ORIGINAL ARTICLE

Garlic Extract Attenuates Immobilization Stress-Induced Alterations in Plasma Antioxidant/Oxidant Parameters and Hepatic Function in Rats*

Syed Kashif Zaidi[1], Shakeel Ahmed Ansari[1], Shams Tabrez[2], Md Nasrul Hoda[3], Ghulam Md. Ashraf[2], Mohd Shahnawaz Khan[4], Sultan Alnohair[5], Naheed Banu[6], and Mohammed Hussain Al-Qahtani[1]

ABSTRACT **Objective**: To investigate the oxidative stress induced by 6 h of immobilization stress in Albino Wistar rats. Further, the pre- and post-treatment of aqueous garlic extract was studied to evaluate its preventive and curative efficacy on stress-induced altered oxidative parameters in rats. **Methods**: Albino Wistar rats were exposed to 6 h of immobilization stress, and received garlic extract (100 mg/kg body weight) treatment pre- or post-stress exposure. The oxidative status of plasma after various treatments were evaluated by determining the levels of reduced glutathione, glucose, uric acid, thiobarbituric acid reactive substances, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and the activities of superoxide dismutase, catalase and glutathione-S-transferase by standardized procedures. **Results**: Immobilization of rats generated oxidative stress in rat plasma, by decreasing the activities of antioxidant enzymes, glutathione levels and glucose, while increasing the lipid peroxidation, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, ALP and uric acid compared to the non-stressed controls (P<0.01). The garlic extract administration both pre- and post-stress exposure significantly prevented the rise in the diagnostic liver enzymes and reverted the decrease of antioxidant enzymatic activities compared to the stressed group (P<0.05 or P<0.01). Post-stress treatment of extract was found more effective than pre-stress treatment in reverting the values back to normal (P<0.01). **Conclusion**: Garlic extract seems promising as a nutritional supplement for scavenging free radicals generated in the plasma and to prevent resulting oxidative stress.

KEYWORDS stress, antioxidant enzymes, aspartate aminotransferase, alanine transaminase, malondialdehyde, garlic

Garlic (*Allium sativum* L.) is extensively used as the flavoring agent and traditional medicine for healing. The aroma in garlic and its medicinal effects are mainly due to the presence of organosulfur compounds present in it.[1,2] Recent studies have demonstrated and validated many medicinal properties attributed to garlic. Different types of garlic supplements like garlic powder (tablets), aged garlic extracts (capsules, tablets and liquid), garlic oils (capsule) are commercially available; each being different in organosulfur compound profile.[3] During the ageing of garlic, unstable and highly odorous compounds are converted into more stable and odorless compounds.[4] Therefore, aged garlic (up to 20 months) is more promising for medicinal purpose and gives the better-known garlic preparations.

Garlic extract contains certain phytochemicals, which prevents oxidative stress-induced damages. Among them, fat and water-soluble organosulfur components, allixin, flavonoids, and selenium are noteworthy. The main organosulfur compound present in garlic are diallyltrisulfide (DATS), S-allylcysteine (SAC), S-allylcysteine sulfoxide (SACS), flavonoids, phenolics and anthrocyanins. It also contains carbohydrates, proteins, fatty acids, glycolipids, phospholipids, fiber, saponins, glycosides, lectins,

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1. Center of Excellence in Genomic Medicine Research, King Abdulaziz University, Jeddah (21589), Kingdom of Saudi Arabia; 2. King Fahd Medical Research Center, King Abdulaziz University, Jeddah (21589), Kingdom of Saudi Arabia; 3. Departments of Medical Laboratory, Imaging and Radiologic Sciences and Neurology, Augusta University, Augusta GA (30912), USA; 4. Department of Biochemistry, College of Science, King Saud University, Riyadh (11451), Kingdom of Saudi Arabia; 5. Family and Community Medicine, College of Medicine, Qassim University, Buraydah (51431), Kingdom of Saudi Arabia; 6. College of Medical Rehabilitation, Qassim University, Buraydah (51452), Kingdom of Saudi Arabia
Correspondence to: Dr. Syed Kashif Zaidi, Tel: 00966-2-6401000, E-mail: kashif_biochem@yahoo.com
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and vitamin B_1, B_2, B_6, C and E.[1,4-6] SAC is the most abundant compound in the aged garlic. Several *in vitro* and *in vivo* studies reported the antioxidant properties of both aged crude garlic extract as well as purified SAC. Considerable number of studies reported their ability to quench reactive oxygen species (ROS) and reactive nitrogen species (RNS).[4,7]

