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QUERCETIN POTENTIATES ANTIRADICAL PROPERTIES OF EPIGALLOCATECHIN-3-GALLATE IN PERIODONTIUM OF RATS UNDER SYSTEMIC AND LOCAL ADMINISTRATION OF LIPOPOLISACCHARIDE OF SALMONELLA TYPHI

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ABSTRACT

Introduction: There has been demonstrated that pharmaceutical effect of epigallocatechin-3-gallate (EGCG), a polyphenol, which is found in green tea *(Camellia sinensis)*, is implemented through the activation of Nrf2 (Nuclear Factor Erythroid 2-Related Factor 2).The importance of Keap1 / Nrf2 / antioxidant response element (ARE) system is determined by the fact that the state of NF-κB- and AP-1-associated pathways depends on its activity. Recent studies have demonstrated the property of quercetin to suppress ubiquitin-dependent proteolysis of complex of NF-κB and its inhibitory protein IκB. All this provides preconditions to eliminate the potentiality of NF-κB-dependent expression of the number of genes of pro-oxidant and pro-inflammatory proteins. However, co-effect produced by quercetin and EGCG on the oxidative nitrosative stress markers in the periodontal tissues is still unclear.

The aim: To investigate the co-effect produced by quercetin and an inducer of the Keap1 / Nrf2 / ARE epigallocatechin-3-gallate on markers of oxidative-nitrosative stress in rats' periodontium under the systemic and local administration of *Salmonella typhi* lipopolysaccharide (LPS).

Materials and methods: The studies were conducted on 30 white rats of the Wistar line, divided into 5 groups: the 1st included intact animals, the 2nd was made up of animals after their exposure to combined systemic and local LPS administration, the 3rd and 4th groups included animals, which were given injections with water-soluble form of quercetin (corvitin) and EGCG respectively, and the 5th group involved rats, which were injected with co-administered corvitin and EGCG. The formation of superoxide anion radical ($\cdot O_2^-$) was evaluated by a test with nitro blue tetrazolium using spectrophotometry of the periodontal soft tissue homogenate. The total activity of NO-synthase and concentration of peroxynitrite in the homogenate of the soft components of periodontium were evaluated spectrophotometrically.

Results: Co-effect produced by corvitin and EGCG under systemic and local LPS administration is accompanied with reduced O_2^- production by NADPH-dependent electron transport chains (microsomal and NOS) by 20.0 % ($p<0.05$) compared with values for the animals received separate corvitin during the experiment. $\cdot O_2^-$ generation by the mitochondrial respiratory chain yielded to comparable data of the 3rd and 4th groups by 27.6 % ($p<0.01$) and 23.8 % ($p<0.05$) respectively. No differences were found between the groups exposed to combined or separate action of the above mentioned agents in the experiment when assessing O_2^- generation by leukocyte NADPH-oxidase. Combined effect of corvitin and EGCG during systemic and local LSP administration showed the decrease in NOS activity and peroxynitrite concentration in periodontal tissues by 53.3 % ($p<0.001$) and 27.0 % ($p<0.02$) compared with the findings in the 3rd group, and by 42.0 % ($p<0.01$) and 22.3 % ($p<0.01$) in the 4th group.

Conclusions: the co-administration of water-soluble form of quercetin and epigallocatechin-3-gallate under systemic and local introducing of lipopolysaccharide *Salmonella* typhi has been proven to be more effective means for preventing and correcting oxidative-nitrosative stress in the periodontal tissues than this occurs at separate administration of each of the polyphenols.

KEY WORDS: signal pathway Keap1 / Nrf2 / ARE, epigallocatechin-3-gallate, quercetin, lipopolysaccharide-induced systemic inflammatory response, oxidative-nitrosative stress, periodontium

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INTRODUCTION

It has been known that impairment of the periodontium structure and functions result from a number of somatic diseases (metabolic syndrome, atherosclerosis, chronic pyelonephritis, traumatic disease, etc), whose development includes lipopolysaccharide (LPS)-induced systemic inflammatory response as a key component of pathogenesis [6]. Free-radical processes, associated with prolonged activation of certain redox-sensitive transcription factors (NF-κB, AP-1), contribute much to this process [2, 27]. This leads to the expression of genes of inflammatory cytokines, metalloproteinases, inducible nitric oxide synthase (NOS), cellular adhesion molecules, cyclooxygenase-2, etc. that can induce production of reactive oxygen and nitrogen species [16, 19, 29].

