.1% naphthylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, USA) in a solution of 2.5% phosphoric acid (Sigma-Aldrich, St. Louis, USA)) was added to the samples, followed by incubation in the dark for 15 min at room temperature. Subsequently, we measured the absorbance of the samples at 550 nm using an ELISA plate reader (ULTimass FC M Microplate PhotoMeter, Thermofisher-Scientific, Waltham, USA). The nitrite concentration in the samples was determined based on the standard solution of NaNO\(_2\) (Sodium nitrite, Centralchem, Bratislava, SR) in a MEM medium in the range of 0-60 \(\mu\)M and expressed as a micromolar nitrite concentration per 2 x 10\(^5\) cells per 24 hours.

Determination of the gap junction intercellular communication

The determination of gap junction intercellular communication (GJIC) using the SL/DT (Scrape Loading/Dye Transfer) technique is a simple functional test that evaluates intercellular communication through interstitial cell junctions in a larger cell population. The principle of this method is the transfer of small molecules (M<900) with cytoplasmic membrane impermeable dyes in viable cells, which are monitored during their intercellular movement through the gap junctions. The dye is caught in the cytoplasm of the damaged cells and is then moved into neighbouring cells connected by functional intercellular connecting channels. The dye transfer is monitored and quantified by fluorescence microscopy, where the amount of dye transferred from one cell to another cell that it is in contact with depends on the number of gap junctions. The distance, or area, over which the dye diffuses over a period after the cell monolayer section is made is a quantitative measure of the GJIC capacity. We followed the protocol specified by BABICA et al. (2016).

The cells for this experiment were seeded and cultured in 6-well plates. 24 hours of preculture and the formation of a dense monolayer of cells was followed by treatment with the exposure substances for a further 24 hours. After the exposition, the culture medium was removed from the wells and the cells were washed with DPBS (Dulbecco’s Phosphate Buffered Saline, Sigma-Aldrich, St. Louis, USA) with the addition of Ca\(^2+\) and Mg\(^{2+}\) (pH 7.2). The low molecular weight luciferase yellow (Sigma-Aldrich, St. Louis, USA) fluorescent dye was used for our experiment. Three horizontal sections were made into the cell’s monolayer in the wells and the cells were incubated with the dye in the dark for 6 minutes. Staining was followed by the fixation of the cells with a 4% formaldehyde solution (Formaldehyde, Sigma-Aldrich, St. Louis, USA). The sections and dye transfer through the cells were evaluated by fluorescence mi-
crosscopy based on the measured area of the diffused fluorescent dye through the gap junction channels using ImageJ software (National Institutes of Health, Maryland, USA).

Statistical analysis of the data

The data for the statistical analyses was obtained from at least four independent experiments (4 repetitions x 16 samples for each experimental group) for each method. The statistical analyses were conducted using the GraphPad Prism program (version 5 for Windows; GraphPad Software, La Jolla, CA, USA, www.graphpad.com). The normality of the variables was assessed using the Shapiro-Wilk normality test followed by the one-way analysis of variance (ANOVA). Dunnett’s test was used as a follow-up test to the ANOVA, based on a comparison of every mean to a control mean, and by computing a confidence interval for the difference between the two means. The level of significance was set at ***(p<0.001); **(p<0.01); *(p<0.05). The comparative analysis was performed as follows: the experimental groups treated with resveratrol alone were compared with the control group without the exposure substances; the resveratrol experimental groups simultaneously subjected to the hydrogen peroxide treatment were compared to the control group exposed to the same concentration of peroxide (300/600 μM H₂O₂ control group).

Results

Total oxidant status

As a parameter of oxidative changes in the cells, the overall oxidative status of the Leydig cells was evaluated. The results of the TOS analysis indicated the antioxidant effect of lower concentrations of resveratrol, when after 24 h of cultivation we observed a slight but not significant decrease in the relevant values in the experimental groups treated with 5 and 10 μM of resveratrol. At a concentration of 25 μM resveratrol, the values of the monitored parameter began to increase, which continued in the cells of the experimental group treated with 50 μM and 100 μM (p<0.05) of resveratrol with significant changes in comparison with the control group (Fig. 1).

