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**RESEARCH ARTICLE**

# Radioprotective Effect of Gliclazide as an Anti-Hyperglycemic Agent Against Genotoxicity Induced by Ionizing Radiation on Human Lymphocytes

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**Abstract:** ***Objectives*:** Gliclazide (GL) is widely used to reduce hyperglycemia in diabetic patients. The aim of this study was to investigate the protective effect of GL against chromosome damage induced by ionizing radiation in human blood lymphocytes.

***Methods*:** In this experimental study, peripheral blood samples were collected from human volunteers and treated with GL at various concentrations (5, 25, 50 or 100 μM) for three hours. Then samples were irradiated to X-ray (1.5 Gy). Blood samples were cultured with mitogenic stimulation. The frequencies of micronuclei in cytokinesis-blocked binucleated lymphocytes were determined in the different samples. The antioxidant activities of GL were assayed by two different methods as 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) free radical scavenging and reducing antioxidant power assays.

***Results*:** GL significantly reduced the percentage of micronuclei in lymphocytes which were irradiated. The maximum radioprotection in the reduction of percentage of micronuclei in lymphocytes was observed at 100 μM of GL with 52% efficacy. GL exhibited excellent free radical scavenging activity and reducing power at concentration dependent activities. The $IC_{50}$ values of GL were lower than ascorbic acid. Higher potencies were observed in the antioxidant activities for GL than ascorbic acid in both methods.

***Conclusion*:** This data exhibits that GL is a powerful radioprotective agent that could protect healthy cells against the chromosome damage induced by ionizing radiation through antioxidant activity. The radioprotective effect is new indication of GL for patients' protection against side effect induced by ionizing radiation.

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## 1. INTRODUCTION

Ionizing Radiation (IR) is widely used for the treatment of malignant diseases such as cancer in patients. IR produces oxidative stress and toxic substances when it is passing into cancerous cells. The highly Reactive Oxygen Species (ROS) can attack critical biomolecules such as DNA leading to deoxyribose oxidation and strand breaks. Cell death is the main result of extensive genotoxicity in the cell [1, 2]. However, IR is able to kill cancerous cell, its side effects on normal tissue are observed during radiation therapy in patients. Also, the people who are unwantedly exposed to IR may suffer from serious side effects such as acute (as hematological toxicity) and/or chronic effects (as cancer) [3]. It is important to protect normal tissue against toxicity induced by IR during radiotherapy or unwanted exposure to IR [4]. Elevated oxidative stress and inflammation process are crucial mechanisms involved in the cellular toxicity. Several studies have shown that antioxidants and anti-inflammatory agents have radioprotective effects against IR-caused injury in cells through scavenging of free radicals produced by IR. Several compounds were evaluated for their radioprotective effects such as *Zataria multiflora*, atorvastatin, mefenamic acid, celecoxib and hesperidin [5-9]. Amifostine is only FDA approved radioprotective agent that is used for the protection of

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patients in radiation therapy, but side effects of this drug are limited usage of this drug in the clinic [4]. Therefore, finding a less toxic medication could be beneficial for patients during radiotherapy [10].

Gliclazide (GL) is an oral anti-hyperglycemic medicine that is used for the reduction of glucose in patients with non-insulin-dependent diabetes mellitus. GL belongs to the sulfonylurea class, which stimulates β cells of the pancreas for releasing insulin [11]. Recently, other pharmacological properties were proposed for GL such as antioxidant and anti-inflammatory and protective effects that were demonstrated *in vitro* and in animal models [12-14]. We hypothesized that GL could also have beneficial effects against genetic damage induced by IR in normal cells beside its anti-diabetic effect. Lymphocytes are sensitive cells to IR. IR induces DNA breaks and chromosome abbreviations in lymphocytes. The micronucleus method for peripheral blood lymphocyte has been extensively used as a genotoxicity method for the assessment of *in vivo* and *in vitro* radiation exposure [15, 16]. Therefore, the aim of the present study was to evaluate the radioprotective effect of GL against genetic damage caused by IR in human normal lymphocytes.

## 2. MATERIAL AND METHODS

### 2.1. Materials

GL was obtained from the Pharmaceutical company (Tehran Daru, Iran). Cytochalasin-B was obtained from Sigma Co. (USA). PHA (phytohemagglutinin), RPMI-1640 (Roswell Park Memorial Institute cultural medium), FBS (fetal bovine serum), penicillin and streptomycin were obtained from Gibco (USA). Giemsa stain, potassium ferricyanide and ferric chloride, methanol, and acetic acid were purchased from Merck Chemical Co. (Germany).