Life exists by maintaining a complex dynamic equilibrium, or homeostasis, that is constantly challenged by intrinsic or extrinsic forces or stressors.[8] We earlier demonstrated that the restraint or immobilization stress is an easy and simple method to induce both physical (muscle work) and psychological stress (escape reaction),[9-12] which results in both, restricted mobility and subsequent aggression. Several studies have reported that oxidative stress is due to enhanced free radicals in various kinds of stresses.[13,14] Generation of free radicals and subsequent oxidative stress like peroxidation of lipids are the potent outcomes of the restraint stress. Moreover, stress has been suggested to decrease the level of reduced glutathione (GSH) and vitamin C, which plays an important role in the protection of tissues from oxidative stress.[15]

Biological systems have developed antioxidant defense mechanisms which disrupt the oxidation chain by providing electrons to the free radicals without becoming reactive themselves.[16] Therefore, the adverse effects of the free radicals depend on the balance between the speed of their generation and dynamics of their inactivation by the defense system, especially endogenous antioxidants. Since both ROS and RNS are highly reactive formed even during our daily activities and stresses, harnessing endogenous protection through up-regulation of endogenous antioxidant system are a promising approach for prevention of their toxicity.

The present study was carried out to investigate the preventive effect of single dose of aqueous garlic extract on the immobilization stress-induced oxidative stress. We investigated and compared activities of superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) as endogenous antioxidant enzymes, and the levels of thiobarbituric acid reactive substances (TBARS), GSH, alkaline phosphatase (ALP), glucose, uric acid, aspartate aminotransferase (AST) [serum glutamic oxaloacetic transaminase (SGOT)] and alanine aminotransferase (ALT) [serum glutamic pyruvic transaminase (SGPT)]. The results of this study will likely contribute in understanding the potential of garlic extract in preventing/alleviating stress-induced diseases involving oxidative stress to cellular constituents.

METHODS

Chemicals

Bovine serum albumin, thiobarbituric acid and 1-chloro-2,4-dinitrobenzene (CDNB), were purchased from Sigma (St Louis, MO, USA); 5-5'-dithiobis-2-nitrobenzoic acid, hydrogen peroxide and pyrogallol were purchased from E-Merck (Darmsradt, Germany). All other chemicals used were of analytical grade and purchased from commercial sources.

Preparation of Garlic Extract

One kilogram of garlic cloves (*Allium sativum* L.) authenticated from Department of Botany, Aligarh Muslim University, were purchased from the local market, peeled and grounded with an electric mincer until an aqueous suspension was obtained. It was diluted in double distilled water at 4 g/mL on the basis of the weight of the starting material and centrifuged (Beckman J20, 15 min at 10,000 × *g* and 4 ℃). The supernatant was aliquot and stored at –80 ℃ until use.

Animal Stress Procedure and Treatments

Adult male Albino Wistar rats weighing 180–200 g were housed in group cages, fed with Purina diets and tap water *ad libitum*. Experimental protocols adhered to the guidelines of the animal welfare committee of the King Abdulaziz University. Prior to commencement and throughout the experiment the rats were housed at 24 ± 3 ℃ room temperature and 12 h light/dark cycles.