In the case of the addition of resveratrol to the Leydig cells which were simultaneously exposed to oxidative stress (300 μM H₂O₂) for 24 h, we observed a very slight decrease in the values of TOS in the experimental groups supplemented with resveratrol at doses of 5 μM and 10 μM. In contrast, statistically significant (p<0.05) elevated values were recorded after an exposure to 100 μM of resveratrol after a comparison with the control cells exposed to H₂O₂ alone. There was also a slight increase of TOS in the cells cultured in parallel with 25 and 50 μM resveratrol (Fig. 1).

After the induction of oxidative stress in the samples exposed to 600 μM H₂O₂ for 24 hours simultaneously with different concentrations of resveratrol, we did not observe statistically significant changes in any of the experimental groups. However, in all the monitored experimental groups, there was an increase in the TOS of the cells (Fig. 1).

Total antioxidant capacity

After 24 hours of in vitro culture with resveratrol alone, the results did not indicate significant changes in the concentrations of 5-50 μM resveratrol, with a slight increase of the values. We observed a statistically significant (p<0.01) decrease in the experimental group that was exposed to the highest dose of resveratrol (100 μM) (Fig. 2).

Fig. 1. The impact of resveratrol alone on the total oxidant status of TM3 Leydig cells and of resveratrol on the total oxidant status of TM3 Leydig cells with H₂O₂ (300/600 μM)-induced oxidative stress after a 24 h exposition. Each bar represents the mean value (±SEM) of the H₂O₂ equivalent concentration (μM) in the experimental groups compared to the control groups (K – untreated cells/cells exposed to 300 or 600 μM H₂O₂). The data was obtained from four (n=4) independent experiments. The level of significance: * p<0.05 between the control and experimental groups.
The TAC values of the cells exposed at the same time to H2O2 together with resveratrol were more or less at the level of the control groups, which were cultured only in the presence of 300/600 μM H2O2. The measured values of the experimental groups with the parallel effect of increasing concentrations of resveratrol did not show statistically significant changes in any of the tested samples (Fig. 2).

NO production

An increased NO production was observed along with increased resveratrol concentrations in the samples. Thus, with a gradual increase of the resveratrol being administered (5, 10 and 25 μM) in the experimental groups, the NO2− values also increased slightly. However, only those cells exposed to the highest concentrations of resveratrol (50 and 100 μM) showed significant (p<0.05) changes after a comparison with the control group (Fig. 3).

The parallel effect of both the tested substances seems to have had a combined stimulatory effect on NO production in the samples. We observed the highest values of the monitored parameter in the samples with the addition of hydrogen peroxide in combination with resveratrol. In the experimental groups that were exposed to 300 μM H2O2 to induce oxidative stress, the administration of resveratrol at the most monitored concentrations (10, 25, 50 and 100 μM) induced NO production, which resulted in increased nitrite concentrations in the samples. However, none of the experimental groups showed statistically significant changes when compared to the H2O2 control group (Fig. 3).

The production of NO by the Leydig cells cultured in the presence of 600 μM H2O2 together with resveratrol was affected, as in the previous experiments, in
terms of an increase in the related values in all the monitored samples with resveratrol. Compared to the H$_2$O$_2$ control group, only the experimental group with 50 $\mu$M of resveratrol achieved statistically significant changes (p<0.05) (Fig. 3).

Gap junction intercellular communication

The level of inhibition or stimulation of intercellular communication by the gap junctions was measured after a medium supplementation with resveratrol alone for 24 h of culture. A significant improvement of this parameter occurred even at the lowest concentration of resveratrol (5 $\mu$M; p<0.05); while at a concentration of 10 $\mu$M, the upward trend continued with a statistically significant (p<0.01) increase in the values. On the contrary, an inhibitory effect of resveratrol was observed at higher concentrations, while at the dose of 100 $\mu$M resveratrol the activity of GJIC decreased significantly (p<0.05) (Fig. 4 and Fig. 5).

