Original Research Article

Ageing induced hyperproduction of reactive oxygen species and dysbalance in enzymatic link of antioxidant defense system of skin and therapeutic efficacy of artichoke extract

ABSTRACT

Skin aging is multitarget persistence processing that immediately involve hyperproduction of free radicals under influence of intrinsic and extrinsic factors and deterioration in intimal antioxidant defense system. The goal of the study was the evaluation of the anti-oxidant potential of artichoke standartizated extracts, 2%, as a protective strategy against skin age-associated oxidative damage caused by D-galactose (D-gal) in rats. 58 female Wistar rats included in the experimental design. D-gal-induced aging was reproduced in 36 animals of main group, and 12 rats included in control group. All animals in main group were randomized for 3 groups: I – animals with skin aging reproduced model receive saline, II – animals with skin aging rats receive artichoke extracts (with content of chloroagenic acid 2.0%) in a dose of intradermal injection 0.13 mg/kg and main III group - animals with skin aging receive 1.3 mg/kg artichoke extract twice at weeks during 4 weeks. Influence of artichoke extracts restores skin relative weight and leads to decreasing the rate of generation of superoxide anion, hydrogen peroxide and lipid peroxidation (LPx), increasing activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and reverse ratio SOD/(catalase+GPx) to the production of H_2O_2 from superoxide dismutation coupling with the decrease ratio of generated O_2^-/H_2O_2. Low-dose of intradermally microinjection of artichoke extracts, 2%, activated the enzymatic link in innate antioxidant defense system in D-gal-induced skin aging model and could be recommended for applications in cosmetics as antiaging mesotherapy.

UNDER PEER REVIEW

Key words: *skin, aging, artichoke extract, oxidant defense system, superoxide anion, glutathione system*.

Abbreviation. GSH - Reduced glutathione, GSSG - oxidized glutathione, GSH-Px – glutathione peroxidase, lipid peroxidation MDA - Malone aldehyde, Mt –Mitochondrial, ROS - Reactive oxygen species, SOD – superoxide dismutase

1. INTRODUCTION

Intrinsic skin aging process mainly includes gloomy skin, relaxation, moisture reduction, thinning, is an inevitable spontaneous process and complex natural phenomenon characterized aging [1-4]. More popular hypothesis that at the molecular level aging is multifactorial gradual biological process associated with diminishes homeostasis, mitochondrial DNA (mtDNA) damage, and progressive decline of innate defense systems of the body, and endogenous antioxidant defense system and oxidative stress formation, particularly [6-7]. Free radical and mitochondrial theories of aging supported by estimation of positive relation between the signs of aging and progression of imbalance of free radical metabolism and oxidative damage affects replication and transcription of mtDNA, which closely accompanied the structure and function deterioration in energy supply systems of tissues and organs of the aging and age-related diseases. The decline or/and disturbances of energy supply system functioning leads to increased mitochondrial reactive oxygen species (ROS) generation, ROS-induced lipid peroxidation in mitochondrial membranes and release of cytochrom C. These together with antioxidant defense systems imbalance results in further greater overproduction of ROS and to a vicious cycle of premature cellular senescence, skin aging and aged related diseases [4,5,8]. As a model for pharmacological studies of age-dependent alterations in skin we have choice one of the most widely used and demonstrated to display similar symptoms to those aging naturally D-galactose (D-gal)-treated animal model [9-14]. At high levels, D-gal, an aldohexose, monosaccharide sugar, is a naturally occurring substance in the body, which is completely