### 2.2. Blood Treatment

This study was approved by research and ethical committees of the Mazandaran University of Medical Sciences (ID Code: 2855). Healthy, non-smoking, non-treated with any medicine for at least one week, four male volunteers, from 20 to 28 years old participated in this study. Whole blood was collected from volunteers in heparinized tubes and aliquoted in microtubes at 0.9 mL each. Subsequently, blood samples were pretreated with 100 µL solution of GL at the final concentrations of 5, 25, 50 or 100 µM. Blood samples were incubated for three hours at 37°C. GL were dissolved in DMSO and diluted in RPMI cultural medium. Control samples received concentration of DMSO (0.1%) similar to the other treated groups.

### 2.3. Ionizing Radiation and Micronucleus Test

The blood samples in microtubes were kept on the plastic box containing water as a phantom and then were exposed to X-ray (6 MV) beam produced by a Linear accelerator (Siemens, Primus, Germany) at a dose of 1.5 Gy. After irradiation, half of millilitre of each sample was added to 4.4 mL of RPMI-1640 culture medium with 10% FBS and 100 µL of PHA. All samples were incubated at 37°C. After 44 h of cell culture, cytochalasin B (30 µl/ 5 ml) was added to each blood sample. Following 72 h of incubation, the samples were centrifuged. After removing up layer of medium, the pelleted cells were re-suspended in cold potassium chloride with a shaker. In this case, red blood cells were disrupted and then lymphocytes were fixed in a fixative solution made of methanol: acetic acid (5:1) two times with pipetting. The small portions of fixed cells were dropped onto clean microscopic slides, and kept for 24 h. Lymphocytes were stained with Giemsa solution in phosphate buffered saline (10%). All slides were evaluated at 1000 × magnification in order to determine the frequency of micronuclei in the cytokinesis-blocked binucleated cells with a well-preserved cytoplasm. The criteria for scoring micronuclei were a diameter between $1/16^{th}$ and $1/3^{th}$ of the main nuclei, not overlapping the main nuclei, non-refractile and not linked to the main nuclei and 1000 binucleate cells were examined for each treated sample from each volunteer to record the percentage of micronuclei in nucleated cells [17]. 4000 binucleated lymphocytes were counted in each treated group from four volunteers. A total of 40,000 binucleated lymphocytes were counted for ten control and treated groups in this study.

### 2.4. Measurement of Free Radical Scavenging Activity

The free radical scavenging capacity of GL was evaluated as bleaching of the stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) [18]. Methanolic solution of DPPH (100 mM) was added at an equal volume to different concentrations of GL (1 to 1000 µM). The mixture of GL and DPPH solutions was kept at room temperature for 15 min. The absorbance of each sample was recorded at 517 nm. Ascorbic acid was used as an antioxidant standard. The experiment was performed in triplicate. The percentage of free radical scavenging activities was calculated using the formula [(Control-Test)/Control] ×100.

### 2.5. Reducing Power Activity

GL was added to potassium ferricyanide (1 mL, 1%) at different concentrations (1 to 1000 µM in methanol). The mixture was incubated at 50°C for 20 min at a bath water. For stopping reaction, tricholoroacetic acid (10%) was added to the mixture and then 1 mL of $FeCl_3$ (0.1%) was added to this solution. Absorbance was measured at 700 nm. The percentage of free radical scavenging activities was calculated using the formula [(Test-Control)/Test] ×100. Ascorbic acid was used as a standard antioxidant.

### 2.6. Statistical Analysis

The data values are presented as means ± Standard Deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), as well as post hoc Tukey multiple comparison tests. $IC_{50}$ (50% of antioxidant activity) values were calculated with Prism 7 Software, 2016 (USA).