A preliminary dose dependent study (*n*=3) was performed to find out the best therapeutic dose of garlic, which can modulate deranged free radical metabolism (data not shown). It was observed that the extract at 100-mg/kg body weight (bw) dose had the most preventive effect on oxidative stress changes in the circulation. To elucidate the effect of immobilization stress-induced pro-oxidant changes and its attenuation by garlic extract, 40 rats were selected and divided into 5 groups (*n*=8 per group). The groups were: a no stress no treatment group (CON-group); a group with 6 h immobilization stress

without any treatments (STR-group); a group only treated with single dose of garlic extract (100 mg/kg bw, Pre-GAR-group); a group pre-treated with single dose (100 mg/kg bw) garlic extract followed by 6 h of stress exposure (Pre-GAR+STR-group); and lastly, a group which was treated with garlic extract after 6 h of stress exposure (STR+Post-GAR-group). The oral dose of extract was given to rats with the help of catheter, 1 h prior to or after the stress treatment. Rats were subjected to immobilization stress between 9 AM to 3 PM for 6 h by placing them in the individual wire mesh cages of appropriate size attached to a wooden board, as reported by earlier.[13] The rats were deprived of food and water during the stress procedure, as it is not feasible also for them to take food or water, similar treatment was given to control or garlic alone treated rats. Animals were sacrificed using pentobarbital (intraperitoneal injection of 50 mg/kg bw) 30 min after the completion of the stress procedure. Non-stressed control animals (with or without garlic extract) were handled at the same time similar to stressed groups but were not immobilized. The three sets of experiment were performed for each figure.

Preparation of Plasma Samples

Immediately after the sacrifice, the heparinized blood was centrifuged (5,000 r/min, 30 min) and the collected plasma was quick frozen and stored at –80 ℃ until assay.

SOD Assay

The circulating SOD activity was measured according to the method of Marklund.[17] This procedure depends upon the autoxidation of pyrogallol (8 nmol/L) in the presence of 0.05 mol/L tris succinate buffer pH 8.2. The inhibition of pyrogallol autoxidation by SOD was monitored at 412 nm. One unit of the enzyme was defined as the amount of enzyme required to inhibit the rate of pyrogallol oxidation by 50%.

CAT Assay

CAT activity was assayed according to the method of Beers and Sizer[18] with hydrogen peroxide (30 mmol/L) as the substrate. One unit of CAT activity is defined as the micromoles of hydrogen peroxide consumed per minute per milligram of protein sample.

GST Assay

GST was assayed according to the method of Habig, et al.[19] CDNB (1.0 mmol/L) was used as a substrate. Enzyme activity was measured by increase in absorbance at 340 nm of CDNB-GSH conjugate generated as a result of GST catalysis between GSH and CDNB.

Lipid Peroxidation Assay

Lipid peroxidation (LPO) was measured according to method of Halliwell, et al.[20] One mol of malondialdehyde (MDA) reacted stoichiometrically with 2 mol of 0.69% 2-thiobarbituric acid at pH 3.5. The pink chromogen was detected spectrophotometrically with an extinction coefficient of 156 mmol/L/cm at 532 nm.

Total GSH Assay

The method of Sedlak and Lindsay[21] was used to measure the circulating GSH. The assay is based on the reduction of 0.01 mol/L 5-5'-dithiobis-2-nitrobenzoic acid (DTNB) by sulfhydryl groups of GSH to form 2-nitro-5-mercaptobenzoic acid per moles of GSH.

ALT, AST, ALP, Glucose and Uric Acid Assays

The assays and activities were measured by using a kit from Reckson Diagnostic Pvt. Ltd. (Delhi, India).

Protein Estimation

The level of protein in the plasma samples were estimated according to method of Lowry, et al[22] using bovine serum albumin as standard.

Statistical Analysis

All the data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Statistical analyses were performed using GraphPad Prism version 4.0 software (GraphPad Software, La Jolla, CA). Since pre-treatment of garlic extract is more logical as the dietary supplement, a 2-garlic extract post-treatment (no versus yes) by a 2 immobilization stress (no versus yes) analysis of variance with interaction was used. One-way analysis of variance (ANOVA) was also used to compare CON-group versus the STR- and STR+Pre-GAR-group, using Dunnett's test. Statistical significance was determined at $P \leqslant 0.05$.

