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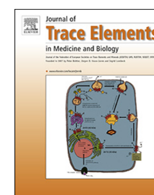

The toxic effect of gallic acid on biochemical factors, viability and proliferation of rat bone marrow mesenchymal stem cells was compensated by boric acid

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ABSTRACT

Objective: Gallic acid (GA) and boron are found in many plants. Our previous studies showed 6 ng/ml boric acid (BA) had positive effect on biochemistry of rat bone marrow mesenchymal stem cells (MSCs) and their osteogenic differentiation. Therefore, we investigate the effect of different doses of GA alone and in the presence of BA on MSCs.

Materials and methods: the viability of MSCs was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue at 12, 24 and 36 h in presence of different concentration of GA. Then 30 and 120 μM of GA as well as 6 ng/ml of BA in 36 h were selected for further study. The proliferation, Morphology, sodium and potassium level, concentration of calcium, activity of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) as well as malondialdehyde (MDA) concentration, total antioxidant capacity (FRAP) and activity of superoxide dismutase (SOD) and catalase (CAT) were estimated.

Result: Results showed GA alone reduced viability, proliferation, nuclear diameter and cytoplasm area. In addition, GA showed anaerobic metabolic shift but no change in MDA and scavenging enzymes. Both concentration of GA caused elevation of FRAP, whereas only at 120 μM increased the sodium-potassium and reduced calcium. The co-treatment of GA and BA improves the viability, proliferation and morphology of the cells. In addition, co-treatment compensated the metabolic shift caused by GA and could balance the potassium level and FRAP as it was raised by GA.

Conclusion: Although GA content of tea is harmful to the cells but simultaneous consumption of fruits and vegetables as a rich source of boron might compensate the damaging effect of GA.

1. Introduction

Gallic acid(GA) or 3,4,5-trihydroxybenzoic acid is a polyhydroxyphenolic compound which is found in plants such as sumac, gall nuts, tea leaves, oak bark, apple peels, green tea, pineapples, bananas, lemons, grapes and strawberries [1,2]. Recent Studies on GA, showed, this natural compound has anti-bacterial, anti-viral, anti- inflammatory, antioxidant and antitumor activities [2] and has been recommended in treatment of malignancies, cardiovascular, and neurodegenerative diseases [3–5]. GA is a natural antioxidant that is extracted from plants especially green tea [6], which scavenges the superoxide and hydroxyl radicals and prevents oxidative stress [7]. In today's world GA is used in daily house hold as plant products, medical treatment because of its medicinal and antioxidant property. Although its usefulness, GA has been reported to induce cell death [8–10], thus for those who consume large quantities of black or green tea in Middle East countries like Iran, GA might cause tissue and cellular damage.

Boron is an essential micronutrient of plants and is found in considerable quantities in dry fruits such as berries, plums, apricots and dates [11,12]. The boron content of banana, broccoli, almonds, celery, tomato, grapes, soy meal and apple ranges from 20.6 to 42.5 μg/g [13], therefore the daily intake of boron is a considerable amount when vegetable meal is taken. Boron is a metalloid element in non-metal group III of the periodic table. When boron is dissolved in water it forms its simplest compound known as boric acid (BA) which is a weak Lewis acid. Studies by Movahedi and Abnosi [14,15] have shown that 6 ng/ml of boric acid not only had positive effect on rat bone marrow mesenchymal stem cells (MSCs) metabolic state but also increased the activity of alkaline phosphatase and matrix deposition of calcium when differentiated to Osteoblast.

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Mesenchymal stem cells are progenitor cells with capabilities of being proliferated to generate itself and also differentiated to other cell lines such as chondrocytes, hepatocytes and osteoblasts [16]. These cells are in the bone marrow and other adult tissues which can go through differentiation process to produce other cell and take part in tissue repair [17]. Since BMCs are in direct contact with peripheral blood, therefore they may come in close proximity with blood born chemicals which are entering the blood via intestinal absorption. Thus one question has to be answered; if a person who consumes quantities of black or green tea and foods rich in vegetables may have considerable amount of GA and boron in their blood. Then what would be the probable effect of these chemicals on the stem cells such as MSCs? In the present research work we try to answer the effect of different concentration of GA on viability of MSCs and then choose a certain dose, the co-treatment effect of GA and BA would be investigated.

