An in vitro evaluation of anti-aging effect of guluronic acid (G2013) based on enzymatic oxidative stress gene expression using healthy individuals PBMCs

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ABSTRACT

Background: Aging is usually associated with increased levels of oxidants, and may result in damages caused by oxidative stress. There is a direct relationship between aging and increased incidence of inflammatory diseases. The present research intended to study the anti-aging and anti-inflammatory effects of the drug G2013 (guluronic acid) at low and high doses on the genes expression of a number of enzymes involved in oxidative stress (including SOD2, GPX1, CAT, GST, iNOS, and MPO) in peripheral blood mononuclear cells (PBMCs) of healthy individuals under in vitro conditions.

Methods: Venous blood samples were taken from 20 healthy individuals, the PBMCs were isolated and their RNAs extracted and their cDNAs were synthesized, and the genes expression levels were measured using the qRT-PCR technique.

Results: Our results indicated that this drug could, at both low and high doses, significantly reduce the expression of the genes for SOD2, GPX1, CAT, and GST compared to the LPS group (p < 0.0001). Moreover, it was noticed that the drug is able to significantly reduce gene expression levels at the high dose and at both doses (low and high), for iNOS and MPO compared to the LPS group (p < 0.0001), respectively.

Conclusions: The present research showed that G2013, as a novel NSAID drug with immunomodulatory properties, could modulate the expression levels of the genes for SOD2, GPX1, CAT, GST, iNOS, and MPO, to the level of healthy gene expression, and possibly it might reduce the pathological process of aging and age-related inflammatory diseases.

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1. Introduction

Free radicals and oxidants are beneficial or toxic compounds and can thus be useful or harmful for the human body. If high levels of free radicals cannot be gradually lowered, their accumulation generates a phenomenon called oxidative stress [1,2]. In other words, oxidative stress is generated due to an imbalance between the production of free radicals and pro-oxidants and the antioxidant defense system [3,4]. Most free radicals obtained from oxygen during aerobic metabolism are called reactive oxygen species (ROS) and reactive nitrogen species (RNS). Free radicals damage many macromolecules including DNA, proteins, lipids, and carbohydrate [5]. The increased ROS and RNS production inflict oxidative damage on body tissues. Oxidative stress is the underlying cause of a number of physiological and pathophysiological phenomena and plays a role in diverse processes including aging, autoimmune disorders, inflammation, cancer and neurodegenerative diseases [2,6]. The human body weakens oxidative stress through antioxidant production [1]. Antioxidants can prevent damages caused by ROS and RNS activities and/or repair these damages and, hence, can strengthen immune defense and anti-aging process activity and reduce the risk of diseases [7–9]. Antioxidant systems are divided into enzymatic and non-enzymatic groups. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and also glutathione S-transferase (GST) are among the most important enzymatic antioxidants [10,11]. SOD is the first line of defense against oxidative stress.