UNDER PEER REVIEW

metabolized at normal concentrations and induced disruption in carbohydrate metabolism pathway and causes oxidative stress via stimulation of free radical production and accumulation, apoptosis and inflammation in beyond normal concentration [8-10]. In according to one of the hypothesis that expressive administration of D-gal could induced damage associate with mitochondrial dysfunction caused by complex I deficiency [8-10, 14] and can accelerate aging was suggested and then confirmed in experimental and clinical data. In order to evaluated the molecular mechanism involved in the controlling of oxidative stress formation we firstly investigated the formation of superoxide anion and hydrogen peroxide and activity of much important components of enzymatic part of antioxidant defense system in D-gal induced skin aging model in experimental animals. Early in clinical practice [16-19] and in experimental studies [20-26], it was shown antioxidant and antitoxic activities [27-28], glycemia-lowering effect [21-22, 24, 28-30], and etc. of artichoke extracts, 5%, but therapeutic properties of artichoke leaves extract on the skin aging process practically have not been investigated. In this study, we examined the possible mesotherapeutic potential of artichoke (*Cynara scolymus* L. (Asteraceae), folium) extract, 2%, to decline the deterioration in skin oxidant defense system in experimental model of skin aging.

2. MATERIALS AND METHODS.

2.1. Plant materials and Authentication

The fresh leaves of the artichoke *C. cardunculus* L. var. *scolymus* (L.), family Aesreraceae, were collected at harvest maturity stage from the June to the middle of October during the 2016-17 years in Mtskhetis region (Rosenthal, Georgia, latitude 41° 56' 02" N and longitude 44° 34' 36" E), average minimum temperature -1°C and maximum 35°C. The plant was identified at the Pharmaceutical Natural Sciences Department of Institute of Pharmacy of Sechenov First Moscow State Medical University (Sechenov University)

UNDER PEER REVIEW

2.2. Preparation of plant extracts and its toxicity study

The leaves the artichoke were separated, washed, cleaned, and drying in according with Eur Ph monograph 01/2008:1866 corrected 6.0. Extraction of dried leaves artichoke, separation and identification of volatiles artichoke was prepared in according with Eur Ph monograph 01/2009:2389 (content of chlorogenic acid <2,5%) as described early [31]. The studying extracts of artichoke, 2%, in ampoule was characterized by the content of chloroagenic acid 1.95% (related to the requirements of assessment report on *C. scolymus*, folium EMA/HMPC/150209/2009), total phenolic content equal 0,31±0.04 mg gallic acid equivalent/100 mg extract, total flavonoids 1.6% and total antioxidant activities determinate as 50% inhibition of 1,1-diphenyl-2-picrylhydrazyl (DDPH) 15.1±0.9%). The toxicity of studding artichoke extracts under i.p. administration is very low, LD50 exceeds 1g/kg body weight and no rats exhibited visible signs of toxicity under 14 days of intradermal injection of extracts of artichoke, 2% including absence of physiologically changes in skin and fur, eyes or mucous membranes. Moderately irritating reactions induced by extracts of artichoke, observed at concentration more than 10% and extracts of artichoke, 2% shows good skin compatibility in patch test [31].

2.3. Animals and experimental study design.

2.3.1. Ethical statement

Animals received humane care in compliance with “Guide for the Care and Use of Laboratory animals” (National Institutes of Health publication 86-23, Revised 1996) and performed with approval of the local Interinstitutional (International Scientific Centre of Introduction of New Biomedical Technology, Department of Medical Pharmacology and Pharmacotherapy, Tbilisi State Medical University, Tbilisi) Animal Care and Use Committee. All animals secured under specific pathogen free conditions according to the Federation of European Laboratory Animal

UNDER PEER REVIEW

Science Associations guidelines in humidity- and temperature-controlled environment, with a daylight environment for at least 1 week before the experiments. Animals were fed commercial laboratory rat's food pellet and allowed drink tap water ad libitum before the experiments.

2.2.2. Study design

Experiments were performed on 58 female Wistar rats weighing 180-200 g. the rats were adapted for 7 days in animal mini clinic and then randomly divided into two groups: control (22 animals) and main (36 animals). Animals in main group after randomization received injection with D-gal (100 mg/kg/day, i.p. [31,32]), while in control group received placebo (0.9% saline, 0.5 ml/day, i.p.), for 8 weeks. At 21 days after injection with D-gal the 3 cm round tattoo area was prefabricated on each side of rats previously disinfected hip under sterile condition and general anesthesia with pentobarbital (40 mg/kg). All animals in main group were secondly randomized into 3 groups in dependence to treatment (twice in week of intradermal injection under general anesthesia) for 5 weeks: control III group animals treated with microinjection of saline (n=12), main I group receive 0.13 mg of 2% lyophilized powder of Artichoke extracts salivated in water for injection (equivalent of average intradermal dose for patients 10 mg, n=12) and main II – animals receive 1.3 mg 2% lyophilized powder of Artichoke extracts (n=12). After the experiments, all the rats euthanized by pentobarbital (60 mg/kg intraperitoneally). Body weight and skin oedema evaluation was investigated as described below [31].