2. Materials and methods

2.1. Cell extraction and isolation

In this experimental study, we used male Wistar rats of 6–8 weeks old and weighing 140 ± 20 g. The rats were purchased from Pasteur Institute (Tehran, Iran) and maintained in the animal house of Arak University in the polyethylene cage under standard conditions of temperature 27 ± 3 °C and convenient access to food and water. Rats were sacrificed by excessive chloroform (Merck, Germany) inhalation according to the animal laboratory ethical committee role and regulation. Then, under sterile conditions, their femora and tibia were surgically removed and cleaned from surrounding connective tissue. The two ends of the bones were cut and bone marrow were flushed out using 2 ml of Dulbecco's Modified Eagles Medium (DMEM, Gibco, Germany) supplemented with 15% fetal bovine serum (FBS, Gibco, Germany) and penicillin/streptomycin (Gibco, Germany). Bone marrow content was centrifuged at 2500 rpm for 5 min, re-suspended in 5 m l culture media, then plated in culture flasks and incubated at 37 °C in an atmosphere of 5% CO2. After 24 h the supernatant containing non-adherent cells were removed and fresh culture media was added. The flasks were incubated for 14 days with replacement of culture media every three days. When bottom of the culture flask was covered with cells, the cells were trepsinized using trypsin-Ethylenediaminetetraacetic acid (Gibco, Germany) and washed with phosphate saline buffer (PBS). Then the fresh culture media was added to the cells and divided in two similar flasks and kept in incubator. To obtain a purity of 90–95 % two more passages were repeated and after the 3rd passage the cells were used for further investigation.

2.2. Exposure to gallic acid

The cells were placed in an appropriate culture dish and allowed to attach for 24 h. Then the cells were treated with 1.87, 3.75, 7.50, 15, 30, 60, 120 μM of GA (Merck, Germany) and incubated for 12, 24, 36 h in presence of control group (treated only with culture media). Each analysis was repeated three times in a bracket model.

3. Cell viability assays

3.1. Trypan blue exclusion assay

MSCs were Cultured at a density of 50,000 cells per well in 24-well culture plates and contaminated culture media contained different concentrations of gallic acid were added to the respective wells. After 12, 24, 36 h the cells were washed with PBS and using trypsin/EDTA and the cells were detached from culture flasks and subsequently collected by centrifugation at 2500 rpm for 5 min. Following centrifugation, the cells were re-suspended in fresh culture media and 50 μl of the cell suspension was mixed with equal volume of trypan blue (Sigma, America) and incubated for 2 min at 37 °C. Using hemocytometer, the percentage of viable and dead cells was determined. Trypan blue is a dye which enters the cell when the cell membrane is damaged cell appears to be blue in color.

3.2. 3-(4, 5-dimethy thiazole-2-yl)-2, 5 diphenyltetrazolium (MTT) assay

3-(4, 5-dimethy thiazo-2-yl)-2, 5 diphenyl tetrazolium or MTT assay also was used to confirm the trypan blue assay as the two assays run on different principles. The MSCs were cultured in a 96 well plates at a density of 10,000 cells per well and treatment was carried out similar to previous test. Then, cells were washed with PBS and 10 μl of MTT (5 mg/1 m l of PBS) was added to 100 μl of FBS free culture media and the plate was incubated for 4 h in 37 °C. In the viable cell, the yellow tetrazolium was converted to blue formazan crystal by mitochondrial succinate dehydrogenase enzyme. The resulting crystals were then dissolved in the 100 μl of dimethyl sulfoxide (DMSO) (Sigma Company, and Germany) and absorbance was measured at 505 nm using ELISA reader (SCO diagnostic, Germany). After plotting the standard graph absorbance vs known number of cells, the number of viable cells was calculated using the linear formula $Y = 0.0158X + 0.030$ with $R^2 = 0.996$ where Y stands for absorbance and X stands for number of the viable cells.

Irrespective of large differences between the GA concentrations (30 and 120 μM) a narrow margin was observed between the mortality caused by them (23–29 %), therefore based on the results of viability tests these two concentrations and 6 ng/ml of boric acid (based on previous study run by Movahedi and Abnosi 2016 [15] at 36 h) were chosen and following experimental groups were designed.

1. C-The control group, treated only with fresh culture media
2. BA group, treated with 6 ng/ml of boric acid
3. GA30, treated with 30 μM of gallic acid
4. GA120, treated with 120 μM of gallic acid
5. GA30 + BA, treated with 30 μM of gallic acid and 6 ng/ml of boric acid
6. GA120 + BA, treated with 120 μM of gallic acid and 6 ng/ml of boric acid

Analysis of each experimental group was repeated three times.

4. Quantification of proliferation ability cells

4.1. Colony forming assay

To investigate the colony formatting ability (CFA), the third passage cells at a density of 50,000 were cultured in 3 cm sterile plates and after 24 h to ensure the cell attachment. Then the cells were incubated for 14 days with every 3 days of culture media replacement according to the above mentioned groups. After 14 days the cells were washed with PBS, and crystal violet stain (0.5 g crystal violet in 100 ml methanol solution) was added to each plate with incubation at room temperature for 15 min. Using a light microscope equipped with a graticule eyepiece the diameter (μm) and colony numbers were determined.

4.2. Population doubling number

To investigate the population doubling number (PDN) 80,000 cells were cultured in 3 cm sterile plates and after 24 h to ensure the cells attachment. Then the cells were treated according to the above mentioned groups with contaminated culture media and incubated for 1, 3 and 7 days. After certain period of time, plates were washed with PBS and detached from bottom of the flasks using trypsin- EDTA. The cells were counted with the help of hemocytometer and the PDN of the cell population was calculated using $PDN = \log N/N0 \times 3.31$ where N0 was the initial number of cells cultured and N was the number of cells

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