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RESVERATROL INHIBITS REACTIVE OXYGEN AND NITROGEN SPECIES FORMATION IN RATS' SALIVARY GLANDS AND THEIR FUNCTIONS UNDER ALCOHOL EXPOSURE AND LIPOPOLYSACCHARIDE-INDUCED SYSTEMIC INFLAMMATORY RESPONSE

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Abstract

Objectives: The aim of this study is to investigate the effects of resveratrol on nitro-oxidative stress and the functions of submandibular salivary glands (SGs) in rats under alcohol exposure during lipopolysaccharide (LPS)-induced systemic inflammatory response (SIR).

Methods: The studies were conducted on 21 Wistar rats weighing 205-220 g, divided into 3 groups of seven animals in each: the 1st group, control group I, included animals, who received isotonic sodium chloride solution intragastrically via gavage twice a day; the 2nd group, control group II, included rats exposed to alcohol (in a dose of 24 mg/kg intragastrically twice a day) for last 2 weeks during LPS-induced SIR; the rats of the 3rd group exposed to alcohol during LPS-induced SIR, who also received resveratrol in a daily dose of 5 mg/kg intragastrically. SIR was induced by intraperitoneal administration of *Salmonella typhi* LPS. During the first week a dose of 0.4 μg/kg of body weight was administered 3 times a week; over the next 7 weeks of the experiment rats received 0.4 μg/kg of body weight once a week.

Results: The administration of resveratrol during the experiment led to a considerable decrease in NADPH-induced superoxide anion radical ($^{\cdot}O_2^-$) production in the submandibular SGs tissues in 1.28 times compared to the control group II. LPS-induced generation of this radical by phagocytes fell in 1.35 times. The total and inducible NO-synthase activity in the submandibular SGs tissues was inferior in 1.34 and 1.40 times, respectively. Under the administration of resveratrol, peroxynitrites and S-nitrosothiols concentration was inferior to the control group II in 1.37 and 1.42 times, respectively. The administration of resveratrol resulted in the changes in alpha-amylase activity and aquaporin-5 concentration in the submandibular SGs tissues.

Conclusion: The use of resveratrol under the combined administration of 40% ethanol solution and LPS considerably limits the development of nitro-oxidative stress in the submandibular SGs tissues. Resveratrol considerably improves the functional status of the submandibular SGs, enhancing the activity of alpha-amylase and concentration of aquaporin-5.

Keywords: resveratrol, alcohol intoxication, systemic inflammation, reactive oxygen and nitrogen species, salivary glands

Introduction

Total alcohol per capita consumption in the world's population over 15 years of age had risen from 5.5 litres of pure alcohol in 2005 to 6.4 litres in 2010, and was still at the level of 6.4 litres in 2016. The highest levels of per capita alcohol consumption are observed in countries of the European Region [1]. The destructive alcohol consequences are among the leading risk factors for population health worldwide and have a direct impact on many health-related conditions, including non-communicable diseases and mental health, infectious diseases, injuries, poisonings etc. Clinical and pathomorphological studies point out the potential alcohol damage to salivary glands (SGs) causing sialadenosis and its complications (dysarthria, dysphagia, dysgeusia, dental caries, periodontal diseases, oral candidosis, acute purulent sialodenitis, etc.) [2-4].

Recent reports have demonstrated the alcohol intake considerably changes NF-kappa B-signalling pathway [5, 6], which uninterrupted functioning is as an important link of pathogenesis, nitro-oxidative stress and chronic low-grade inflammation, which are also known as links to pathogenesis of a number of highly prevalent diseases (metabolic syndrome, cardiovascular diseases, diabetes, periodontitis, traumatic injury and intoxications, etc.) [7-10]. Numerous studies have found out the relationship between them and inflammatory or dystrophic SGs diseases [11-13].

Recent experiments on white rats exposed to systemic inflammatory response (SIR) have revealed metabolic and functional disorders of SGs that are supposed to occur due to NF-kappa B-associated increasing concentrations of reactive oxygen and nitrogen species (ROS / RNS) [14].

Previously, we have provided evidence that 40% ethanol administered through gavage with gastric cannula during lipopolysaccharide (LPS)-induced SIR course led to an increase in pro-inflammatory factors (tumour necrosis factor-alpha, interleukin-6, C-reactive protein) that exceeded the respective values when *Salmonella typhi* and alcohol were administered separately [15].