The redox-sensitive signaling pathway Keap1/Nrf2 plays a key role in the highly regulated cell systems. It activates gene expression through the interaction Nrf2 (Nuclear Factor Erythroid 2-Related Factor 2) and cis-acting enhancer sequence, known as antioxidant response element (ARE) [21, 24].

It has been reported that Nrf2 signalling is important in controlling oxidative stress in healthy periodontium

that helps to maintain integrity of periodontium despite of its constant exposure to aggressive factors as bacteria, neutrophiles, and macrophages [6].

Recently there has been demonstrated that pharmaceutical effect of epigallocatechin-3-gallate (EGCG), a polyphenol, which is found in green tea *(Camellia sinensis)* [12], is implemented through the activation of Nrf2 due to proteolysis of an inhibitory protein Keap1 [10, 22].

We have demonstrated than the introduction of EGCG in the lipopolysaccharide-inducible systemic inflammatory response is an effective mean to correct oxidative and nitrosative stress in the tissues of periodontium of the rats: it reduces the generation of superoxide anion radicals, in particular, its production by mitochondrial electron transport chain, microsomal and NOS electron transport chains, leukocyte NADPH-oxidase, as well as reduces the total activity of NOS and peroxynitrite concentration [30]. The administration of EGCG under modeled systemic inflammatory response is an effective means of preventing and correcting the disruption of connective tissue of periodontium in rats: it reduces collagenolysis and depolymerization of proteoglycans and glycoproteins [28].

The importance of Keap1 / Nrf2 / ARE system is determined by the fact that the state of NF-κB- and AP-1-associated pathways depends on its activity. In this ways Nrf2 inductors phenethyl isothiocyanate, sulforaphane and curcumin suppress the NF-κB activation [15].

Recent studies have demonstrated the property of quercetin to suppress ubiquitin-dependent proteolysis of complex of NF-κB and its inhibitory protein IκB that impairs the IκB degradation due to the proteasome activity [9]. All this provides preconditions to eliminate the potentiality of NF-κB-dependent expression of the number of genes of pro-oxidant and pro-inflammatory proteins (cytokines, matrix metalloproteinases, inducible NOS, etc.) [18].

However, co-effect produced by quercetin and EGCG on the oxidative and nitrosative stress markers in the periodontal tissues is still unclear. Solving this problem will enable to evaluate the role of this polyphenols as a potential means of pathogenetic therapy for inflammatory and dystrophic diseases of periodontal tissues.

THE AIM

The aim of the present study was to investigate the co-effect produced by quercetin and an inducer of the Keap1 / Nrf2 / ARE epigallocatechin-3-gallate on markers of oxidative and nitrosative stress in rats' periodontium under systemic and local administration of *S. typhi* LPS.

MATERIALS AND METHODS

The studies were conducted on 30 white rats of the Wistar line weighing 180-220 g, divided into 5 groups: the 1st included intact animals, the 2nd was made up of animals after the combined systemic and local LPS administration, the 3rd and 4th groups included animals, which were given injections with water-soluble form of quercetin (corvitin) and EGCG respectively, and the 5th group involved rats, which were injected with co-administered corvitin and EGCG.

For systemic administration, *S. typhi* LPS (pyrogenalum, "Medgamal", Russia) was injected intraperitoneally in a dose, which stimulated rise in temperature by 1.5 °C according to the scheme [30]: during the first week, 4 minimum pyrogenic doses (MPD) of 0.4 μg / kg of rat body weight were given 3 times a week. During the following 7 weeks of the experiment, rats were given 4 MPD / kg of body weight once a week.

For local administration, LPS *S. typhi* was performed once in as dose of 1 μg / kg, equally divided into four injections into the gum at the level of the second molars 7 days prior to the decapitation (acute gingivitis model).

Water-soluble form of quercetin (corvitin, "Borshchahivskiy CPP", Ukraine) was administered intraperitoneally in a daily dose of 10 mg / kg recalculated for quercetin [11], and EGCG (Sigma-Aldrich, Inc., USA) was administered in a dose of 21.1 mg / kg [28] 3 times a week, starting on the 30th day of the systemic LPS administration.

The research was conducted in compliance with the standards of the Convention on Bioethics of the Council of Europe 'European convention for the protection of vertebrate animals used for experimental and other scientific purposes' (Strasbourg, 18.III.1986). The animals were decapitated with ethereal anesthesia. Soft components of periodontium (gingiva and periodontal ligament) served as the objects of the study.