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under physiological and pathological conditions [12]. Based on tissue distribution and differences in metal cofactors, four groups of SOD have been identified. Superoxide dismutase 2, mitochondrial (SOD2), which is often referred as manganese-dependent SOD (MnSOD), is encoded by the SOD2 gene on chromosome 6q25.3 in humans [12]. In a research conducted by Liu on C57BL/6N(C/N) mice, it was found that the lack of SOD in the brain could increase the protein oxidation and lipid peroxidation, so that the effect of aging was appeared much earlier compared to healthy mice [13]. The GPX1 enzyme, which plays the most important role in cellular defense against oxidative stress [14], is encoded by the GPX1 gene on chromosome 3 in humans [15]. Normally, the concentration of lipofuscin, and lipid peroxidation increase in the aging process, but the activity of GPX decreases. Bala et al. showed in their research that the concentration of lipofuscin, and lipid peroxidation, in the brain area, substantially decreased, but the activity of GPX considerably increased, during the treatment of old rats with curcumin [16]. CAT is one of the most important enzymes in the protection of cells against oxidative stress caused by hydrogen peroxide \( (H_2O_2) \) [17]. In humans, the CAT gene is located on chromosome 11p13 [17]. Normally, CAT is more active in old rats compared to young ones. Palomero et al. demonstrated in their study that CAT activity substantially decreased during the treatment of old rats with cyclosporine [18]. The activity of the enzyme GST is induced in many organisms that are exposed to various stresses including oxidative stress [19]. Cytosolic GST has an extensive genetic polymorphism in human populations. In humans, the GSTM1 gene is located on chromosome 1p13.3 [20]. In research conducted by Fano concerning the effects of aging on skeletal muscles in men and women, it was found that aging influenced only the GSH-related isoenzymes (GST) and reduced its activity [21]. Some enzymes, including inducible nitric oxide synthase (iNOS) and myeloperoxidase (MPO), produce free radicals (that are involved both in inflammation and in aging). The genes encoding iNOS and MPO in humans are located on chromosomes 12 and 17, respectively [22,23]. Ferrini et al. noticed in their research that the iNOS protein (and NOS performance in general) increased in the hypothalamus and in other regions of the brain in rats during aging [24]. It was shown in another research that the inflammation caused by lipopolysaccharide (LPS) increased the activity of MPO and its protein content in the kidneys of young rats. It is thought that the increased activity of MPO in old age is related to increases in the number of inflammatory cells [25].

NSAIDs play an important role in the management of inflammatory diseases [26]. During recent years, researchers have tried to identify the safer and more effective types of anti-inflammatory and immunomodulatory drugs. The patented (DE-102016113017.6) G2013 (guluronic acid) molecule is an agent with the lowest molecular weight and has no toxicity compared with other NSAIDs [27,28]. G2013 is a novel designed drug which could be classified as a non-steroidal anti-inflammatory drug (NSAID), with immunomodulatory property [29].

Regarding, the pivotal role of oxidative stress enzymes in aging phenomenon, the present study aimed to investigate the anti-aging and anti-inflammatory efficacy of G2013 in both low and high doses on gene expression of enzymes involved in oxidative stress (including SOD2, GPX1, CAT, GST, iNOS, and MPO) in peripheral blood mononuclear cells (PBMCs) of healthy individuals under in vitro conditions.

2. Material and methods

2.1. Blood collection and isolation of PBMCs

The venous blood of twenty healthy individuals (between 25 and 45 years, were recruited at Blood Transfusion Center of Rafsanjan-Iran, after informed consent protocol of the declaration), were collected on sodium heparin as an anticoagulant. The healthy volunteers were from no smoking group and without using illicit drugs or suffering from diabetes, hypertension or metabolic disease and they had no history of the chronic or autoimmune disease. The PBMCs were isolated from blood sample through Ficoll-Hypaque (Mediatech Cellgro) density gradient centrifugation from buffy coats provided from the healthy blood. The PBMCs were then resuspended in RPMI-1640 supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (all reagents provided by Gibco, USA). The cell viability was determined by trypan blue 0.4% (Sigma-Aldrich) exclusion at 1: 4 dilution (1 part of cells: 4 parts of dye). The samples were only used when viability was >95%. This experiment was approved by Ethic Committee of Rafsanjan University of Medical Sciences (RUMS).

2.2. Preparation of G2013

The guluronic acid (G2013) with molecular formula \( (C_{6}H_{10}O_{7}) \) and IUPAC name [(2R/3S/4S/5S)-2/3/4/5-tetrahydroxy-6-oxohexanoic acid] were prepared from alginic acid sodium salt (Sigma-Aldrich, St. Louis, MO) as a reference sample. The purification method was carried out based on a modified procedure of the acid hydrolysis method [30]. Briefly, alginic acid sodium salt (100 g) was dissolved gently in 20% \( H_2SO_4 \) at 0 °C and the mixture was thoroughly stirred at room temperature. This solution was heated at 80 °C until its color was changed from a creamy color to light brown. The hydrolysate was cooled to room temperature and precipitated by centrifugation (3700 × g). The precipitate was re-dissolved by neutralization using 1 M \( Na_2CO_3 \). The solution was then adjusted to pH 2.85 with 0.1 M HCl. The precipitate was collected and washed once with distilled water. The final precipitate (guluronic acid) was spread over and dried out in Petri dishes [31]. The method was validated by characterizing the hydrolytic products using Fourier Transform Infrared (FT-IR) spectroscopy and carbon-13 nuclear magnetic resonance \( (^{13}C \text{ NMR}) \) spectroscopy for confirming its molecular weight (194.139 g/mol) and exact/molecular mass (194.043 g/mol).