2.3. Determination of activities of enzymatic part of endogenous antioxidant defense system of skin of rats

Isolation of mitochondria incubated with buffer (6 mM succinate, 70 mM sucrose, 220 mM mannitol, 2 mM, Hepes, 25 mM KH_2PO_4, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 5 µg/ml catalase, pH 7.4) at 37˚C and immediately measured of velocity of superoxide anion generation, superoxide dismutase (total), catalase, gluthatione peroxidase and malone aldehyde (MDA) were described

UNDER PEER REVIEW

[33,34]. Rate of H_2O_2 production was determinate as described below [35,36]. The activity of glutathione redox system including determination of glutathione peroxidase (GSH-Px) and glutathione reductase by velocity of redox $NADP^+$ formation, and redox glutathione in homogenate of lyophilized in liquid nitrogen skin tissue in according to [34-36]. The protein concentration was determined with BSA protein assay kit.

2.4. Statistical analysis

Statistical analysis of presented data as mean ± standard deviation of mean (SD) was performed using the Statistical Sciences (SPSS, version 23.1). The significance level of the differences between the control and main groups assessed using Student t-test and $p < 0.05$ considered as a significant.

RESULTS

The studying water artichoke (*C. cardunculus*, cultivated in Georgia, Mtskhetis region) extracts, 2% content of chloroagenic acid and about 10% of total phenolic acids and confirmed the requirements of the Assessment report on *C. scolymus*, folium EMA/HMPC/150209/2009 for medicinal using artichoke preparation.

3.1. Changes in body weight and skin oedema during D-gal-induced skin aging and influence of artichoke extracts, 2%

Prolonged 8 weeks D-gal-treated animals characterized by a unique skin appearance, with wrinkling's and furrows, which indicated that developed the evident symptoms of aging. Prior to euthanized, no morbidity/mortality and clinically relief differences in food intake and water consumption in subgroups of main group were not observed. The relative weight of skin markedly decrease in D-gal model of aging. Artichoke at the doses of 0.13 and 1,3 mg/kg

UNDER PEER REVIEW

155 improved body weight of D-gal-induced aging rats (table 1). While the administration of
156 artichoke extracts in normal rats for 8 weeks did not change, the body weight compared to the
157 control group. Thus, treatment with artichoke extracts, 2% restores the water disbalanced in the
158 aging skin in both doses.
159
160 Table 1. **Therapeutic efficacy of different doses of artichoke extracts for maintenance the**
161 **activity of endogenous enzymatic antioxidant defense system D-galactose induced aging**
162 **skin in experimental animals.**

Groups	**Control I, n=10**	**Control II, n=12**	**D-galactose agin sking rats, n=36**		
			Control III, n=12	**Liophylized extract artichoke, dose, mg/kg intradermally**	
				0.13, n=12	**1.3, n=12**
Body weight, g	187±22	312±23	245±25$^{*\#\#}$	278±24**	268±21*
Relative weight, mg dry/100 mg wet weight	31.5±2.1	32.8±1.4	23.5± 2.3$^{**\#\#}$	29.2± 1.8^{x}	29.7± 2.1^{x}
Velocity of O_2^- generation	0.27±0.02	0.31±0.03	0.48± 0.06$^{**\#\#}$	0.35± 0.04^{*x}	0.36± 0.05^{*x}
H_2O_2, µmol/L · min	1.59±0.14	1.80±0.14	5.15± 0.23$^{***\#\#\#}$	3.02± 0.32$^{***\#\#\#xxx}$	3.17± 0.21$^{***\#\#\#xxx}$
SOD, U/mg protein/min	0.33± 0.04	0.32± 0.03	0.26± 0.02$^{*\#}$	0.39± 0.03$^{\#xxx}$	0.32± 0.03$^{\#x\S}$
Catalase, nMol H_2O_2/mg protein/min	64±9	67±8	42±4$^{**\#\#}$	68±6	59±6$^{\#}$
Glutathione redox	3.18±	2.90±	1.83±	2.41±	2.23±

