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Prophylactic Effects of DL-β Hydroxybutyrate against Hepatic Cellular Senescence Induced by D-galactose or γ-irradiation via Partial Modulation of Antioxidants and Trace Elements in Male Rats

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AGING of cells is a normal feature but, it is important to delay this process and improve the life style. The present work aims at investigating the possible potential inhibitory effect of DL-β-hydroxybutyrate (βOHB) against hepatic cellular senescence induced by D-galactose or γ-irradiation in rats based on antioxidant and certain trace elements estimation in the liver. Six groups of male rats were used as follows: the control, irradiated group (acute dose 5 Gy, 2 weeks), D-galactose (150 mg/kg b.wt, 6 weeks), βOHB (72.8 mg/kg b.wt, 14 days), γ-irradiation plus βOHB, and D-galactose plus βOHB. Results show that neither βOHB nor D-galactose administration had a significant effect relative to the normal control value on Hb, MCV, and RBC's. βOHB along with D-galactose or γ-irradiation significantly increased G6PD activity compared with the control group. In D-galactose, or γ-irradiation groups, liver MDA levels and SOD activity were significantly increased. Meanwhile, NO and GSH levels were significantly increased in the γ-irradiation group relative to the normal control levels. The findings show that βOHB alleviated hematological, antioxidant alterations and modulated the change in Cu, Fe, and Zn elements in D-galactose or γ-irradiation group. The results concur well with histological alteration in our previous findings. It could be concluded that these findings highlight a role for βOHB as a potent protective agent against hepatic cellular senescence associated liver injury through enhancing RBC's G6PD activity, reduction of oxidative stress and partial modulation of trace elements.

Keywords: D-galactose, Gamma irradiation, Oxidative stress, β-hydroxybutyrate, Trace elements.

Introduction

Senescence, often known as aging, is a biological process that is unavoidable. Senescence is involved in normal development, tissue homeostasis, and tumor progression, but it is also linked to a variety of chronic conditions such as cancer, neurological disorders, and cardiovascular disease (Franceschi et al., 2018). Senescence occurs at the organ and cellular levels. Most senescent cells are characterized by morphological changes resulting in large, flat, and multinucleated phenotypes. In addition, senescence cells, a stable growth arrest and other phenotypic alteration, include proinflamatory secretome (Hasan, 2018). D-galactose or γ-irradiation can cause the accumulation of reactive oxygen species (ROS) with a final oxidative stress (Chen et al., 2007; Kim & Lee, 2014). Oxidative stress is considered to be significant in the pathophysiology of various diseases, including, cancer, Parkinson's and

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cardiovascular diseases and the aging process. ROS damage cellular lipids, proteins, and DNA resulting in disturbance in their normal functions. An excess of ROS can result in cell aging and death (Butterfield & Boyd-Kimball, 2018).

As an inducer of aging in vivo, D-galactose (D-gal) is a naturally reducing sugar which is totally metabolized at normal levels in the body. Conversely, excess D-galactose is converted to galactitol by galactose reductase, which can induce osmotic stress. Additionally, galactose oxidase induces the metabolism of high D-galactose levels to produce hydrogen peroxide. Consequently, high levels of hydrogen peroxide increase the oxidative stress and finally disturb the function of macromolecules and cells (Kumar et al., 2010). Therefore, D-galactose-treated rats have been demonstrated to display similar symptoms to those aging naturally. Thus, D-gal injection has been widely used to establish a model for anti-aging research (Liu et al., 2013 a).

Exposures of the organism to ionizing radiation (IR) such as X-, γ- or cosmic rays and alpha-particles from radon decay as well as ultraviolet A (UVA) and ultraviolet B (UVB) solar light are among the environmental sources that induce oxidative stress (Alcocer et al., 2020). High-dose exposure to ionizing radiation results in deleterious biological adverse effects. Its direct and indirect activities cause the adverse effects of radiation. Both acts induce biomolecule damage that often involves enzymatic repair. The indirect effects of the generation of reactive free radicals induce oxidative damage to cell biomolecules, affecting their normal functions (Butterfield & Boyd-Kimball, 2018).