Promising approaches in the SIR pathogenetic correction may consist in applying polyphenols capable of modulating the activity of NF-kappa B or transcription factors of synergistic interaction, and, in particularly, activator protein 1 (AP-1) or signal transducer and activator of transcription 3 (STAT-3); or transcription factors of antagonistic interaction, for instance, nuclear factor-erythroid 2-related factor 2 (Nrf2) or peroxisome proliferator activated receptor gamma (PPAR-gamma), with subsequent inhibition of expression of genes encoding pro-inflammatory cytokines, acute phase proteins, nitro-oxidative stress markers, etc. [14-16]. A positive effect of some alcoholic beverages (wine) on the human body, and particularly their antiatherogenic effect, can be explained by the polyphenol content [17-19].

Numerous experimental and clinical studies have elucidated a role of resveratrol (3,4′,5-trihydroxy-trans-stilbene), a natural phytoalexin and a member of the stilbene family of polyphenols, to serve as a scavenger of superoxide radical anion, peroxide and hydroxyl radicals [20]. The ability of this polyphenol to hinder the transmission of inflammatory signals by inhibiting phospholipase A2 has also been proven [21].

The literature review shows that at present, protein deacetylation by SIRT1 is the most closely studied mechanism of pharmacological action of resveratrol. It has been proven that resveratrol reduces SIRT1 deacetylation of RelA / p65, a heterodimer of a NF-kappa B family member [22]. Researches have confirmed the capability of resveratrol to suppress signs of inflammatory process caused by tumour necrosis factor-alpha through SIRT1 that supports the fact that SIRT1 is an effective target to regulate inflammation [22]. It is the inhibition of NF-kappa B that is induced by LPS or proinflammatory cytokines that is associated with the antioxidant properties of resveratrol in monocytes, endothelial cells, myeloid and dendritic cells [23]. In addition to NF-kappa B, there are some other SIRT1 targets including transcription factors FOXO 1 and 3 (Forkhead box protein O), STAT-3, p53, HEY2 (Hairy/enhancer-of-split related with YRPW motif protein 2), PPAR gamma, etc. [24-26].

However, the efficiency of resveratrol as an agent for pathogenetic therapy of alcoholism under the SIR condition has not been elucidated yet.

The aim of this study is to investigate the effects of resveratrol on nitro-oxidative stress and the functions of submandibular SGs in rats under alcohol exposure during LPS-induced SIR.

Methods

The studies were conducted on 21 Wistar rats weighing 205-220 g, divided into 3 groups of seven animals in each: the 1st group, control group I, including animals, which received isotonic sodium chloride solution intragastrically via gavage twice a day; the 2nd group, control group II, including rats exposed to alcohol for last 2 weeks during LPS-induced SIR; the rats of the 3rd group exposed to alcohol during LPS-induced SIR, who also received resveratrol ("Sigma-Aldrich, Inc.", USA) in a daily dose of 5 mg/kg [27] intragastrically via gavage.

To simulate the pattern of alcohol consumption, 40% ethanol solution in a dose of 24 mg/kg was given intragastrically twice a day for 14 days [28]. SIR was induced by intraperitoneal administration of *Salmonella typhi* LPS (pyrogenalum, "Medgamal", Russia) according to the following scheme: during the first week a dose of 0.4 μg/kg of body weight was administered 3 times a week; over the next 7 weeks of the experiment rats received 0.4 μg/kg of body weight once a week [29].

The research is consistent with the standards and policies of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes drawn up by the Council of Europe (Strasbourg, 18.III.1986). The rats were decapitated under ethereal anesthesia. The SGs were removed and rinsed with 0.9% sodium chloride solution. Then there were homogenized with 0.1 M Tris-buffer (pH 7.4) in cold to obtain 10% tissue homogenate.

The production of superoxide anion radical ($\cdot O_2^-$) was evaluated by a test with nitroblue tetrazolium using spectrophotometry of the tissue homogenate with following inductors: nicotinamide adenine dinucleotide reduced (NADH) was used to assess $\cdot O_2^-$ production by the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate reduced (NADPH) was used to evaluate $\cdot O_2^-$ production by endoplasmic reticulum and NO-synthase (NOS), and *S. typhi* LPS was used to assess $\cdot O_2^-$ production by phagocytic NADPH oxidase [30].