UNDER PEER REVIEW

potential, GSH/GSSG	0.38	0.29	0.23$^{***\#\#}$	0.19^{**xx}	0.15^{**x}
Glutathione peroxidase, nMol NADP/mg protein	2.44± 0.22	2.69± 0.33	1.73± 0.23$^{**\#\#}$	2.51± 0.20^{x}	1.97± 0.13$^{*\#}$
Gluthatione reductase, µMol NADPH/g wet tissue	0.10± 0.02	0.19± 0.03*	0.29± 0.04$^{***\#\#}$	0.18± 0.04^{*x}	0.11± 0.03$^{\#xxx}$
MDA, µmol/mg protein	0.88± 0.08	0.92± 0.10	1.48± 0.16$^{***\#\#}$	0.96± 0.06^{xxx}	1.09± 0.09^{xx}

Note: *- compared with control 1, # - with control 2 group, x - with control 3 and § - between artichoke extracts treatment groups; significance of difference of comparison: one symbol – $p<0.05$, two – $p<0.01$, three - $p<0.001$, absence of symbol indicated that differences is not significance ($p>0.05$).

3.2. D-gal-induced aging changes in skin and activity of total SOD and generation of superoxide anion.

D-gal in dose 100 mg/kg i.p. during 8 weeks cause to significant decreased in total SOD activity in skin in comparison with control I and control II, while differences in SOD activity between control I and control II groups did not mentioned (table 1). At the same time, the velocity of superoxide anion generation increased by 15% in control II group when comparing the rate of O_2^- production in 240 days rats (table 1). Treatment with 2% artichoke extract from the 21 days after D-galactose induced aging in rats leads to increase SOD activity by 50% and by 23% in comparison with control III groups and this accompanied with markedly decreasing in velocity of O_2^- generation by 27% and 25% in low and high doses of extracts, respectively. The velocity of superoxide anion generation at the end of the treatment in both doses of artichoke extracts did not differences from the level in placebo (control II) group.

UNDER PEER REVIEW

3.3. D-gal-induced aging changes in skin and activity of catalase and generation of hydrogen peroxide

There were no significant differences in catalase activity between control groups. Exposure to D-galactose did not induced changes in catalase activity in skin tissue (table 1). However, the production of H_2O_2 increased under treatment of D-gal and exceeds control II level by 186%. Treatment with 2% artichoke leaf extract increased the level of catalase activity, and decrease the level of H_2O_2 production by 42% in dosage of 0.13 mg and by 25% under higher doses.

3.4. D-galactose-induced aging changes in skin and activity of glutathione redox system

Exposure to D-gal reduced the GSH content in skin tissue from 1.20±0.13 nmol/mg/protein to 0.74±0.13 nmol/mg/protein ($p< 0.01$ *vs*. control III). Treatment with artichoke extract at doses 0.13 and 1.3 mg/kg significantly recovered the GSH content up to 0.98±0.09 and 0.89±0.09 nmol/mg/protein ($p<0.01$ and $p<0.05$, respectively) when compared to D-gal-treated animals. Simultaneously the GSH/GSSG ratio is proportionately decreased in D-gal-induced skin aging model by 37%. Treatment with artichoke extracts in doses of 1.3 mg/kg restored the glutathione redox and it has reached level in the same aging groups while at higher doses treatment the GSH/GSSG ratio increased only by 22% (table 1). Due to D-gal-treatment observed significantly decreasing of GSH-Px activity, with out any differences in GR activity (table). Treatment with artichoke extracts in dose of 0.13 mg increased the level of GSH-Px by 31% and only by 14% (NS) at doses of 13 mg/kg. Ratio of activities of SOD/(Catalase + GSH-Px), which represents equilibrium between formation of hydrogen peroxide from superoxide dismutation and its utilization by catalase and GSH-Px equal $5.0\pm0.3\times10^{-3}$ in rats at the beginning of the experiments and $4.6\pm0.2\times10^{-3}$ in control II group. In D-gal model of aging skin ratio