IR is an oxidative stress and DNA damage inducer, which induces tissue damage. In addition, IR enhances cell cycle growth arrest both *in vitro* and *in vivo* via p53 induced by DNA damage response (Mavragani et al., 2017; Nguyen et al., 2018). Interestingly, *in vivo* IR response is tissue-specific and dose-dependent, and the proliferative capacity and structural function of the affected tissues seem to be determining factors regulating IR responses (Schofield & Kondratowicz, 2018).

The body responses to the prolonged reduction of glucose availability, insulin decline, and liver glycogen depletion by increasing the ketone bodies to act as alternate metabolic substrates for extra-hepatic tissues, in many cases such as hunger, fasting, intense exercise, calorie restriction, or the ketogenic diet (KD). Although KD has a strong therapeutic potential, its efficacy and usefulness as a metabolic therapy for widespread clinical use is restricted by several factors. Due to severe dietary restriction, patient compliance with KD can be limited, and the diet is widely shown as unpalatable and intolerant due to high-fat ingestion. As a result, ketosis may be difficult to manage as even intake of a small amount of carbohydrates or excess protein may readily inhibit ketogenesis (Amari et al., 1995; Halevy et al., 2012). Moreover, it may take several weeks for the ketone body synthesis to be consumed by extra-hepatic tissues (keto-adaptation), and patients may develop moderate hypoglycemic symptoms during this transitional phase (Zhang et al., 2013).

Given both the great therapeutic potential and limitations of KD, an oral exogenous ketone supplement capable of inducing sustained therapeutic ketosis without the need for dietary restriction would serve as a practical alternative. Furthermore, several supplements of natural and synthetic ketones capable of inducing nutritional ketosis have been identified. Desrochers et al. (1995) demonstrated that exogenous ketone supplements: (R, S)-1, 3 butanediol and (R, S)-1, 3 butanediol-acetoacetate monoesters and diester have been confirmed to elevate pig blood ketone bodies (>0.5 mM). Clarke et al. (2012) demonstrated the safety and efficacy of chronic oral administration of a ketone monoester of βOHB in rats and humans. Subjects maintained high blood ketones without dietary restriction and recorded little to no adverse side effects, demonstrating the potential to eliminate the restrictive diet typically needed to achieve therapeutic ketosis.

Numerous studies suggest that certain KD benefits are responsible for the effects of ketone body metabolism. Interestingly, in research on type 2 diabetes patients, improved glycemic regulation, enhanced lipid markers, and retraction of insulin and other medicines occurred before weight loss became important. In parallel, the growth of mitochondrial ROS species is reduced by both βOHB and acetoacetate (Kim et al., 2007; Maalouf et al., 2007). In addition, it has been shown that ketone bodies improve

the heart hydraulic performance by 28% while decreasing oxygen consumption and elevating ATP production (Kashiwaya et al., 1994). High ketone bodies have thus increased metabolic efficiency by reducing superoxide production and enhancing glutathione levels (Veech et al., 2001).

Sullivan et al. (2004) found that rats fed KD for 10–12 days showed increased hippocampal uncoupling proteins, suggesting a decreased ROS produced by mitochondria. In addition, mitochondrial biogenesis increased in rats maintained on KD for 4–6 weeks (Bough & Rho, 2007; Bough et al., 2006). Consequently, Shimazu et al. (2013) stated that βOHB acts as a class-I histone deacetylases inhibitor, which provides protection against oxidative stress by enhancing stress responsive gene such as FOXO3. Ketone bodies have also been shown to suppress inflammation by reducing inflammatory biomarkers (e.g., tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-8 and E-selectin), (Ruskin et al., 2009; Paoli et al., 2015). It is also assumed that exogenous ketone bodies themselves impart many of the advantages associated with KD. Our previous research paper (Habieb et al., 2021) has confirmed that either D-galactose administration or γ-ray exposure successfully elevated hepatic cellular senescence biomarker including $P16^{INKa}$ and $p21^{CIP1}$ gene and protein expression, in addition to elevation of certain senescence-associated secretory phenotype (SASP) such as IL-6 and TNF-α. Thus, the present research is an extension to our previous study to spot a new light on prophylactic effect of βOHB on hematological alteration and hepatic injury associated with hepatic cellular senescence induced by chronic administration of D-galactose or by an acute dose of γ-irradiation in liver of male rats through its effect on antioxidants and certain trace elements.

