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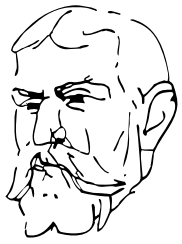

Memory of
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VOLUME LXXV, ISSUE 3, MARCH 2022

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ORIGINAL ARTICLE

BIOFLAVONOIDS AS AGENTS FOR CORRECTING NITRO-OXIDATIVE STRESS AND SALIVARY GLAND FUNCTIONS IN RATS EXPOSED TO ALCOHOL DURING MODELED LIPOPOLYSACCHARIDE-INDUCED SYSTEMIC INFLAMMATORY RESPONSE

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Rita Kozaeva[1], Mykola O. Klymenko[1], Oleksandr V. Katrushov[2], Vitalii O. Kostenko[2]
[1] PETRO MOHYLA BLACK SEA NATIONAL UNIVERSITY, MYKOLAYIV, UKRAINE
[2] POLTAVA STATE MEDICAL UNIVERSITY, POLTAVA, UKRAINE

ABSTRACT

The aim: To investigate the effects of bioflavonoids (curcumin, epigallocatechin-3-gallate and quercetin) on nitro-oxidative stress and the functions of submandibular SGs in rats under alcohol exposure during SIR.

Materials and methods: The studies were conducted on 35 rats of the Wistar line weighing 205-220 g, divided into 5 groups of seven animals in each: the 1st group, control group I, included animals receiving isotonic sodium chloride solution intragastrically twice a day; the 2nd group, control group II, included rats exposed to alcohol (in a dose of 24 mg/kg intragastrically through gavage a twice a day) for last 2 weeks during lipopolysaccharide (LPS)-induced SIR; the rats of the 3rd, 4th and 5th groups exposed to alcohol during LPS-induced SIR, which also received bioflavonoids. The bioflavonoids ("Sigma-Aldrich, Inc.", USA) were as following: curcumin (in a daily dose of 200 mg/kg), epigallocatechin-3-gallate (in a daily dose of 40 mg/kg), and quercetin (in a daily dose of 200 mg/kg), respectively. 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; during the next 7 weeks of the experiment rats received 0.4 μg/kg of body weight once a week. The formation of superoxide anion radical ($O_2^{\cdot-}$), activity of NO-synthase – total (NOS), its constitutive and inducible isoforms (cNOS, iNOS), and concentration of peroxynitrites and S-nitrosothiols were evaluated spectrophotometrically. To assess the functional status of submandibular SGs in their homogenate we determined α-amylase activity (spectrophotometrically) and the aquaporin-5 concentration (by enzyme-linked immunosorbent assay). through gav-age with orogastric cannul.

Results: When applying bioflavonoids 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 by 36.8% under administering curcumin, by 34.5% under administering epigallocatechin-3-gallate, and by 41.3% under administering quercetin. The total NOS activity in SGs tissues was inferior by 42.8% to the relevant data in the control group II (under curcumin administration), by 33.7% (under epigallocatechin-3-gallate administration) and by 46.6% (under quercetin administration); and the iNOS activity decreased by 47.0, 38.3 and 52.0%, respectively. Under the administration of bioflavonoids peroxynitrites concentration in the submandibular SGs tissues was inferior to the control group II by 35.6% (under curcumin administration), by 37.4% (under epigallocatechin-3-gallate administration), and by 39.3% (under quercetin administration); the content of S-nitrosothiols was lower by 34.5, 31.1 and 35.3%, respectively. The administration of bioflavonoids led to the changes in α-amylase activity in the submandibular SGs tissues: its values exceeded the relevant data in the control group II by 40.4% (under curcumin administration), by 38.2% (under epigallocatechin-3-gallate administration), and by 34.1% (under quercetin administration); under those conditions aquaporin-5 concentration grew in 2.66, 2.61 and 2.55 times, respectively.

Conclusions: The use of bioflavonoids (curcumin, epigallocatechin-3-gallate, and quercetin) 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. The administration of the bioflavonoids increases the level of cNOS coupling, and improves the functional status of the submandibular SGs under the combined administration of alcohol and LPS enhancing the activity of α-amylase and concentration of aquaporin-5.