2.3. Treatment of PBMCs with G2013 and LPS

PBMCs were cultured in 4-well of culture plate with RPMI-1640 supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (all reagents from Gibco, USA). In this way, the first well contains \( 2 \times 10^6 \) cells in the absence of LPS and G2013 (negative control) and second well contains \( 2 \times 10^6 \) cells in the presence of LPS-EB (LPS from \( E. coli \) O111: B4) (1 \( \mu g/ml \)) in order to stimulate PBMCs and absence of G2013 (positive control). The third and fourth wells of culture plate contain \( 2 \times 10^6 \) cells and LPS-EB (1 \( \mu g/ml \)), so that after 4 h incubation at 37 °C, we added 5 \( \mu g/well \) of G2013 (the low dose of the test well) and 25 \( \mu g/well \) of G2013 (the high dose of the test well). Cells were incubated for 18 h at 37 °C in humidified 5% carbon dioxide \( (CO_2) \) incubator.

2.4. RNA extraction

RNA was extracted from control and treated cells using Hybrid-R™ Mini kit (GeneAll, Republic of Korea) according to the manufacturer's guidelines. The extracted RNA quality was identified by electrophoresis on the GelRed™ (Biotium, USA) containing agarose gel and measured its absorption on A260/280 nm by NanoDrop® ND1000 spectrophotometer (Isogen Life Science, Netherlands).
2.5. cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Total RNA from control and treated cells was reverse transcribed using HyperScript™ First-strand Synthesis Kit (GeneAll, Republic of Korea) and the manufacturer’s instructions. The cDNA synthesis reactions included 10 µl total RNA, 1 µl Random hexamer, 1 µl dNTPs, 2 µl Nuclease-free water, 2 µl RT reaction buffer (10×), 2 µl MDTT (0.1), 2 µl HyperScript Reverse Transcriptase (200 U/µl), 1 µl ZymAll™ RNase Inhibitor. The total mentioned agents (20 µl) were incubated for 5 min at 65 °C, 1 min on ice, 40 min at 50 °C, 5 min at 60 °C, and held at 4 °C. Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Dalian, Takara Co., Ltd), produced cDNA and appropriate primers (Table 1). Twenty microliters of real-time PCR reactions included 1 µl genomic template DNA, 10 µl SYBR® Premix Ex Taq™ II, 0.4 µl Rox, 7.6 µl nuclease-free water, 0.5 µl forward primer, 0.5 µl reverse primers. The following program was run on the ABI StepOne Plus real-time PCR system (Applied Biosystems Company, USA): one cycle of 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 30 s, 70 °C for 15 s. Real-time PCR was carried out in optical 8-cap strips (MicroAmp™ Applied Biosystems, Singapore) and the β-actin as housekeeping gene was used for normalizing the amplification. The relative amounts of PCR product were calculated using the 2-ΔΔct method. The quality of graphs, melting curves and quantitative analyses of the data were performed using StepOne Software Version 2.2.2 (Applied Biosystems, USA).

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism Software Version 6.02 (GraphPad Software, San Diego California USA; www.graphpad.com). Repeated measures one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons post hoc tests was utilized to analyze the expression of the genes between treated and untreated (control) group. The results were expressed as the mean value ± standard deviation (SD). A p-value < 0.05 was considered to be a statistically significant difference.