Material and Methods

Chemicals and reagents

Both D-galactose and β-hydroxybutric acid sodium salt were purchased from Sigma-Aldrich Co, ST. Louis. Mo, USA. Urethane was purchased from Sigma-Aldrich Co, ST. Louis. Mo, USA.

Experimental animals

Throughout the experiment in the current study, 48 male albino rats aged 3 months (120-150 g) were used and collected from the animal house of the Egyptian Atomic Energy Authority. In addition, the animals were fed commercial pellets and given tap water *ad libitum* throughout the experimental stages. Rats from the Animal Ethics Committee (IAEC) of the Faculty of Science, Tanta University, Tanta, Egypt, were treated in compliance with the proposed national ethical guidelines instructions.

Irradiation process

Acute 5 Gy (0.708 rad/s) whole-body γ-irradiation dose (Seol et al., 2012) was performed using the facilities provided by the National Center for Radiation Research and Technology. The Cs^{137} irradiation unit (gamma cell, 40) provided by the Candian Atomic Energy was used. It is defined by a uniform distribution of rays to small biological materials that do not pose any external hazards to operators.

Treatments

D-galactose (D-Gal) was supplied to the animals at a dose of 150 mg /kg b.wt diluted in saline (Liu et al., 2009). A ketone body, DL-β hydroxybutyrate (β-OHB) was supplied to certain groups of rats at a dose of 75.5 mg/kg b.wt dissolved in saline(Choragiewicz et al., 2008).

Experimental design

Following two week of acclimatization to the home cage, the rats were randomly divided into six groups, each comprising 8 rats, as follows: Group I: normal control group injected with the same volume of saline; Group II: rats received 72.8 mg DL-β hydroxybutyrate/kg b.wt intraperitoneally for 14 consecutive days; Group III: rats received 150 mg D-galactose/kg b.wt intraperitoneally for 42 consecutive days; Group IV: rats received 150 mg D-galactose /kg b.wt for 28 consecutive days followed by D-galactose and intraperitoneal injection of β-hydroxybutyrate 72.8 mg/kg b.wt for consecutive 14 days; Group V: rats were exposed to 5 Gy, as a single dose whole body γ- irradiation; Group VI: rats exposed to γ- irradiation 5 Gy (whole body irradiation) as in group V, and treated at the same time by intraperitoneal injection by β-hydroxybutyrate 72.8 mg / kg b.wt for 14 consecutive days; On the day after the final treatment, rats were anesthetized by intraperitoneal injection of 1.2 mg/kg b.wt urethane (Flecknell, 1993). Consequently, the rats were sacrificed after the whole blood and liver tissues were collected for biochemical analyses.

Samples collection

Blood sampling

Whole blood was collected via cardiac puncture after anesthesia. In an ethylene diamine tetraacetic acid (EDTA) tube, blood was collected from each rat. In addition, EDTA blood samples were used for the determination of activity and a complete blood count of glucose-6-phosphate dehydrogenase (G6PD), where hematological parameters including red blood cells (RBC's), white blood cells (WBC's), mean corpuscular volume (MCV), platelet (PLT) counts, and hemoglobin (Hb) concentration were evaluated using blood counters (Techo, Miami, FL, USA).