The protective effect of resveratrol in the cells with induced oxidative stress (300 $\mu$M H$_2$O$_2$) was also observed in terms of intercellular communication when compared to the H$_2$O$_2$ control group. There was a significant increase of the values in the samples co-treated with 10 $\mu$M resveratrol (p<0.05). GJIC was inhibited after coculturing with 100 $\mu$M resveratrol, when intercellular communication was significantly (p<0.05) reduced compared to the control (Fig. 4, Fig. 6). For this parameter, we omitted the experiment with a higher dose of hydrogen peroxide (600 $\mu$M) with the parallel action of different resveratrol concentrations due to the low density of adhered viable cells after the H$_2$O$_2$ exposure at this concentration, which could lead to a misinterpretation of the results.

Fig. 4. The impact of resveratrol alone on the gap junction intercellular communication of TM3 Leydig cells and of resveratrol on the gap junction intercellular communication of TM3 Leydig cells with H$_2$O$_2$ (300 $\mu$M)-induced oxidative stress after a 24 h exposition. Each bar represents the mean value ($\pm$SEM) of the gap junction intercellular communication as a percentage of the control groups (K – untreated cells/cells exposed to 300 $\mu$M H$_2$O$_2$), which represents 100%, and the data is expressed as a % of the control groups. The data was obtained from four (n=4) independent experiments. The level of significance was set at *** (p<0.001), ** (p<0.01) and * (p<0.05) between the control and experimental groups.

Fig. 5. Gap junction intercellular communication of the Leydig cells after a 24 h resveratrol exposure. Ctrl – control group; A – 5 $\mu$M resveratrol; B – 10 $\mu$M resveratrol; C – 25 $\mu$M resveratrol; D – 50 $\mu$M resveratrol; E – 100 $\mu$M resveratrol.

Fig. 6. Gap junction intercellular communication of the Leydig cells after a 24 h H$_2$O$_2$ (300 $\mu$M) exposure with the parallel action of resveratrol. Ctrl – 300 $\mu$M H$_2$O$_2$; A – 300 $\mu$M H$_2$O$_2$ + 5 $\mu$M resveratrol; B – 300 $\mu$M H$_2$O$_2$ + 10 $\mu$M resveratrol; C – 300 H$_2$O$_2$ + 25 $\mu$M resveratrol; D – 300 $\mu$M H$_2$O$_2$ + 50 $\mu$M resveratrol; E – 300 $\mu$M H$_2$O$_2$ + 100 $\mu$M resveratrol.
Discussion

Hydrogen peroxide occurs in the standard metabolism of mammalian cells, and it is an essential metabolite in oxidative stress (SIES et al. 2017). Its physicochemical properties allow it to act as an effective messenger that carries signals from the site of its generation to a target site in the redox signalling pathways (MARINHO et al. 2014; FORMAN et al. 2010). On the other hand, H₂O₂ is considered to be one of the major ROS, in which overproduction is related to the development of oxidative stress. It is easily diffused into cells and reacts with the metal ions, such as copper or iron, present in the intracellular environment, leading to the production of highly reactive hydroxyl radicals that attack the cellular biomolecules including DNA, lipids and proteins, resulting in different forms of oxidative damage (CHANG et al. 2008). Even though oxidative stress plays an important role in male infertility and male reproductive disorders, it has not been routinely investigated in this respect (SALEH & GARWAL 2002). The process of steroidogenesis in Leydig cells, despite the existence of complicated antioxidant systems in the testicular tissue, appears to be highly prone to oxidative stress damage, with oxidative damage currently being considered as one of the most important aspects of testicular dysfunction (AITKEN & ROMAN 2008). The steroid hormones biosynthesised in Leydig cells are essential for the homeostasis of key physiological functions and control crucial phases of development. The Leydig cells of the testis are mainly responsible for producing testosterone, which is necessary for spermatogenesis. In addition, testosterone is a key hormone for the maintenance of secondary sexual functions in males (WANG et al. 2017). Studies have shown that in most males, there is decreasing trend in the levels of total and bioactive testosterone with aging, even in the absence of pathological conditions (HARMAN et al. 2001).