The formation of superoxide anion radical ($\cdot O_2^-$) was evaluated by a test with nitro blue tetrazolium using spectrophotometry of the periodontal soft tissue homogenate by the following inductors: NADH was used to evaluate O_2^- production by the mitochondrial electron transport chain, NADPH was used to evaluate O_2^- production by endoplasmic reticulum and NOS, and *S. typhi* LPS was used to assess O_2^- production by NADPH oxidase of white blood cells [13].

The activity of NOS was determined by the difference in the concentration of nitrite ions before and after the incubation of homogenate into the medium containing L-arginine (NOS substrate) and NADPH. The concentration of nitrite ions was assessed by the formation of diazo-compounds in the reaction with sulfanilic acid, and then we carried out the reaction with α- naphthylethylenediamine, resulting in the production of red color derivatives [1]. The concentration of peroxynitrite in the homogenate was evaluated spectrophotometrically by wavelength of 355 nm [1].

The findings obtained were statistically processed. To verify the normality distribution, the Shapiro-Wilk test was applied. If they corresponded to the normal distribution, then the Student's t-test was used to compare independent samples. When the results ranges were not subject to normal distribution, statistical processing was performed using a nonparametric method, the Mann-Whitney test. Statistical calculations were performed using the "StatisticSoft 6.0" program.

RESULTS AND DISCUSSION

Systemic and local LPS co-administration led to significant changes in the O_2^-generation in the tissues of periodontium (Table I). Thus, the $\cdot O_2^-$ production by NADPH-dependent electron transport chains (microsomal and NOS) increased by 71.0 % ($p<0.001$), while the $\cdot O_2^-$ production by the mitochondrial respiratory chain increased by 85.5 % ($p<0.001$). Generation of $\cdot O_2^-$ by NADPH oxidase of white blood cells also elevated by 85.4 % ($p<0.001$).

According to the literature, the largest amount of $\cdot O_2^-$ is produced by the mitochondrial electron transport chain as a result of the one-electron reduction of oxygen at the level of the following complexes: NADH:ubiquinone oxidoreductase, ubiquinol:cytochrome c oxidoreductase and cytochrome bc1 [5].

Resent studies have demonstrated the activation of NF-κB and AP-1 enhances O_2^- generation by NADH- and NADPH-dependent electron transport chains (mitochondrial, microsomal and NOS) in the periodontal tissues homogenate [14, 27]. This process is accelerated by the formation of peroxynitrite in the tissues [3, 14].

At the same time, a powerful level of $\cdot O_2^-$ production under the conditions of LPS-inducible systemic inflammatory response is provided by NADPH-dependent electron transport chains: in the reactions of microsomal oxidation (with cytochrome P450) [7] and by NOS itself, which is capable of switching from NO to $\cdot O_2^-$ under unfavourable conditions [20]. When the bacterial LPS are introduced into the mammalian body, the $\cdot O_2^-$ generation by leukocyte NADPH oxidase naturally increases [4]. In addition to LPS, proinflammatory cytokines, whose synthesis depends on the activation of NF-κB [8, 23], are also known as effective stimulants of $\cdot O_2^-$synthesis.

Combined systemic and local LPS administration increased NOS activity and peroxynitrite concentration (Table II) in periodontal tissues in three times ($p<0.001$) and 85.5 % ($p<0.001$) respectively. This can be explained by the ability of LPS to provide NF-κB-dependent activation of inducible NOS [23].

It was found that the separate administration of both corvitin and EGCG reduced $\cdot O_2^-$ production by NADPH-dependent electron transport chains by 27.0 % ($p<0.001$) and by 31.0 % ($p<0.001$) (Table I). Under the same conditions, O_2^- production by the mitochondrial respiratory chain declined by 31.2 % ($p<0.01$) and 34.6 % ($p<0.001$) respectively. Generation of O_2^- by leukocyte NADPH-oxidase in periodontal tissues was lessened by 34.5 % ($p<0.001$) and 37.9 % ($p<0.001$) respectively to comparable data of the 2nd group.

Under these conditions, NOS activity in periodontal tissues decreased by 32.8 % ($p<0.01$) and 45.9 % ($p<0.001$), peroxynitrite concentration declined by 35.1 % ($p<0.001$) and 39.0 % ($p<0.001$) respectively to comparable data of the 2nd group (Table II).

Co-effect produced by corvitin and EGCG under systemic and local LPS administration is accompanied with reduced O_2^-production by NADPH-dependent electron transport chains (microsomal and NOS) by 20.0 % ($p<0.05$) compared with values for the animals received separate corvitin during the experiment (Table I). $\cdot O_2^-$ generation by the mitochondrial respiratory chain yielded to comparable data of the 3rd and 4th groups by 27.6 % ($p<0.01$) and 23.8 % ($p<0.05$) respectively.