KEY WORDS: bioflavonoids, curcumin, epigallocatechin-3-gallate, quercetin, lipopolysaccharide-induced systemic inflammatory response

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INTRODUCTION

More than a half (57%, or 3.1 billion people) of the global population aged 15 years and over have abstained from drinking alcohol in the previous 12 months. Some 2.3 billion people are current drinkers. Alcohol is consumed by more than a half of the population in only three World Health Organization regions – the Americas, Europe and Western Pacific [1].

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 increasing concentrations of reactive oxygen and nitrogen species [2, 3]. Numerous studies have found out the relationship between inflammatory and dystrophic SGs diseases and systemic diseases, pathogenesis of which is accompanied by the SIR devel-

opment (metabolic syndrome, cardiovascular diseases, diabetes, etc.) [4, 5, 6, 7].

Promising approaches in the SIR pathogenetic correction may consist in applying bioflavonoids capable of modulating the activity of redox-sensitive transcription factors NF-kappa B and Nrf2 with subsequent inhibition of expression of genes encoding pro-inflammatory cytokines, acute phase proteins, nitro-oxidative stress markers, etc. [8, 9, 2].

However, the efficiency of polyphenols as agents for pathogenetic therapy of alcoholism against the background of systemic inflammatory response has not yet been elucidated.

THE AIM

The aim of this study is to investigate the effects of bioflavonoids (curcumin, epigallocatechin-3-gallate and quercetin) on nitro-oxidative stress and the functions of submandibular SGs in rats under alcohol exposure during SIR.

MATERIALS AND METHODS

The studies were conducted on 35 rats of the Wistar line weighing 205-220 g, divided into 5 groups of seven animals in each: the 1st group, control group I, included animals receiving isotonic sodium chloride solution intragastrically through gavage twice a day; the 2nd group, control group II, included rats exposed to alcohol for last 2 weeks during lipopolysaccharide (LPS)-induced SIR; the rats of the 3rd, 4th and 5th groups exposed to alcohol during LPS-induced SIR, which also received bioflavonoids intragastrically through gavage. The bioflavonoids ("Sigma-Aldrich, Inc.", USA) were as following: curcumin (in a daily dose of 200 mg/kg), *epigallocatechin-3*-gallate *(in a daily dose of* 40 mg/kg), and quercetin (in a daily dose of 200 mg/kg), respectively.

To simulate the pattern of alcohol consumption, 40% ethanol solution in a dose of 24 mg/kg was administered intragastrically through gavage a twice a day for 14 days [10]. SIR was induced by intraperitoneal administration of *Salmonella typhi* LPS (pyrogenalum, "Medgamal", RF) 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; during the next 7 weeks of the experiment rats received 0.4 μg/kg of body weight once a week [2].

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 production of superoxide anion radical (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 [11].

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 [12]. To evaluate the activity of constitutive isoforms (cNOS), we added 1% solution of aminoguanidine hydrochloride (98%, "Sigma Aldrich") [13]. 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 [14].

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 [12]. 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 [15].

To assess the functional status of submandibular SGs in their homogenate we determined α-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

Reactive oxygen species (ROS) production in the tissues of submandibular SGs significantly elevated under the administration of alcohol during LPS-induced SIR. At that $\cdot O_2^-$ production (Table I) by mitochondrial respiratory chain exceeded the relevant parameters in the control group I by 84.6%; $\cdot O_2^-$ production by microsomal oxygenases and NOS grew by 70.2%, and by phagocyte NADPH-oxidase by 74.1%.

Significant growth in $\cdot O_2^-$production under these 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 signaling

Table I. Effect of bioflavonoids on the production of superoxide anion radical in submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response (M±m, n=35)

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 SIR (control group II)	33,17±1,49 *	24,77±0,37 *	3,03±0,07 *
+ Curcumin	20.95±0.81 *,**	16.94±0.66 *,**	1.86±0.07 **
+ Epigallocatechin-3-gallate	21.74±0.60 *,**	17.57±0.46 *,**	1.92±0.07 **
+ Quercetin	19.46±1.05 **	15.79±0.84 **	1.70±0.10 **

Note (in table 1-4): * – p<0.05 compared with values in the control group I; ** – p<0.05 compared with values in the control group II.