The supplementation of natural antioxidants as a strategy to avoid or manage the pathological conditions associated with oxidative stress has gained a huge amount of interest in recent years. Bioactive compounds isolated from herbal plants are known to exert a beneficial impact in biological systems by scavenging free radicals and modulating the comprehensive antioxidant defence system. Many in vivo and in vitro studies have suggested that resveratrol has remarkable antioxidant effects in the fight against oxidative stress. As MENG et al. (2020) determined, this effect is mainly attributed to the ability of resveratrol to reduce the lipid peroxidation processes in cells, reduce the number of apoptotic cells, improve the cells’ functional properties and provide effective protection against environmental toxins. Research has also shown that the effect of resveratrol is dose-dependent in various cell models (CABALRESE et al. 2010). However, in terms of reproduction, the previously collected data assessing the impact of resveratrol on male reproductive functions is inconsistent, mainly due to the structural similarities of resveratrol with oestrogen, which places it in the group of phytoestrogens. Its phytoestrogenic nature defines resveratrol’s antiandrogenic properties, because it can enter gene transcription via the oestrogen and androgen receptors, where it acts as an agonist and antagonist of those receptors (BOwers et al. 2000; SUPORNSILCHAI et al. 2005).

Both the results of the present study and the data obtained in our previous study (GREIFOVÁ et al. 2020) indicate that resveratrol exhibits protective effects on the structural integrity of TM3 Leydig cells and their functional activity under physiological in vitro conditions, as well as when there is increased oxidative stress in the TM3 Leydig cells. These parameters also correlate with the results of the quantification of hormone production by the Leydig cells after exposure in this study. In the case of the Leydig cells treated with resveratrol alone, we observed a significant increase in the production of both progesterone and testosterone in the experimental group at a concentration of 10 μM, with the same result occurring even after induction of oxidative stress by 300 μM of hydrogen peroxide. With respect to the higher concentration of hydrogen peroxide (600 μM), both the progesterone and testosterone levels were significantly increased at a concentration of 5 μM resveratrol compared to the H₂O₂ control group, with a more pronounced increase after the exposure to 10 μM resveratrol. Although we cannot unambiguously confirm the antioxidant activity of resveratrol based on the results of the current study, the data suggests a potentially positive effect of low doses of resveratrol under the condition of induced oxidative stress and also under normal culture conditions when evaluating the parameters of total antioxidant capacity, total oxidative status and intercellular communication of the exposed cells, even if those changes are not significant, especially in the case of the TOS and TAC analysis. By contrast, higher doses of resveratrol exhibited a negative impact even on the Leydig cells that were not simultaneously exposed to oxidative stress, indicating a dose-dependent biological activity of resveratrol.

There are several mechanisms of cytoprotective activity for resveratrol in biological systems. It is a very efficient scavenger of free radicals, including the reactive forms of oxygen and nitrogen, as well as the secondary organic radicals which are formed in reactions with ROS and RNS (GERSZON et al. 2014). Another well-documented mechanism of resveratrol’s antioxidant effect is its ability to induce the expression of the enzymes responsible for maintaining the balance of redox reactions in cells. These are mainly SOD, catalase, heme oxygenase and glutathione peroxidase (KINCAID & BOSSY-WETZEL 2013). Resveratrol is further characterised by an effective reduction in the activity of the enzymes that play a crucial role in ROS production, such as xanthine
oxidase (Delmas et al. 2005). Like other polyphenols, it is an effective chelator of metal ions, which is manifested by blocking the Fenton reaction. Resveratrol is even thought to be an effective scavenger of other radicals with a similar structure (OOR), such as peroxy and protein peroxy radicals. Furthermore, it has been shown to effectively remove reactive nitrogen species by reacting directly with peroxynitrite, resulting in a reduction in the nitrosylation of cysteine and tyrosine residues in proteins (Yang et al. 2015). The inhibition of lipid peroxidation in the cell membranes is another essential antioxidant mechanism of resveratrol. In addition, it is able to prevent the oxidation of the polyunsaturated fatty acids present in low-density lipoproteins (LDL) (Gerszon et al. 2014).

The induction of antioxidant enzyme expression by resveratrol is best described in the case of superoxide dismutase. Resveratrol has been shown to activate the PI3K/AKT and GSK-3β-catenin signalling pathways. Resveratrol is also able to translocate to the cell nucleus, where it activates sirtuin 1, which directly stimulates the mitochondrial SOD2 expression (Fukui et al. 2010). Carrizzo et al. (2013) additionally showed that resveratrol increases the mitochondrial superoxide dismutase expression by a mechanism that is dependent on nuclear factor Nrf2, which regulates the cellular resistance to oxidants. Experiments have also documented the activation of the genes for ‘second phase enzymes’, such as glutathione S-transferase, oxidoreductases and NAD(P)H-quinone reductase (Scapagnini et al. 2014).