UNDER PEER REVIEW

SOD/(Catalase + GSH-Px) increased to 6.0±0.2 x10^{-3}, and decreased to 5.5a±0.2 and 5.2±0.2 after artichoke extracts treatments in low and high dosage, respectively. Simultaneously, the redox potential, ratio of generation O^{2-}/H_2O_2 which equal in intact group 0.17±0.04 decrease to 0.09±0.01 in D-gal treated control III group and increase to 0.12±0.2 ($p<0.01$) after artichoke treatment. There were no correlation between the level of ratio SOD/(Catalase + GSH-Px) and MDA content in skin (r=0,37, NS).

3.5. D-galactose-induced aging changes in skin MDA content

Despite that level of MDA also determinate as a marker of lipid peroxidation in skin and other tissues, MDA content, as a final product of lipid peroxidation, could not reflects the disturbances in the sensitivity of lipid to oxidation [37]. In the model of D-gal-induced aging levels of MDA in skin significant elevated, when compared to the control group ($p < 0.001$) following 42 days of exposure to D-gal, but not in aging group without D-gal-treatment (table). Interestingly, treatment of rats with artichoke at doses of 0.13 and 1.3 mg/kg significantly decreased the levels of MDA in skin in both cases.

4. DISCUSSION

D-gal is pharmacological adaptive aging model, because D-gal primary roles in pathogenesis of aging. Skin aging is a complicated multitargets misbalancing progression in the epidermis and dermis which documented by rising in superoxide anion production in D-gal-induced skin aging model in rats. Influence of artichoke extracts restored skin relative weight and leads to an increase of solubility in neutral salt, acid, and decreased pepsin solubility collagen fraction, restored the hexosamine/collagen (hydroxyproline) ratio and decreased the activity of nuclear transcription factor (NF-kB). Local prolonged treatment with artichoke extracts improved collagen metabolism and attenuated the progression of inflammation in D-gal-induced skin aging model [29]. Early it was shown, that chronic (6-8weeks) administration of D-gal blocking of

UNDER PEER REVIEW

glycometabolism (hyperproduction of advanced glycation products), misbalanced and loses of antioxidant activity of tissue (decreasing the level of SOD and gluthatione peroxidase activity) and increased level of MDA in dose dependent manner (50-500 mg/kg i.p. or subcutaneously) [10, 32, 38-40]. Rats in the model group exhibited the typical changes of aging skin compared with the control group, rats in the model group had significantly increased MDA content, and decreased serum SOD and GSH-Px activities ($P<0.05$). The end product of free radicals oxidizing of unsaturated lipids of biological membranes is MDA which can influence exchange of substances between cells, and finally lead to rupture and death of cells. Extract of artichoke is rich in phenolic and flavonoids and gives a powerful antioxidant activity [14-16, 40]. Pre-clinical and clinical investigations have suggested that the artichoke leaf extract has potential lipid-lowering and hepatoprotective effects [16-19, 21,22, 24,25]. The beneficial effects of artichoke could mainly attribute to its antioxidant components: the main substances are mono- and dicaffeoylquinic acid (cynarin and chlorogenic acid), caffeic acid (1%) and volatile sesquiterpene and flavonoids (1%) that include the glycosides luteolin-7-beta-rutinoside (scolymoside), luteolin-7-beta-D-glucoside and luteolin-4-beta-D-glucoside [14-16, 39]. Several *in vitro* studies have shown that the antioxidant potential of artichoke extracts is dependent on radical scavenging and metal ion chelating effect of its constituents such ascynarin, chlorogenic acid and flavonoids. However, pure constituents of artichoke extracts shown to produce less inhibitory activity on free radical production than the extract itself [14,15]. Interestingly, that artichoke is favors that synthesis of coenzymes NAD (NADH$_2$) and NADP(NADPH$_2$) and mainly of the NADP(NADPH$_2$) pair, which take key plays in the regulation of antioxidant/prooxidant status of the cell and its including in the antioxidant properties of artichoke extracts could be included. Preincubation of HUVEC cells or human leukocytes with the artichoke extract at concentrations of 25–100 µg/mL for 24 h abolished ROS generation induced by lipopolysaccharide and oxidation of low density lipoproteins [20, 40]. Early it was shown that artichoke (*C. scolymus*) in dosage 20, 40 80 mg/kg daily per os in D-gal (40 mg/kg