RESULTS

The 6 h of restraint stress caused a significant decline in the enzymatic activities of SOD, CAT and GST and the levels of GSH and glucose, and a concurrent significant increase in TBARS, AST, ALT, ALP and uric acid levels as compared to the non-

stressed control group (P<0.01). Both pre- and post-garlic extract treatments resulted into a significant increase in the plasma antioxidant enzymatic activities as compared to the stressed group (P<0.05 or P<0.01). However, the post-stress oral administration of garlic extract was more effective in restoring stress-induced loss in SOD, CAT and GST activities, and GSH and glucose levels, and in attenuating the increased levels of TBARS, AST, ALT, ALP and uric acid than the pre-stress garlic extract treatment as compared to the stressed group (P<0.01, Figures 1–3).

DISCUSSION

The key findings from the present work are that the immobilization stress causes detrimental oxidative stress via alterations in the pro-oxidant/antioxidant status. Moreover, dietary supplement with active antioxidants such as aged garlic extract are promising conventional treatment to harness the endogenous protection and defend against the oxidative stress.

Restraint stress has been reported to cause

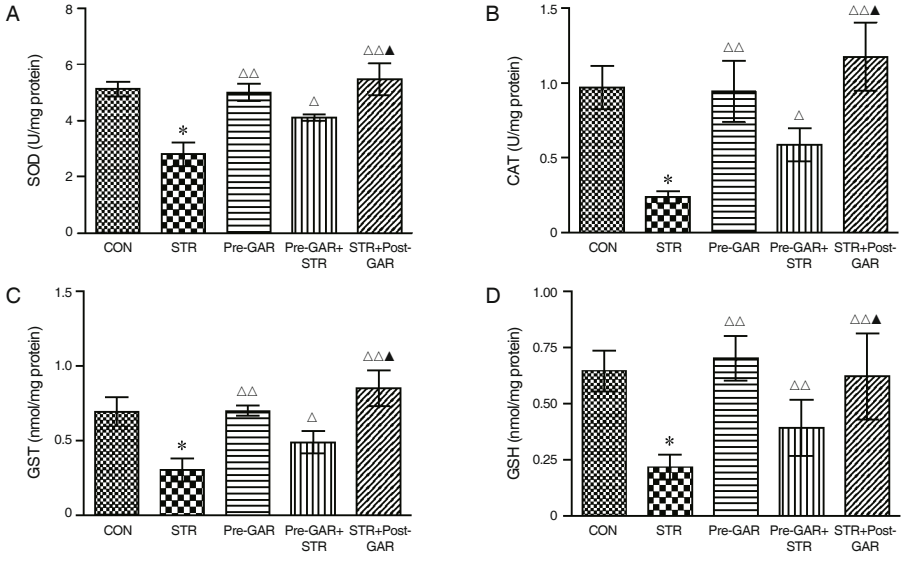

Figure 1. Effect of Aqueous Extract of Garlic on Immobilization Stress-Induced Changes in Circulating (Plasma) Levels of SOD, GST, CAT and GSH

Notes: $^{*}P$<0.01, compared with CON-group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01, compared with STR-group; $^{\blacktriangle}P$<0.01, compared with Pre-GAR+STR group