As was mentioned above, the results of previous studies observing the impact of resveratrol on male reproductive functions are inconsistent. The same cell model was used to investigate the antioxidant effects of resveratrol on nicotine-induced oxidative stress in the MA-10 Leydig cells after the induction of oxidative stress by benzo(a)pyrene during 24 hours of exposure. The results showed an increase in the activity of SOD and GPx, with a concomitant decrease in the concentration of malondialdehyde. Despite the well-described antioxidant biological properties of this polyphenol, it is well known that resveratrol readily undergoes an autooxidative process at high concentra-
tions in the tissue, resulting in the production of H$_2$O$_2$ and a complex mixture of semiquinones and quinones that can be cytotoxic. In addition, the resveratrol molecule can form complexes with copper, which therefore causes DNA fragmentation in the cells (HADI et al. 2010).

NO is generated in most cell types by NO synthases as well as by non-enzymatic reactions, which has also been confirmed in both human Leydig cells and rodent Leydig cells, including the MA-10 and TM3 cell lines (DAVIDOFF et al. 1995). In addition to the normal physiological properties of nitric oxide, an increased NO production may trigger side signalling mechanisms to activate the inflammatory-immune pathways in tissues. The biological activity of NO is regulated at several levels, involving mechanisms that control the localisation and activation of NO synthases and various states of the oxidative metabolism of NO, resulting in the production of bioactive reactive nitrogen species (RNS) (BOVE & VAN DER VLIET 2006). Ultimately, RNS can impair cell functions by the oxidation or nitration of different cellular targets. Tyrosine residues nitration has been considered as a potential mechanism of protein regulation by RNS, which is believed to be of increased significance during inflammation when the prooxidant mechanisms that promote RNS formation are enhanced (ISCHIROPoulos & BECKMAN 2003; RADI 2004). Furthermore, NO is produced within normal testis, where it is believed to be involved in regulating the Leydig cell functions and spermatogenic development. A number of recent in vitro studies have indicated that the inflammatory vasodilator nitric oxide is capable of inhibiting steroidogenesis by the Leydig cells, the granulosa luteal cells and the adrenal cortex (O’BRYAN et al. 2000). The production of NO occurs through the action of one of three nitric oxide synthase (NOS) enzymes. Immunohistochemical studies have revealed that the endothelial isoform is present in both human and rodent Leydig and Sertoli cells (ZINI et al. 1996), and that the neuronal isoform is present in human and rat testes (DAVIDOFF et al. 1995). The administration of a broad-spectrum NO inhibitor to adult rodents resulted in an elevation in serum testosterone levels, indicating that NO is involved in regulating normal testosterone production (ADAMS et al. 1996). In addition, the research unexpectedly found that inducible nitric oxide synthase is actually expressed in normal testis, which suggests a unique role for this induced enzyme in the functions of normal tissue. The NO produced by Leydig cells acts in a paracrine but also in an autocrine fashion, and may influence the activity of the neighbouring Leydig cells, muscle cells and pericytes of the testicular blood vessels and the peritubular/tunica albuginea myofibroblasts. In this way, NO may participate in the regulation of the testicular activity related to the functions of the Leydig cells and the interrelationships between the blood vessel, interstitial, peritubular and probably intratubular compartments of the testis (O’BRYAN et al. 2000). Nowadays, it is well known that there is an age-dependent increase in NO production, which is not only specific to the Leydig cells but also occurs in other tissues, as is documented by a progressive accumulation of nitrite in the serum. Studies have also pointed to the fact that the influence of nitric oxide on the Leydig cell’s functions could be pharmacologically manipulated. These findings are not only clinically relevant, but also open an avenue to further basic and clinical investigations on the effects of different bioactive molecules on steroidogenesis in aging males and the mechanism by which they affect gene expression (SOKANOVIC et al. 2013).