3. Results

3.1. Effects of G2013 on mRNA expression of SOD2, GPX1, CAT, and GST

Our data demonstrated that the gene expression of SOD2 in PBMCs of healthy donors was decreased 237% ± 8 and 247% ± 6 by G2013 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (362% ± 24) (Fig. 1). The statistical analyses revealed that the difference between G2013 at low dose + LPS and control-treated with LPS (1 µg/ml) were significant (p < 0.0001). Moreover, the difference between G2013 at high dose + LPS compared to LPS group was significant (p < 0.0001). The results also illustrated that the mRNA expression of GPX1 in PBMCs of healthy donors was decreased 269% ± 31 and 318% ± 10 by G2013 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (489% ± 47) (Fig. 2). The statistical analyses revealed that the difference between G2013 at low dose + LPS and control-treated with LPS was significant (p < 0.0001). Moreover, the difference between G2013 at high dose + LPS and LPS group was also significant (p < 0.0001).

Our results indicated that the gene expression of CAT in PBMCs of healthy donors was decreased 72% ± 37 and 342% ± 15 by G2013 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (417% ± 44) (Fig. 3). The statistical analyses revealed that the difference between G2013 at low dose + LPS and control-treated with LPS (1 µg/ml) was not significant (p = 0.27), while the difference between G2013 at high dose + LPS compared to LPS group was significant (p < 0.0001). The results also showed that the mRNA expression of GST in PBMCs of healthy donors was decreased 255% ± 9 and 265% ± 7 by G2013 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (349% ± 23) (Fig. 4). The statistical analyses revealed that the

![Table 1](Image)

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difference between G2013 low dose + LPS and control-treated with LPS was significant (p < 0.0001). Moreover, the difference between G2013 at high dose + LPS and LPS group was significant (p < 0.0001).

3.2. Effects of G2013 on mRNA expression of iNOS and MPO

Our findings showed that the mRNA expression of iNOS in PBMCs of healthy donors was decreased 72% ± 31 and 286% ± 9 by G2013 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (401% ± 36) (Fig. 5). The statistical analyses revealed that the difference between G2013 at low dose + LPS and control-treated with LPS (1 μg/ml) was not significant (p = 0.18), while the difference between G2013 at high dose + LPS and LPS group was significant (p < 0.0001). The results also indicated that the gene expression of MPO in PBMCs of healthy donors was decreased 265% ± 27 and 261% ± 7 by G2013 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (356% ± 27) (Fig. 6). The statistical analyses revealed that the difference between G2013 at low dose + LPS and control-treated with LPS (1 μg/ml) were significant (p < 0.0001). Moreover, the
Fig. 6. The effects of G2013 on the level of MPO mRNA in PBMCs of healthy volunteers. The groups were untreated control, LPS-treated (1 μg/ml) and G2013 with two doses, low (5 μg/ml) and high (25 μg/ml). The MPO mRNA expression was measured by qRT-PCR using total RNA extracted from control and treated cells. Values were normalized to the β-actin content of the samples. The results were expressed as the target/reference ratio of the treated samples divided into the target/reference ratio of the untreated control sample and expressed as mean ± SD. *p < 0.0001 vs. LPS-treated group and ****p < 0.0001 vs. untreated control group.

difference between G2013 at high dose + LPS and LPS group was significant (p < 0.0001).

4. Discussion

Aging is typically associated with the increased level of oxidants; it may occur due to the damage caused by ROS or oxidative stress in a cell, which impairs cell function. The oxidative stress that leads to aging varies among organisms, tissues, and even various cell types [32]. The Oxidative Stress Theory of aging expresses that ROS, which is the initial result of the natural metabolism of mitochondria, leads to a progressive damage called “aging” [33]. The chronic oxidative stress and inflammation are directed toward many age-related diseases such as arthritis, atherosclerosis, Alzheimer’s, and Parkinson’s [34].