The NOS activity was determined by the difference between the concentration of nitrite ions before and after the incubation of homogenate into the medium containing L-arginine and NADPH [31]. To evaluate the activity of constitutive isoforms (cNOS), we added 1% solution of aminoguanidine hydrochloride (98%, "Sigma Aldrich") [32]. The activity of inducible NOS (iNOS) was evaluated by subtracting the cNOS activity from the overall NOS activity.

The cNOS coupling index was calculated as the ratio between the cNOS activity and the $\cdot O_2^-$ generation rate by the NADPH-dependent electron transport chains. This index points out the presence of substrates (L-arginine, O_2) and tetrahydrobiopterin for NO production, but not for $\cdot O_2^-$ generation under oxidative metabolism of L-arginine [33].

Peroxynitrites of alkali and alkali-earth metals concentration was measured by using their reaction with potassium iodide under pH 7.0 in 0.2 M phosphate buffer with the same pH [31]. The content of low molecular weight S-nitrosothiols was determined by the difference between the concentration of nitrites before and after oxidation of nitrosothiol complexes with a mercury chloride solution [34].

To assess the functional status of submandibular SGs in their homogenate we determined alpha-amylase activity (spectrophotometrically) and the aquaporin-5 concentration (by enzyme-linked immunosorbent assay using the Rat Aquaporin 5 ELISA Kit, MyBioSource, USA).

The findings obtained were statistically processed using Microsoft Office Excel software pack and Real Statistics add-in. To verify the normality distribution, the calculation of the Shapiro-Wilk test was applied. When the ordered sample values corresponded to the normal distribution, then the Student's t-test was used to compare independent samples. When the result ranges were not subject to normal distribution,

statistical processing was performed using a non-parametric method, the Mann-Whitney test.

Results

ROS production in the tissues of submandibular SGs significantly elevated under the administration of alcohol during LPS-induced SIR. The $\cdot O_2^-$ production (Table I) by mitochondrial respiratory chain exceeded the relevant parameters in the control group I in 1.85 times (P<0.001); $\cdot O_2^-$ production by microsomal oxygenases and NOS grew in 1.70 times (P<0.001), and by phagocyte NADPH-oxidase in 1.74 times (P<0.001).

When applying resveratrol under the conditions of alcohol administration during SIR, NADH-induced $\cdot O_2^-$ production decreased and yielded to the result in the control group II in 33.0 times (P<0.001).

The administration of resveratrol during the experiment also led to a considerable decrease in NADPH-induced $\cdot O_2^-$ production in the submandibular SGs tissues in 1.28 times (P<0.001) compared to the control group II. LPS-induced generation of this radical by phagocytes fell in 1.35 times (P<0.001).

The exposure to alcohol during SIR resulted in an increase in NOS activity (Table 2) as evidenced by the homogenate of submandibular SGs tissues. At the same time, results of the total NOS activity and the activity of iNOS exceeded the values in the control group I in 2.14 and 2.60 times (P<0.001), respectively, while the cNOS activity lowered in 1.41 times (P<0.02). Under the alcohol administration during SIR modelling, cNOS coupling index was in 1.66 times (P<0.001) lower than in the control group I. This indicates that cNOS produces $\cdot O_2^-$, instead of producing NO, thus creating a vicious circle of mutual strengthening between the level of oxidative stress and cNOS uncoupling.

When applying resveratrol under the alcohol administration during modelled SIR, the indicators of nitrosative stress significantly changed. The total NOS and iNOS activity in the submandibular SGs tissues was inferior in 1.34 and 1.40 times (P<0.001), respectively, to the relevant data in the control group II. However, this compound used in the study did not significantly change the cNOS activity in submandibular SGs compared with the control group II. The calculation of cNOS coupling index revealed that applying this polyphenol notably improved the cNOS coupling in the tissues of the submandibular SGs. The value of cNOS coupling index exceeded the values in the control group II in 2.23 times (P<0.05).

The alcohol exposure during modelled SIR resulted in the considerable growth in the content of important RNS, peroxynitrites and S-nitrosothiols, in the tissues (Table 3) in 1.79 and 1.59 times (P<0.001), respectively, compared with the control group I.