## 3. RESULTS

### 3.1. Micronucleus Assay

A micronucleus in binucleated lymphocyte is shown in Fig. (**1**). The mean percentage of micronuclei in irradiated blood lymphocytes was 8.50 ± 1.09, while it was 0.55 ± 0.06 in non-irradiated control samples. Exposure of blood samples to IR significantly increased the frequency of micronuclei

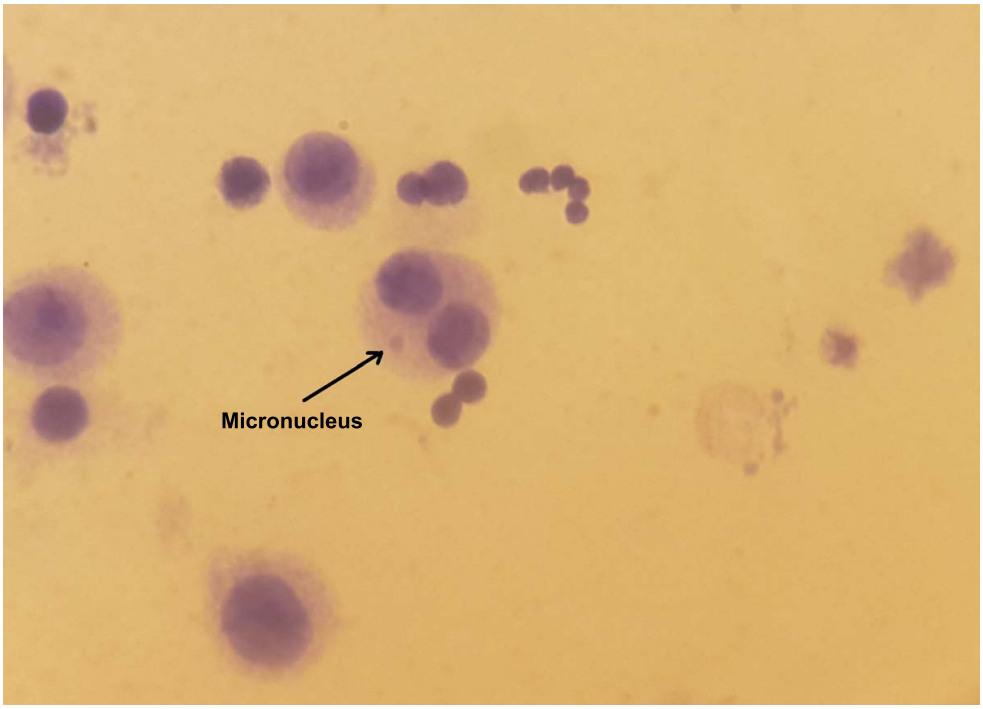


**Fig. (1).** A typical binucleated lymphocyte with micronucleus in this study.

**Table 1. The frequency of micronuclei induced *in vitro* by 150 cGy X-ray radiation (IR) in cultured blood lymphocytes from human volunteers examined treated at different doses of gliclazide (GL).**

| Group / Sample | %Micronuclei | | | | |
|---|---|---|---|---|---|
| | I | II | III | IV | Mean ± SD |
| C | 0.6 | 0.5 | 0.5 | 0.62 | 0.55 ± 0.06 |
| IR | 9.8 | 8.2 | 8.8 | 7.2 | 8.5 ± 1.09[a] |
| 5GL+IR | 6 | 6 | 6.2 | 5.8 | 6 ± 0.16[b, C] |
| 25GL+IR | 5.2 | 5.4 | 5.6 | 5.5 | 5.42 ± 0.17[b] |
| 50GL+IR | 4.8 | 5 | 4.8 | 5.2 | 4.95 ± 0.19[b] |
| 100GL+IR | 4 | 4.4 | 4 | 3.8 | 4.05 ± 0.25[b] |
| 5GL | 0.8 | 0.9 | 0.7 | 0.7 | 0.77 ± 0.09[d] |
| 25GL | 0.9 | 0.9 | 1 | 0.8 | 0.9 ± 0.08[d] |
| 50GL | 0.9 | 1.2 | 1.2 | 1 | 1.07 ± 0.15[d] |
| 100GL | 1.2 | 1.4 | 1.6 | 1.2 | 1.35 ± 0.19[d] |

1000 BN cells were examined in each sample.
[a] $P < 0.0001$ compared to control, [b] $P < 0.001$ compared to IR, [c] $P < 0.05$ compared to 50GL+IR and 100GL+IR, [d] non-significant compared to control (ANOVA analysis with Tukey's Multiple Comparison Test was applied for comparison of data).