Our results concerning the effect of resveratrol on NO production are inconsistent with the previous findings of BANERJEE et al. (2016), where it was reported that resveratrol down-regulated testicular NO production by suppressing the MAPK and ATF2 activation related to improving the oxidative status of the testis and prevented apoptosis. In accordance with this data, another in vivo study with male albino mice reported a remarkable reduction in NO levels in the cisplatin and resveratrol co-administered group in comparison to the control group with induced testicular damage using cisplatin (SINGH et al. 2017). The research of JALILI et al. (2017) also showed decreasing NO levels in mice with morphine-induced infertility after the administration of resveratrol. However, the therapeutic effect of resveratrol was more related to an increased amount of antioxidants in their study, because there is a presumption that antioxidants can disrupt the system of NO generation (protein, enzymes, substrates and cofactors) (DAS et al. 2005). On the contrary, according to KLINGE et al. (2008), nutritionally relevant concentrations of resveratrol rapidly activate the plasma membrane-associated ER$\alpha$ in HUVECs, leading to NO production via the activation of Gsa, Cav-1, Src and MAPK in a manner similar to that elicited by oestradiol.

The communication between the same cells of the same tissue occurs through very small junctions. This is called gap junctional intercellular communication. The cells share ions and electrolytes through these junctions that are made up of connexin molecules which contain genes (YAMASAKI et al. 1999). The connexin genes regulate many of the cellular responses to oxidative stress and inflammation (TROSKO & RUCH 2002). However, GJIC can be disrupted, especially in cases where there is extensive inflammation, oxidative stress and cellular damage during chronic non-optimal and pathological conditions such as carcinogenesis (STAHL & SIES 2007). Thus, there is a direct link between oxidative stress and impaired intercellular communication through the gap junctions, with extensive studies showing that there are many nutraceuticals and antioxidants that are able to repair or enhance GJIC (HALIWELL & GUTTERIDGE 2015).
The results of our analyses showed that, depending on the concentration, resveratrol can affect the GJIC communication of exposed Leydig cells in a positive sense at low concentrations. In the cells with oxidative stress induced by 300 μM H₂O₂, the cells that were co-exposed to 5 μM resveratrol showed a slight increase in GJIC, while at a concentration of 10 μM there was a significant (p<0.05) increase in these values compared to the H₂O₂ control group. Our results are consistent with the results presented by KIM et al. (2009a) in an experiment with WB-F344 rat liver epithelial cells. After the induction of oxidative stress in cells using H₂O₂ (300 μM), a pretreatment of the cells with resveratrol at concentrations of 5 μM (p<0.05) and 50 μM (p<0.01) showed a significant reduction in the H₂O₂-induced inhibition of GJIC, which was also supported by a blocking of the H₂O₂-induced phosphorylation of ERK1/2 by resveratrol in these experimental groups. The same cell model (WB-F344) was used in another in vitro study in which GJIC was suppressed in the cells by gallic acid with evidence of H₂O₂ overproduction. In that case, resveratrol (50 μM) was able to significantly (p<0.01) stimulate intercellular communication by suppressing the phosphorylation of Connexin 43, an essential protein in GJIC modulation (KIM et al. 2007). UPHAM et al. (2007) reported similar results with resveratrol in relation to intercellular communication. Here, resveratrol (50 and 100 μM) acted preventively in the WB-F344 cells in which GJIC was inhibited by an exposure to dicumyl peroxide, with MAPK suppression occurring after the treatment of the cells with resveratrol.

The issue of the long-term side effects of synthetic medicaments and antioxidants has increasingly been turning attention to the use of natural bioactive substances in the prevention of diseases associated with oxidative stress. Our results indicate that resveratrol, at a low concentration, could represent a promising strategy for reducing the oxidative stress that results in a dysfunction of the male reproductive system associated with damage to the Leydig cells. However, in the context of male reproduction, a further detailed identification of the interactions between resveratrol and the oestrogen and androgen receptors in the Leydig cells, resulting in impaired steroidogenesis and ultimately a decreased testosterone biosynthesis, is required, because maintaining a normal testosterone level is the key to male reproductive functions.

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Author Contributions


Conflict of Interest

We declare that we have no proprietary, financial, professional or personal interest of any nature in any product, service and/or company in relation to the publication of this paper.

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