Liver tissue sampling

Liver tissue was prepared following the method of Li et al. (2014). Excised liver tissue was washed with ice-cold isotonic 0.9% NaCl after dissection, blotted between two filter papers, and weighed. Liver tissue was divided into two portions (portion 1 of the liver tissue, 10% (*W*/*V*) homogenate in ice-cold 0.9% NaCl was prepared for antioxidant (SOD, GSH) and oxidant (NO, MDA) biochemical analysis using a Tri-R STIR-R model K41 homogenizer; portion 2 of the liver, was washed for determination of certain elements with deionized water).

Biochemical analysis

Lipid peroxidation was quantified in liver homogenate according to Yoshioka et al. (1979). The estimation of nitric oxide was according to Montgomery & Dymock (1961). Liver glutathione (GSH) was calculated according to the updated procedure of Ellman (1959). Moreover, superoxide dismutase (SOD) activity in tissue homogenates was assessed according to the method of Masayasu & Hiroshi (1979). Consequently, the biochemical analysis was conducted using a Helios γ UV-VIS spectrophotometer.

Preparation of tissue samples for atomic absorption

Trace elements have been determined in the liver tissue of rats. Following digestion at 5:1 ratio (*v*/*v*) in pure concentrated nitric acid and hydrogen peroxide (Nada et al., 2012), a Landmark MLS-1200 Super High-Performance Microwave Digestor Unit was used for sample digestion. Moreover, the selected elements were estimated using Thermo Science ICE 3000 Series Atomic Absorption Spectrometry, fitted with deuterium background correction. All the solutions were prepared with ultrapure water obtained from ELGA company with a specific resistance of 18 U/cm. Consequently, the biochemical analysis was performed using a Helios γ UV-VIS spectrophotometer.

Statistical analysis

Statistical analysis was conducted using one-way variance analysis (ANOVA) followed by the honestly relevant difference test of post hoc Tukey using the GraphPad Prism 5 software package. Data were provided with an appropriate significance level of $P \leq 0.05$ as mean ± SE ($n = 8$ rats/group). The approach used for analyzing the findings was given by Milton et al. (1986).

Results

The βOHB administration per se resulted in a substantial increase ($P \leq 0.05$) in the PLT count by 54.39% relative to the standard control values (Table 1). The administration of D-galactose resulted in a significant 45.28% rise ($P \leq 0.05$) in WBC's relative to the control value. βOHB administration along with D-galactose restored all hematological parameters to normal control levels.

Neither βOHB nor D-galactose administration had a significant effect relative to the normal control value on Hb, MCV, and RBC's. Moreover, βOHB administration along with γ-irradiation significantly increased ($P \leq 0.05$) WBC's compared with the irradiated group, achieving a percentage increase of 124.35%.

Figure 1 shows that G6PD activity was significantly reduced by 22.14% in rats treated with D-galactose ($P \leq 0.05$) compared with the control group. In addition, βOHB administration along with D-galactose resulted in a substantial 21.29% increase in G6PD activity relative to the D-galactose group.

In contrast with the control group, γ-irradiation caused a significant decrease ($P \leq 0.05$) in blood G6PD activity among γ-irradiated animals by 34.98%. In contrast with the γ-irradiated group, βOHB administration with γ-irradiation significantly increased ($P \leq 0.05$) G6PD activity by 46.4%. Furthermore, G6PD activity was restored to normal control levels by βOHB administration along with D-galactose or γ-irradiation.

TABLE 1. Effect of DL-β-hydroxybutyrate (72.8 mg/kg) on complete blood count (CBC) on hepatic cellular senescence induced by D-galactose (150 mg/kg b.wt) or γ-irradiation (5 Gy)