(15-fold rise) in irradiated lymphocytes ($P < 0.0001$) (Table **1**). The frequency of micronuclei in irradiated samples with GL pre-treatment at the concentrations of 5, 25, 50, or 100 µM were 6.00% ± 0.16, 5.42% ± 0.17, 4.95% ± 0.19 and 4.05% ± 0.25, respectively (Table **1**). The data demonstrated that the pre-treated samples with GL at all concentrations exhibited a significant decrease in the frequency of micronuclei as compared to irradiated samples without GL treatment ($P < 0.001$). Total micronuclei frequencies in irradiated samples pre-treated with GL were reduced to 29%, 36%, 42% and 52% at concentrations of 5, 25, 50, or 100 µM, respectively, compared to irradiated samples (Table **1**). The maximum protection of lymphocytes was observed at a concentration of 100 µM through GL treatment. The frequency of micronuclei was significantly reduced in irradiated sample treated by GL at a concentration of 5 µM as compared to irradiated samples with 50 and 100 µM GL concentration ($P < 0.05$). Non-irradiated samples with GL treatments at all

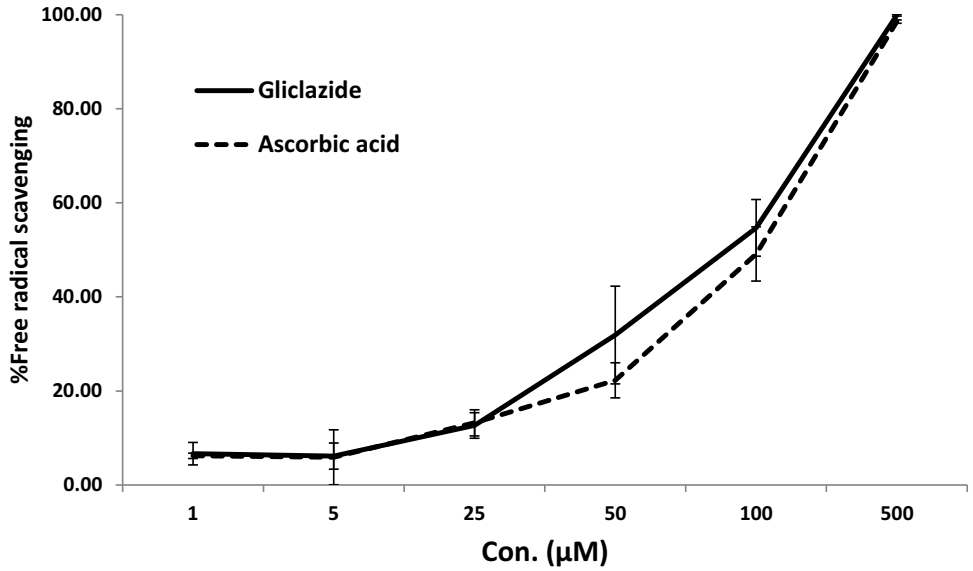


**Fig. (2).** Free radical scavenging effect of different concentrations of gliclazide and ascorbic acid on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical at 517 nm.