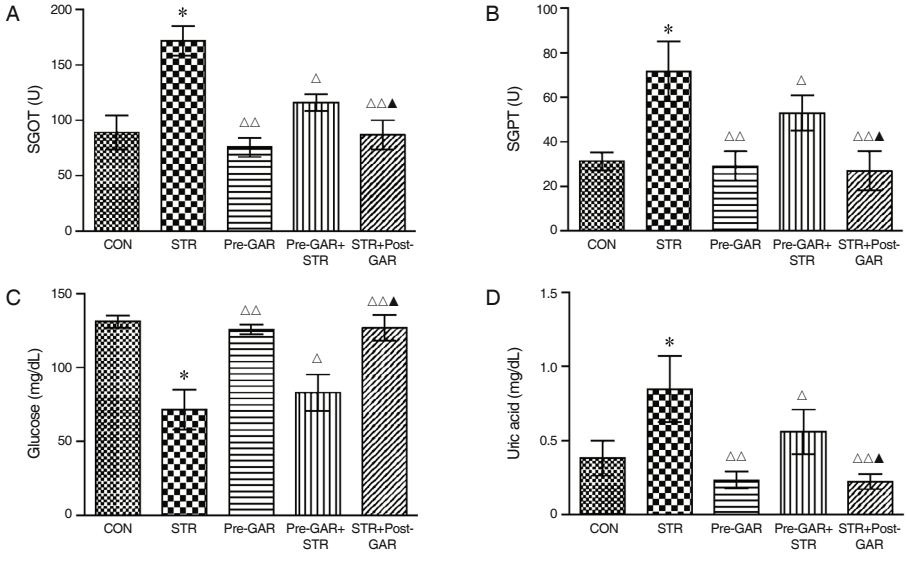

Figure 2. Effect of Crude Extract of Garlic on Immobilization Stress-Induced Changes in Circulating (Plasma) Levels of SGOT, SGPT, Glucose and Uric Acid

Notes: $^{*}P$<0.01, compared with CON-group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01, compared with STR-group; $^{\blacktriangle}P$<0.01, compared with Pre-GAR+STR group

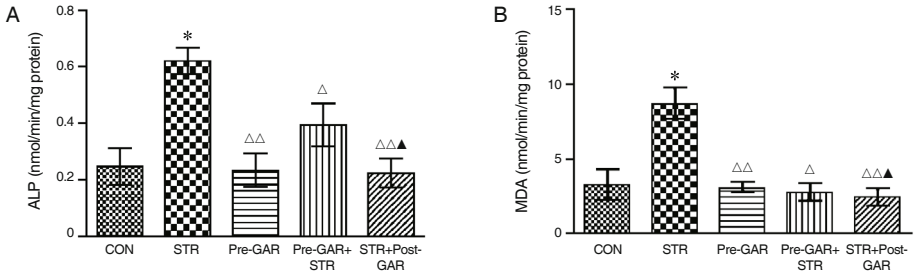

Figure 3. Effect of Crude Extract of Garlic on Immobilization Stress-Induced Changes in the Circulating (Plasma) Levels of ALP and MDA

Notes: $^{*}P<0.01$, compared with CON-group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, compared with STR-group; $^{\blacktriangle}P<0.01$, compared with Pre-GAR+STR group

alterations in antioxidant defensive profile in the plasma.[8] SOD, GST and CAT play an important role in the scavenging of oxy-radicals and their products.[23] In order to maintain the stability in living organism, it is necessary to maintain the redox balance. In our study, 6 h of immobilization stress resulted in the generation of oxidative stress/ROS in the rat plasma, which resulted into decline in GSH and glucose levels, and decrease in antioxidant enzymatic activities. Furthermore, ROS enhances the level of pro-oxidants such as uric acid and TBARS, an indicator of peroxidation of lipids. Moreover lipoxidative damage can also cause loss in the membrane integrity which leads to cellular disorganization. Indeed, membrane LPO is considered as the major factor involved in the mechanism of oxidative injury.[23] The enhancement in circulating lipid peroxide has been shown to correlate with depletion of endogenous GSH.[24] It has been proposed that the combined action of GSH and SOD forms an integral component of cellular anti-high levels of catecholamines and glucagon, which is an oxidant defense and is one of the key protective factors against oxidative stress.[24] There has been reports that both short- (1 h) and long-term (24 h) immobilization stresses results into an increase in TBARS content in the brain, liver and heart, accompanied by a decrease in circulating GSH.[25,26] The depletion in the GSH content observed in the present study might be the result of decreased activities of the free radical scavenging enzymes SOD, CAT and GST, further causing increased LPO. In the last decade, a number of natural products and neutraceuticals have been investigated and reported for their beneficial effects in humans. However, the anti-stress potential of garlic extract has not been clearly outlined as yet. As far as our knowledge goes, the present study reports modulatory effects and subsequent biochemical adaptive role of garlic extract against stress-induced ROS especially in plasma.[27,28]