Under the administration of resveratrol, peroxynitrite and S-nitrosothiol concentration in the submandibular SGs tissues was inferior to the control group II in 1.37 and 1.42 times (P<0.001), respectively.

We investigated the activity of alpha-amylase and the concentration of aquaporin-5 as markers reflecting the functional state of submandibular SGs tissues. The alcohol administration during LPS-induced SIR considerably restricts alpha-amylase activity and lowers the aquaporin-5 concentration (Table 4) in the submandibular SGs homogenates in 1.35 and 1.65 times (P<0.001) compared with control group I. That is, the level of functional impairment of SGs is consistent with the above demonstrated indices of nitro-oxidative stress progression.

The administration of resveratrol led to the changes in alpha-amylase activity and aquaporin-5 concentration in the submandibular SGs tissues: its values exceeded the respective findings in the control group II in 1.40 and 2.77 times (P<0.001), respectively.

Discussion

Significant growth in $\cdot O_2^-$ production under experimental conditions is apparently due to the emergence of additional ways of ROS generation under the combined action of ethanol and the SIR progression. On the one hand, alcohol causes 1-electron O_2 reduction in the mitochondrial and microsomal electron transport chains. On the other hand, the influx of LPS as a pathogen-associated

molecular pattern through the activation of Toll-like receptors 4 and their dependent NF-kappa B- and AP-1-associated signalling pathways promotes ROS formation by various sources and enables to induce other pro-inflammatory mediators in an easier way [35]. The change in the redox potential under these conditions, in turn, further activates redox-sensitive transcription factors, and, in particular, NF-kappa B [10]. At the same time, moderate oxidative stress becomes intensified and enhances more intense formation of pro-oxidant and inflammatory mediators.

Compared to the values obtained under separate injections of LPS and alcohol, the tissue of the submandibular salivary glands in the animals under the ethanol administration during SIR course showed higher production of $^{\cdot}O_2^-$ with the mitochondrial respiratory chain, microsomal monooxygenases, cNOS and phagocyte NADPH-oxidase; the activity of iNOS, peroxynitrites and S-nitrosothiols concentration were significantly raised [15]. These changes in the submandibular SGs homogenate were accompanied by more marked decrease in alpha-amylase activity and the concentration of aquaporin-5 that impairs water and protein excretion by the SGs. It allows us to conclude that the administration of 40% ethanol during LPS-induced SIR results in more pronounced development of oxidative-nitrosative stress in the submandibular SGs causing their more marked dysfunction compared with separate use of LPS and alcohol [15].

The previous works have reported on the dependence of between the development of nitro-oxidative stress in the SGs under SIR and the activity of the NF-kappa B-dependent signalling pathway. The administration of pyrrolidine dithiocarbamate, a potent NF-kappa B inhibitor, lowers NOS activity, $^{\cdot}O_2^-$ production, and the level of lipid peroxidation in SGs, and enhances antioxidant protection [14]. Thus, ROS / RNS formed are the means of redox-sensitive transcription factors (NF-kappa B, in particular) regulation, and alterations in their activity affect not only oxidative metabolism in SGs, but also in other organs through the SIR development. The latter is known as an important mechanism of damaging SGs tissues because it induces nitro-oxidative stress [14].

Many studies suggest the main physiological function of polyphenols is to correct this process. We have found out the administration of resveratrol under the experimental conditions increases the level of cNOS coupling in the submandibular SGs tissues. The investigated polyphenol considerably improves SGs functional status, enhancing the activity of alpha-amylase and concentration of aquaporin-5, essential for water transport through biological membranes in salivary glands. Aquaporin-5 in SGs is known to form water channels transporting fluid through biological membranes [36].

Resveratrol, primarily available in peanuts, berries, grapes, and red wine, has been broadly studied due to its protective properties, such as antioxidant, anti-inflammatory, and anti-aging effects, as well as cardio- and neuroprotective action [37, 38]. As a direct antioxidant agent, resveratrol scavenges diverse ROS / RNS as well as secondary organic radicals with mechanisms of hydrogen atom transfer and sequential proton loss electron transfer, thereby protecting cellular biomolecules from oxidative damage [20]. Resveratrol also enhances the expression of various antioxidant defensive enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and heme oxygenase 1, as well as increases glutathione level responsible for maintaining the cellular redox balance. Such defences could be achieved by regulating various signalling pathways including sirtuin 1, NF-kappa B, and Nrf2 [20, 39]. Nrf2 regulates the expression of antioxidant response element, which is an enhancer for a number of genes including genes of most antioxidant enzymes and genes of many enzymes of phase II metabolism of xenobiotics, in particular, heme oxygenase 1, NAD(P)H-quinonoxidoreductase, glutathione transferases, UDP-glucuronyltransferase that are important for antioxidant cell protection [40].