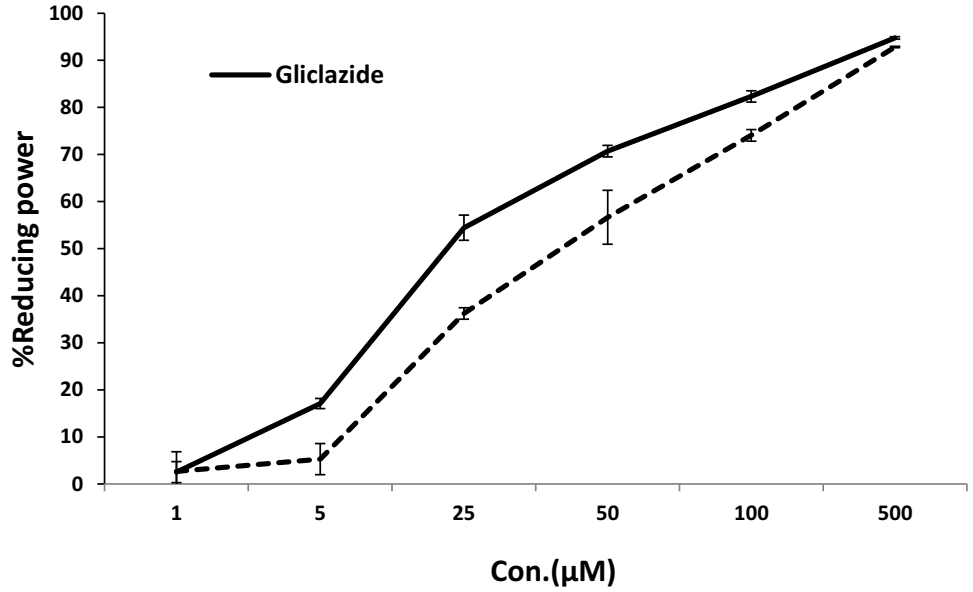


**Fig. (3).** Reducing power of different concentrations of gliclazide and ascorbic acid with ferric reducing power system.

concentrations have not shown any increased genotoxicity as compared to control group that were compared with Tukey multiple comparison tests between them.

### 3.2. Antioxidant Activity

The free radical scavenging activity and reducing power of GL were evaluated as main mechanisms are involved in mitigation of oxidative stress. In free radical scavenging activity method, DPPH is a stable free radical with purple colour that enables to accept an electron or hydrogen radical. The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidant. GL showed an excellent free radical inhibition at a concentration dependent manner, similar to acid ascorbic as a well-known antioxidant. The maximum free radical scavenging activities were observed at about 55% and 100% for GL at concentrations 100 and 500 µM, respectively, while these activities were 49% and 98% for ascorbic acid at the same concentrations of GL (Fig. **2**). The $IC_{50}$ (the concentration of antioxidant which eliminate 50% of DPPH radicals) values were found to be 115.9 µM and 101.5 µM for ascorbic acid and GL, respectively.

Reducing power determination is based on the principle in which substances with reduction potency, react with potassium ferricyanide ($Fe^{3+}$) to form potassium ferrocyanide ($Fe^{2+}$), which can react with ferric chloride to form ferric ferrous complex with a maximum absorption at 700 nm [19]. The reducing power of the GL increases in proportion to the increase in amount of GL (Fig. **3**). The maximum reducing power activities were observed at about 82% and 95% for

GL at concentrations 100 and 500 μM, respectively, while these activities were 74% and 93% for acid ascorbic at the same concentrations of GL (Fig. **3**). The $IC_{50}$ (the concentration of antioxidant which have 50% reducing power activity) values were found to be 40.2 μM and 19.6 μM for ascorbic acid and GL, respectively.