Table II. Effect of bioflavonoids on the activity of NO-synthase isoforms in submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response (M±m, n=35)

Groups of test animals	NOS activity, μmol (NO2-) / 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 SIR (control group II)	16,41±0,71 *	1,04±0,23 *	15,37±0,53 *	0,042±0,009 *
+ Curcumin	9.39±0.53 *,**	1.25±0.18 *	8.14±0.59 *,**	0.073±0.010 *,**
+ Epigallocatechin-3-gallate	10.88±0.51*,**	1.40±0.14	9.48±0.46 *,**	0.080±0.008 *,**
+ Quercetin	8.76±0.46 **	1.38±0.16	7.38±0.40 *,**	0.089±0.011 *,**

Table III. Effect of bioflavonoids on the reactive nitrogen species content in submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response (M±m, n=35)

Groups of test animals	Peroxynitrites of alkali and alkali-earth metals concentration, μmol/g	S-nitrosothiols, μmol/g
Administration of isotonic sodium chloride (control group I)	0,91±0,04	0,75±0,02
Administration of ethanol during SIR (control group II)	1,63±0,06 *	1,19±0,03 *
+ Curcumin	1.05±0.03 *,**	0.78±0.02 **
+ Epigallocatechin-3-gallate	1.02±0.04 **	0.82±0.03 **
+ Quercetin	0.99±0.05 **	0.77±0.04 **

pathways promotes ROS formation by various sources and enables to induction other pro-inflammatory mediators in an easier way [16]. The change in the redox potential under these conditions, in turn, further activates redox-sensitive transcription factors, and in particular, NF-kappa B [17]. At the same time, moderate oxidative stress becomes intensified and enhances more intense formation of pro-oxidant and inflammatory mediators.

When applying bioflavonoids 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 by 36.8% under administering curcumin, by 34.5% under administering epigallocatechin-3-gallate, and by 41.3% under administering quercetin.

The administration of polyphenols during the experiment also led to a considerable decrease in NADPH-induced $\cdot O_2^-$production in the tissues of the submandibular SGs compared to the control group II. Accordingly, NADPH-induced production of $\cdot O_2^-$by microsomal monooxygenases and NOS in the tissues of the submandibular SGs went down by 31.6% when applying curcumin, by 29.1% when applying e*pigallocatechin-3*-gallate, and 29.1 % when applying quercetin. LPS-induced generation of this radical by phagocytes fell by 38.6, 36.6 and 43.9%, respectively.

The exposure to alcohol during SIR resulted in an increase in NO-synthase activity (Table II) as evidenced by the homogenate of submandibular SGs tissues. At the same time, results of the total NOS activity and the activity of NOS inducible isoform exceeded the values in the control group I in 2.14 and 2.6 times, respectively, while the cNOS activity lowered by 40.9%.

Among the mechanisms ROS generating along with such sources as mitochondria, microsomal monooxygenases and NOS, NADPH-oxidase of white blood cells, xanthine oxidase, lipo- and cyclooxygenase, considerable attention has recently been paid to the functioning of non-conjugated cNOS [18]. Under the alcohol administration during SIR

Table IV. Effect of bioflavonoids on parameters of functional state of submandibular salivary glands under alcohol exposure during lipopolysaccharide-induced systemic inflammatory response (M±m, n=35)

Groups of test animals	α-Amylase activity, mg/min × g	Aquaporin-5 concentration, pg / ml
Administration of isotonic sodium chloride (control group I)	68.18±0.95	0.51±0.02
Administration of ethanol during SIR (control group II)	44.42±0.95 *	0.18±0.01 *
+ Curcumin	62.38±1.55 *,**	0.48±0.02 **
+ Epigallocatechin-3-gallate	61.37±0.80 *,**	0.47±0.02 **
+ Quercetin	59.55±1.45 *,**	0.46±0.02 **

modelling, cNOS coupling index was 65.6% 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 bioflavonoids under the alcohol administration during modeled SIR, the indicators of nitrosative stress significantly changed. The total NOS activity in the submandibular SGs tissues was inferior by 42.8% to the relevant data in the control group II (under curcumin administration), by 33.7% (under epigallocatechin-3-gallate administration) and by 46.6% (under quercetin administration); and the iNOS activity decreased by 47.0, 38.3 and 52.0%, respectively.

However, the bioflavonoids 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 these polyphenols 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 by 73.8% (under curcumin administration), by 90.5% (under epigallocatechin-3-gallate administration) and 111.0% (under quercetin administration).

The alcohol administration during modeled SIR resulted in the considerable growth in the content of important effectors of nitrosative stress, peroxynitrites and S-nitrosothiols, in the tissues (Table III) by 79.1 and 58.7%, respectively, compared with the control group I.