MnSOD-lacking mice better show the harmful effects of superoxide, resulting in accumulation and formation of mitochondrial ROS and RNS [35]. Van Remmen et al. proved that 50% of MnSOD enzyme activity decrease in SOD2 +/- phenotype mice and oxidative damage occurs in all tissues and mitochondrial DNA, and this phenotype increases cancer incidence in older mice [36]. Tanaka et al. proved that high concentration of glucose in diabetes might lead to intracellular ROS accumulation in islets of Langerhans and consequently the increase of GPX performance in beta cells [37]. In a Study using diabetic rats, Jang et al. demonstrated the GPX activity reduces in liver mitochondria and increases in its expression in pancreas and kidney mitochondria [38]. The decrease catalase activity and increased levels of hydrogen peroxide are observed in the epidermis of the patients with vitiligo, the conditions that lead to epidermal oxidative stress which finally damages pigment cells [39]. Another study shows that catalase activity reduces when it meets RNS under laboratory conditions [40]. Several studies in animal models proved the effect of GST activity in preventing liver toxicity induced by chemical agents [41]. GSTM1 null polymorphism is associated with leukemia, cancer, rheumatoid arthritis, and asthma. Bekris et al. have proved that the environmental toxic agents and oxidative stress are important in type 1 diabetes (autoimmune) progression and GSTM1 isoform may also involve in the disease. It was reported that the lack of GSTM1 activity is in agreement with type 1 diabetes because it can reduce the inflammatory responses [42]. Mikul et al. have shown that GSTM1 null genotype involves in an increased risk of autoimmune rheumatoid arthritis [43]. The patients with RA have demonstrated an increased expression in iNOS and also an increased nitric oxide (NO) formation, which is associated with disease activity [44]. In addition, the overproduction of NO by iNOS is a major mediator of apoptosis in RA joints and a major regulator of Th1/Th2 balance in autoimmune diseases [45,46]. Studies have proved that the injection of iNOS inhibitors has beneficial effects on mouse models of RA [47]. Many studies have mentioned the role of MPO in atherosclerosis. For instance, Eiserich et al. showed that a mouse with no MPO is more resistant to acetylcholine-dependent vascular relaxation induced by LPS treatment than a healthy mouse [48]. Moreover, MPO acts as the major catalyst of lipid oxidation at the site of inflammation; this phenomenon forms the basic process of formation and spread of plaques in atherosclerosis [49]. Choi et al. proved that the MPO gene expression increases significantly in the individuals with Parkinson’s disease [50]. Green et al. have shown that the MPO gene expression increases in the nerve cells of patients with Alzheimer’s disease [51]. Therefore, it can increase the probability that this enzyme assists oxidative damage in multiple disorders of chronic neurological diseases [50]. According to the research conducted on G2013, this molecule has shown the significant positive effects on the experimental model of multiple sclerosis (MS) [27]. Advantages of G2013 compared to other NSAIDs include its low molecular weight and natural base, immunomodulatory and anti-aging effects with its high safety property [28,29,52].

Regarding the recent studies have proved a major role for the enzymes involved in oxidative stress in aging and autoimmune and inflammatory diseases, therefore, this study aimed to study the anti-aging and anti-inflammatory effects of the G2013 at low and high doses on gene expression of the enzymes involved in oxidative stress (including SOD2, GPX1, CAT, GST, iNOS, and MPO) in the PBMCs of healthy individuals under in vitro conditions. The results of this study revealed that the drug at both (low and high) doses is able to reduce SOD2, GPX1, CAT and GST gene expression significantly as compared with the LPS-treated control group (Figs. 1–4). This study also showed this drug at the high dose and both doses (low and high) significantly reduce iNOS and MPO gene expression, respectively as compared with the LPS-treated control group (Figs. 5 and 6).

5. Conclusion

The enzymatic oxidative stress and its related parameters are one of the most important pathophysiological phenomena which play an essential role in the progression of in the aging process. The investigators attempt to control the progression of inflammatory diseases and aging using antioxidant-based drugs. This research showed that G2013, as a novel NSAID with immunomodulatory properties, could adjust the oxidative stress through reducing the gene expression of SOD2, GPX1, CAT, GST, iNOS, and MPO to the level of healthy control which possibly, it might be able to reduce the process of aging and age-related inflammatory diseases.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

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