No differences were found between the groups exposed to combined or separate action of the above mentioned agents in the experiment when assessing O_2^- generation by leukocyte NADPH-oxidase.

Combined effect of corvitin and EGCG during systemic and local LSP administration showed the decrease in NOS activity and peroxynitrite concentration in periodontal tissues by 53.3 % ($p<0.001$) and 27.0 % ($p<0.02$) compared with the findings in the 3rd group, and by 42.0 % ($p<0.01$) and 22.3 % ($p<0.01$) in the 4th group.

The results obtained point out the synergic effect produced by water-soluble form of quercetin and EGCG on

Table I. Co-effect produced by corvitin and epigallocatechin-3-gallate on generation of superoxide anion-radical in periodontium soft tissues under local and systemic administration of S. typhi lipopolysaccharide (M+m, n=35)

Groups of the animals studied	Inductors of superoxide anion radical production, nmol / g • s		
	NADPH	NADH	LPS
Control group I (intact animals)	12.47±0.87	15.41±1.08	1.58±0.12
Control group II (combined systemic and local LPS introduction)	21.32±0.41 *	28.59±1.41 *	2.93±0.12 *
+ water-soluble form of quercetin (corvitin)	15.57±0.85 */**	19.68±1.32 */**	1.92±0.13 **
+ EGCG	14.72±0.81 **	18.69±1.31 **	1.82±0.14 **
+ corvitin + EGCG	12.45±0.83 **/***	14.24±0.90 **/***/****	1.72±0.10 **

Note (in table I-II):

* – $p<0.05$ compared with values in the control group I (intact rats);

** – $p<0.05$ compared with values in the control group II (combined systemic and local LPS administration);

*** $p<0.05$ compared with values in the animals received corvitin only under systemic and local LPS administration;

**** $p<0.05$ compared with values in the animals received EGCG only under systemic and local LPS administration

Table II. Co-effect produced by corvitin and epigallocatechin-3-gallate on nitrosative stress markers in periodontium soft tissues under local and systemic administration of S. typhi lipopolysaccharide (M+m, n=35)

Groups of the animals studied	Total activity of NO-synthase, μmol / min ·g· of protein	Concentration of peroxynitrite- ions, μmol / g, of homogenate
Control group I (intact animals)	4.20±0.22	0.83±0.04
Control group II (combined systemic and local LPS administration)	12.53±0.61 *	1.54±0.06 *
+ water-soluble form of quercetin (corvitin)	8.42±0.64 */**	1.00±0.08 **
+ EGCG	6.78±0.54 */**	0.94±0.05 **
+ corvitin + EGCG	3.93±0.48 **/***/****	0.73±0.03 **/***/****

the correction of oxidative and nitrosative stress markers in the periodontal tissues.

Resent studies have demonstrated the potential of Keap1 / Nrf2 / ARE system to control other redox-sensitive elements, including NF-κB i AP-1 [15, 25]. We have found out the activation of NF-κB is an important component in the mechanism of free radical damage to the periodontal tissues during systemic inflammatory response. The use of an inhibitor of the nuclear translocation of NF-κB ammonium pyrrolidine dithiocarbamate in these conditions limits the manifestations of oxidative and nitrosative stress in the periodontium [29].

Previously it was shown that LPS-induced NF-κB activation could be attenuated by certain Nrf2 activators, such as phenethyl isothiocyanate, sulforaphane and curcumin [15]. The administration of these compounds significantly inhibits phosphorylation in the site of IκB kinase (*IKK*) / IκB and p65 NF-κB subunit nuclear translocation, consequently alleviating NF-κB signaling [26].

Co-administration of water-soluble form of quercetin and EGCG allows to limit NFκB-dependent mechanisms of oxidative and nitrosative stress more effectively as well as to enhance antioxidant potential of periodontal tissues associated with ARE-dependent gene expression.

Non-toxicity of quercetin and EGCG is a benefit, which distinguishes them from most NF-κB activation inhibitors, whose applying is known to cause a number of adverse reactions [17].

Thus, the co-administration of water-soluble form of quercetin and epigallocatechin-3-gallate under systemic and local introducing of *S. typhi* lipopolysaccharide has been proven to be more effective means for preventing and correcting oxidative and nitrosative stress in the periodontal tissues than this occurs at separate administration of each of the polyphenols.

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Authors' contributions:
According to the order of the Authorship.

Conflict of interest:
The Authors declare no conflict of interest.

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