The intra-gastric administration of garlic extract significantly increased the circulating activities of SOD, CAT and GST, and the levels of glucose and GSH while the circulating levels of TBARS (LPO), AST, ALT, ALP and uric acid was found to be decreased. Garlic extract was found to prevent and normalize oxidative stress generated by immobilization stress, which was evident by the reversal of deranged antioxidant enzymatic activities and liver functions including glucose and uric acid towards their normal values. The possible reason behind this result might be due to the organosulfur contents present in the garlic viz. allicin, alliin, and two major organosulfur compounds SAC and SAC S-allylmercaptocysteine which are potent free radical scavengers.[29] These antioxidant compounds present in the garlic might act as double-edged swords. They could upregulate the antioxidant enzymatic activities during stress as well as GSH to scavenge the free radicals, and could down regulate LPO too.[13] Indeed, similar benefits of garlic extract has been also reported in cardiac muscles with an increased GSH content, SOD, CAT and GST activities.[30]

The activity of AST and ALT are sensitive indicators of acute hepatic functional impairment and the level of ALP is known as an indicator of hepatobiliary disease.[31] The decrease in these surrogate biomarkers in the plasma indicates degenerative changes, metabolic alterations and hypo function of heart and liver, which are adversely effected by immobilized stress.[32] Our findings further support the notion that the intra-gastric garlic extract treatment (both pre- and post-stress) could reverses the hypo function of heart and liver.

Plasma level of glucose is found to be significantly decreased in response to immobilization stress. This could be due to the enhanced catecholamine levels in

immobilization stress. The catecholamines secretion evokes an initial repression of insulin secretion, followed by rebound hyper secretion of pancreatic hormone, subsequently leading to hypoglycemia, which can decrease plasma glucose level observed during immobilization stress, either by enhancing peripheral glucose uptake or by interacting directly with β-cells of the pancreas.[33] In our study 6 h of stress resulted into decreased in plasma glucose level,which was reverted to their control values by both pre- and post-stress garlic extract treatment.

Uric acid is considered as non-enzymatic antioxidant, but increased production in response to immobilization stress can cause increase in free radical generation due to activation of xanthine oxidase enzymes system. This increase could be detrimental under depleted GSH level, which often happens due to immobilization stress. The treatment with garlic extract resulted in a significant decrease in the uric acid level in both pre- and post-extract treatments with a relative dominance by later. The increase in uric acid concentration in immobilization stress could be due to body's natural response to combat enhanced free radicals produced due to decreased activities of scavenging enzymes, increased xanthine oxidase activity and/or also due to high levels of catecholamines during oxidative stress, as some studies show that catecholamines increase purine catabolites.[34]

Immobilization stress was found to induce oxidative stress through decrease in the activities of SOD, CAT, GST and GSH, glucose levels, while increase in uric acid, AST, ALT, ALP and LPO levels. The pre and post-stress oral administration of aqueous extract of garlic were effective in protecting immobilization stress-induced oxidative changes. The extract treatment alone did not showed any effect, but the post-stress extract treatment was found to be comparatively more effective than pre-stress extract treatment in preventing/restoring the stress-induced decrease in SOD, CAT, GST and GSH, glucose levels and increase in the levels of uric acid, AST, ALT, ALP and MDA.

Conflict of Interest

All the authors of the manuscript do not have any financial relation with the commercial identities mentioned in the article and there are no conflicts of interest to declare.

Author Contributions

SKZ and NB conceived and designed the experiments; SKZ performed the experiments; SKZ, ST and GMA interpreted the results; SKZ and SA drafted and edited the manuscript; MSK prepared the figures. MHA provided the reagents and facilities. MNH and MHA did the statistical analysis. SKZ, SAA, NB and ST approved the final version of the manuscript.

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