## 4. DISCUSSION

In this study we demonstrated the protective effect of GL as an antidiabetic agent, against genetic damage induced by IR in human healthy lymphocytes. This is the first study that demonstrates the protective effect of GL on DNA damage induced by IR in human blood lymphocytes. GL at concentrations 5, 25, 50, 100 μM significantly reduced chromosome damage induced by IR in lymphocytes. The maximum radioprotection was observed at concentration 100 μM of GL. Micronucleus is a biomarker of chromosome fragmentation resulting from exposure to clastogenic agent or IR. The human lymphocyte is a sensitive normal cell to IR and susceptible to chromosome damage; this cell type is widely used for genotoxicity assessment *in vitro* and *in vivo* experiments that exposed to IR or chemical hazardous [20, 21]. IR produces toxic substances that attack macromolecules into cells and lead to DNA damage. The genotoxicity is one of the main reasons of cancer if it is wrongly repaired or unrepaired. It is demonstrated that antioxidants enable to scavenge free radicals leading to protect DNA and other cellular biomolecules against deleterious effects of ROS in the cells. Antioxidant activity is the main mechanism that contributes to radioprotective effects of chemical or natural compounds [22, 23]. In this study, GL showed excellent antioxidant property that was evaluated with two different systems. The antioxidant activity of GL is correlated to its concentrations. Antioxidant interacts with DPPH radical and either transfer an electron or hydrogen atom to it resulting in neutralizing the free radical character. The colour of DPPH solution changes from purple to yellow with a decrease in the 517 nm absorbance. The degree of absorbance reduction indicates the free radical scavenging potential of antioxidants. GL efficiently scavenges DPPH free radical more than ascorbic acid as a well-known antioxidant at some concentration. The ferric reducing power assay measures the electron donating ability of antioxidants. In reducing power assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples [24]. Our study showed that GL efficiently scavenges free radical DPPH and is more effective than the ascorbic acid as a well-known antioxidant at some concentrations. Lower $IC_{50}$ value shows more antioxidant potential. The $IC_{50}$ value of GL (101.5 μM) was lower than ascorbic acid (115.9 μM), then GL is more potent than ascorbic acid in free radical scavenging. In addition, The $IC_{50}$ value of GL (19.6 μM) was lower than ascorbic acid (40.2 μM), then GL is more potent than ascorbic acid in reducing the power activity. The antioxidant activities of GL have been demonstrated in the *in vitro* and *in vivo* studies. GL attenuated oxidative stress in cells exposed to hydrogen oxide and it appears to have anti-apoptotic effect [25]. GL exhibited neuroprotective effect in rats through antioxidant activities [26]. Administration of GL to type 2 diabetic patients resulted in a reduction of 8-isoprostanes as a marker of lipid oxidation. GL increased the antioxidant parameters as total plasma antioxidant capacity, superoxide dismutase and thiols levels in patients [12]. GL, with antioxidant activity, is able to protect vascular complication in animals [13]. GL is able to decrease the lipid peroxidation and increase the levels of glutathione peroxidase (Gpx), catalase (CAT) and glutathione S-transferase (GST) activities as antioxidant biomarkers in patients with type 2 diabetes mellitus [27]. In our study, the concentration dependent GL was validated for its antioxidant activity and radioprotective effects against chromosome damage induced by IR in lymphocytes. It is determined that the capacity of GL for scavenging of free radicals is correlated with radioprotection efficacy of GL in lymphocytes. Amifostine as a synthetic compound is used for radioprotection in human, but it has several side effects (such as hypotension, nausea, vomiting) that is limited usage of it in patients or human during exposure to IR. It should be administrated in clinical under special cautions [4]. New medications are interesting radioprotective agents with regards to their low toxicity. GL is used as an antidiabetic agent that is widely used in patients without any severe cautions and side effects [28]. A single oral administration of GL at doses of 40 to 120 mg results in a $C_{max}$ of 2.2 to 8.0 μg/ml within 2 to 8 hours. GL has a molecular weight equal to 323, then the plasma concentrations of GL are about 7-25 μM [29]. In the present study, GL exhibited significant radioprotective effect at concentration of 5-25 μM that is close to plasma concentrations of GL. Further study is needed to provide appropriate doses of GL to have little side effect on patients for significant clinical benefit. For the first time, our study reports new indication of GL as radioprotective agent for protection of human normal lymphocytes against genotoxicity induced by IR.

## CONCLUSION

In this study it was observed that GL with antioxidant property can contribute to reduce genotoxicity induced by IR in human lymphocytes, with regards to its low toxicity as an antidiabetic agent; it can help defense body against side effects induced by IR in human.

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| GL | = | Gliclazide |
| IR | = | Ionizing Radiation |
| ROS | = | Reactive Oxygen Species |
| DMSO | = | Dimethyl Sulfoxide |
| PHA | = | Phytohemagglutinin |
| RPMI 1640 | = | Roswell Park Memorial Institute cultural medium |
| FBS | = | Fetal Bovine Serum |
| DPPH | = | 1,1-Diphenyl-2-picryl hydrazyl radical |
| Gpx | = | Glutathione peroxidase |
| CAT | = | Catalase |
| GST | = | Glutathione S-Transferase |

## AUTHORS' CONTRIBUTIONS

Designed research; Seyed Jalal Hosseinimehr, performed research; Maysa Pouri, Zahra Shaghaghi, Arash Ghasemi, contributed important reagents; Seyed Jalal Hosseinimehr, collected data; Maysa Pouri, Zahra Shaghaghi, Arash Ghasemi, analyzed data; Maysa Pouri, Seyed Jalal Hosseinimehr, wrote paper, Seyed Jalal Hosseinimehr' approval final manuscript; all authors.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was *in vitro* on blood. This study was approved by Mazandaran University of Medical Sciences, Iran with ID Code: 2855.

## HUMAN AND ANIMAL RIGHTS

No Animals were used in the study. All reported experiments on humans were in accordance with the ethical standards of the committee responsible for human experimentation (Institutional and National), and with the Helsinki Declaration of 1975, as revised in 2013.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

Not applicable.

## FUNDING

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## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

## ACKNOWLEDGEMENTS

Declared none.

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