Our study has evidenced that applying resveratrol for the correction of nitro-oxidative stress in SGs results in an improvement of their functions, therefore further in-depth investigation of this polyphenol as an agent to prevent and treat SG diseases under the conditions accompanied by SIR seems to be very promising.

Conclusions

1. The use of resveratrol under the combined administration of 40% ethanol solution and LPS considerably limits the development of nitro-oxidative stress in the tissues of the submandibular SGs. This is confirmed by a significant decrease in the superoxide anion radical production by microsomal monooxygenases, mitochondrial respiratory chain, phagocyte NADPH-oxidase and cNOS, lowered iNOS activity and RNS concentration (peroxynitrites and S-nitrosothiols).

2. Resveratrol considerably improves the functional status of the submandibular SGs under the combined administration of alcohol and LPS, enhancing the activity of alpha-amylase and concentration of aquaporin-5, which is essential for water transport through biological membranes in SGs.

Conflict of interest: no conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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Table 1. Effect of resveratrol on the production of superoxide anion radical in submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response, nmol/s per g of tissue (M±m)

Experiment conditions	Sources of the superoxide anion radical production		
	NADH-dependent (mitochondrial) electron-transport chain	NADPH-dependent electron-transport chains	NADPH-oxidase of white blood cells
Administration of isotonic sodium chloride (control group I)	17.97±1.02	14.55±0.82	1.74±0.10
Administration of ethanol during systemic inflammatory response (control group II)	33.17±1.49 *	24.77±0.37 *	3.03±0.07 *
Administration of resveratrol under alcohol exposure during systemic inflammatory response	22.23±1.14 *.**	17.76±0.80 *.**	1.96±0.12 **

Note: * – P<0.05 compared with values in the control group I; ** – P<0.05 compared with values in the control group II.

Table 2. Effect of resveratrol on the activity of NO-synthase isoforms in submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response (M±m)

Experiment conditions	NOS activity, μmol (NO_2^-) / min·g of protein			cNOS coupling index
	Total	cNOS	iNOS	
Administration of isotonic sodium chloride (control group I)	7.67±0.39	1.76±0.09	5.91±0.34	0.122±0.007
Administration of ethanol during systemic inflammatory response (control group II)	16.41±0.71 *	1.04±0.23 *	15.37±0.53 *	0.042±0.009 *
Administration of resveratrol under alcohol exposure during systemic inflammatory response	10.88±0.56 *.**	1.61±0.29	9.27±0.58 *.**	0.094±0.019 **

Note: * – P<0.05 compared with values in the control group I; ** – P<0.05 compared with values in the control group II.

Table 3. Effect of resveratrol on the reactive nitrogen species content in submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response (M±m)

Experiment conditions	Peroxynitrites of alkali and alkali-earth metals concentration, μmol/g of tissue	S-nitrosothiols, μmol/g of tissue
Administration of isotonic sodium chloride (control group I)	0.91±0.04	0.75±0.02
Administration of ethanol during systemic inflammatory response (control group II)	1.63±0.06 *	1.19±0.03 *
Administration of resveratrol under alcohol exposure during systemic inflammatory response	1.03±0.05 **	0.69±0.03 **

Note: * – P<0.05 compared with values in the control group I; ** – P<0.05 compared with values in the control group II.

Table 4. Effect of resveratrol on parameters of functional state of submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response (M±m)

Experiment conditions	Alpha-amylase activity, mg/min × g of tissue	Aquaporin-5 concentration, pg / ml of homogenate
Administration of isotonic sodium chloride (control group I)	68.18±0.95	0.51±0.02
Administration of ethanol during systemic inflammatory response (control group II)	44.42±0.95 *	0.18±0.01 *
Administration of resveratrol under alcohol exposure during systemic inflammatory response	62.3±0.40 *.**	0.50±0.01**

Note: * – P<0.05 compared with values in the control group I; ** – P<0.05 compared with values in the control group II.