UNDER PEER REVIEW

body weight) daily for 36 days increase activity of SOD in brain and liver, GSH-Px in brain, and catalase activity in liver [32]. In present article for the first time was study influence of local intradermal action of *C. scolymus* extracts on restoration the ability of endogenous antioxidant defense system to prevent free radical injury development in skin of D-gal-treated (100 mg/kg daily for 8 weeks, i.p.) rats. D-gal (100 mg/kg daily for 8 weeks, i.p.) skin aging in rats characterized increasing in superoxide anion generation in and hydrogen peroxide in widely applied to anti-aging pharmacology studies sub-acutely aging models of rodents induced by chronic injection of D-gal [39]. States of skin in this model accompanied with decrease in the activity of SOD, catalase and GSH-Px, and increased production of superoxide anion and hydroperoxide. Hyperproduction of hydrogen peroxide in aging occurs in response disturbances in aerobic respiration and one molecule of catalase can inactivate about 6 million hydrogen peroxide molecule per min by combined them two a time. Thus, the less increased in catalase activity under treatment of artichoke really could sufficient to neutralized produced hydrogen peroxide under decreasing of superoxide anion generation and as a result its oxidation to H_2O_2 by SOD. Oxidative damage was concomitant to an imbalance in the principal antioxidant cytoplasmic agent - a significant reduction in cellular GSH, which exerts antioxidant activity by acting as a free-radical scavenger during the reductive detoxification of hydrogen peroxide and lipid peroxide is one of the important target of skin-whitening effect of aging. Exposure to D-gal reduced the GSH content in skin tissue, while artichoke extract at doses 0.13 and 1.3 mg/kg significantly recovered the GSH content. Due to D-gal-treatment observed significantly decreasing of GSH-Px activity, with_out any differences in GR activity (table). Treatment with artichoke extracts in dose of 0.13 mg markedly increased the level of GSH-Px by 45% and 13% in dose of 13 mg/kg. The data suggest that oxidative stress reduces gluthathione redox potential and that prevention disturbances in GSH redox cycle activity appears to be an important component of the antiaging phenomenon.

UNDER PEER REVIEW

5. CONCLUSION

In conclusion the redox potential of the $O_2/2H_2O$ redox system could play a key role in the "Free Radical Theory of Aging" , seems to address a key facet of intrinsic biological instability of living systems throughout unavoidably formed ROS in the course of metabolism and arising due to the action of various exogenous factors, damage biomolecules [1-5, 37-39]. Obtained data indicate that the concomitant use of 2% artichoke extract improve reserve ability of antioxidant defense system and exert antiaging action in this model of skin aging in experimental animals. The increased reserve ability of intrinsic antioxidant defense system of skin after course of local treatment with artichoke extracts emphasizes artichoke dry extract efficacy in cosmetic formulation and its beneficial effects for anti-aging skin care.

CONSENT

Is not applicable

ETHICAL APPROVAL

Authors declared that the all procedures with animals meet the requirements of Declaration of Helsinki, Finland in its seven revisions (General Assembly, October, 2013) [Declaration of Helsinki History Website". Ethical Principles For Medical Research. The JAMA Network. Retrieved 26 July 2015] and European Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes

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UNDER PEER REVIEW

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COMPETING OF INTEREST

The authors declare that they have no conflict of interests regarding the publication of this paper. The authors alone are responsible for the content and writing of this article.

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REVIEW

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UNDER PEER REVIEW

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PEER REVIEW

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