Under the administration of bioflavonoids peroxynitrites concentration in the submandibular SGs tissues was inferior to the control group II by 35.6% (under curcumin administration), by 37.4% (under epigallocatechin-3-gallate administration), and by 39.3% (under quercetin administration); the content of S-nitrosothiols was lower by 34.5, 31.1 and 35.3%, respectively.

We investigated the activity of α-amylase and the concentration of aquaporin-5 as markers reflecting the functional state of submandibular SGs tissues. Aquaporin-5 in SGs is known to form water channels transporting fluid through biological membranes [19]. The alcohol administration during LPS-induced SIR considerably restricts α-amylase activity and lowered the aquaporin-5 concentration (Table IV) in the submandibular SGs homogenates by 34.8 and 64.7% 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 bioflavonoids led to the changes in α-amylase activity in the submandibular SGs tissues: its values exceeded the relevant data in the control group II by 40.4 % (under curcumin administration), by 38.2 % (under epigallocatechin-3-gallate administration), and by 34.1 % (under quercetin administration); under those conditions aquaporin-5 concentration grew in 2.66, 2.61 and 2.55 times, respectively.

DISCUSSION

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 signaling 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 [3].

It has been shown that SIR modeled in the SGs tissues also manifests by impaired functioning of the nuclear factor erythroid 2–related factor (*Nrf2*), a transcription factor, which binds to the antioxidant response element (ARE) and thus regulates the expression of a large battery of genes involved in the cellular antioxidant and anti-inflammatory defence, and mitochondrial protection as well [2].

Reactive oxygen and nitrogen species formed are the means of redox-sensitive transcription factors (NF-kappa B, in particular) regulation, whose alterations in the 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 [3]. Many studies suggest the main physiological function of bioflavonoids is to correct this process. Among the principal mechanisms, which provide protective effects of polyphenols, along with their high antiradical activity, their ability to interact with the Nrf2 / ARE signaling system is known to play a critical role [20, 21]. Nrf2 regulates the expression of ARE, 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, NAD(P)H-quinonoxidorefutase, hemoxigenase-1, glutathione transferases, UDP-glucuronyltransferase that are important for antioxidant cell protection.

Moreover, curcumin and quercetin serve as inhibitors of NF-kappa B activation demonstrating different mechanisms of action: the first is able to block the phosphorylation and degradation of the inhibitory IκB protein [22], while the latter inhibits the proteasome formation [23]. Curcumin can also impact the activity of the AP-1 transcription factor (activator protein 1) by inhibiting c-Jun N-terminal kinases that is explained by the predominant inhibitory effect on c-jun gene expression [24-27].

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

CONCLUSIONS

1. The use of bioflavonoids (curcumin, epigallocatechin-3-gallate, and quercetin) under the combined administration of 40% ethanol solution and *S. typhi* lipopolysaccharide considerably limits the development of nitro-oxidative stress in the tissues of the submandibular salivary glands. This is confirmed by a significant decrease in the superoxide anion radical production by microsomal monooxygenases, mitochondrial respiratory chain, phagocyte NADPH-oxidase, lowered activity of inducible isoform of NO-synthase and concentration of reactive metabolites of nitrogen (peroxynitrites and S-nitrosothiols).
2. The administration of bioflavonoids (curcumin, epigallocatechin-3-gallate, and quercetin) under the experimental conditions increases the level of cNOS coupling in the tissues of the submandibular salivary glands.
3. The investigated bioflavonoids (curcumin, epigallocatechin-3-gallate, and quercetin) considerably improve the functional status of the submandibular salivary glands under the combined administration of alcohol and *S. typhi* lipopolysaccharide, enhancing the activity of α-amylase and concentration of aquaporin-5, essential for water transport through biological membranes in salivary glands.

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ORCID and contributionship:
Rita Kozaeva [B,C,D]
Mykola O. Klymenko [C,D,F]
Oleksandr V. Katrushov [A,E,F]
Vitalii O. Kostenko [A,E,F]

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CORRESPONDING AUTHOR
Mykola O. Klymenko
Petro Mohyla Black Sea National University,
68 Desantnykiv St., 54003 Mykolayiv, Ukraine
tel: +38 512 769286
e-mail: mykola.klymenko@chmnu.edu.ua

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A - Work concept and design, **B** – Data collection and analysis, **C** – Responsibility for statistical analysis, **D** – Writing the article, **E** – Critical review, **F** – Final approval of the article