Groups	Hb (g/dl)	MCV (fl)	PLT (*10⁹/l)	RBC's (*10¹²/l)	WBC's (*10⁹/l)
Control	17.04 ± 0.199	50.78 ± 0.852	555.6 ± 38.38	8.916 ± 0.07	4.56 ± 0.318
βOHB	16.44 ± 0.625 - 3.52 %	53.08 ± 0.62 - 4.52 %	857.8 ± 45.56[a] 54.39 %	8.566 ± 0.22 - 3.9 %	3.67 ± 0.27 - 19.51 %
D- Gal	16.94 ± 0.297 - 0.58 %	50.92 ± 0.755 - 0.27 %	532 ± 23.01 - 4.24 %	8.806 ± 0.193 - 1.23 %	6.625 ± 0.396[a] 48.54 %
D-Gal + βOHB	15.56 ± 0.367 - 8.68 % **- 8.14 %**	52.32 ± 0.575 - 3.03 % **2.74 %**	535.8 ± 32.15 - 3.56 % **0.71 %**	8.75 ± 0.16 - 1.86 % **- 0.63 %**	5.82 ± 0.503 27.63 % **- 12.15 %**
IR	11.95 ± .5 [a] - 29.87 %	70.87 ± 1.1[a] 39.56 %	133.3 ± 4.6[a] - 76.00 %	5.78 ± 0.229 [a] - 35.17 %	1.56 ± 0.067[a] - 65.78 %
IR+ βOHB	14.1 ± 0.28 [ac] - 17.25 % **(17.99 %)**	77.86 ± 2.89 [ac] 53.32 % **(9.86 %)**	347 ± 16.62 [ac] - 37.54 % **(134.77 %)**	6.8 ± 0.2 [ac] - 23.73 % **(17.64 %)**	3.5 ± 0.122 [c] - 23.24% **(124.35 %)**

Data are presented as mean ± SE (n = 8). **a** indicates significant changes from the control, **b** indicates significant changes from D-Gal, **c** indicates significant changes from IR at P≤ 0.05 using ANOVA followed by Tukey–Kramer as a post-ANOVA test. Normal percentage indicates percentage change from the control group. Underlined percentage indicates percentage change from the D-galactose group. Percentage in round brackets indicates percentage change from the IR group. βOHB β-hydroxybutyrate, D-Gal D-galactose, IR γ-irradiation, Hb hemoglobin, MCV mean corpuscular volume, PLT platelets, RBC's red blood cells, WBC's White blood cells.

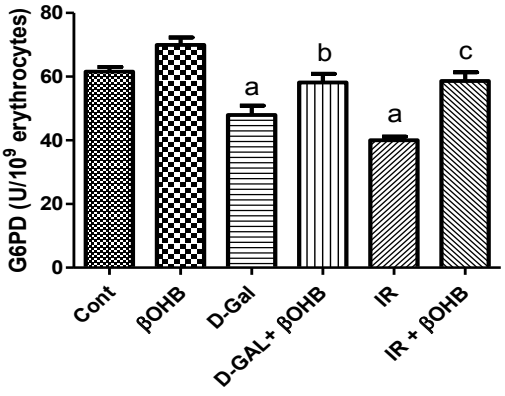

Fig. 1. Effect of DL-β-hydroxybutyrate (72.8 mg/kg) on blood glucose-6-phosphate dehydrogenase activity (G6PD) on hepatic cellular senescence induced by D-galactose (150 mg/kg b.wt) or γ-irradiation (5 Gy) [Data are presented as mean ± SE (n= 8). a indicates significant changes from the control, b indicates significant changes from D-Gal, c indicates significant changes from IR at P≤ 0.05 using ANOVA followed by Tukey–Kramer as a post-ANOVA test. βOHB: β-hydroxybutyrate , D-Gal: D-galactose, IR: γ-irradiation]

As highlighted in Fig. 2, βOHB administration *per se* did not significantly alter liver GSH and nitric oxide (NO) contents. Meanwhile, it significantly ($P \leq 0.05$) increased malondialdehyde (MDA) levels and SOD activity by 318.31% and 26% relative to the normal control value, respectively. Administration of D-galactose significantly ($P \leq 0.05$) increased hepatic MDA content and SOD activity by 302.19% and 28.2%, respectively, in comparison with the normal control levels. However, D-galactose-treated groups with βOHB induced a significant ($P \leq 0.05$) decrease in hepatic MDA and SOD activity by 45.76% and 66.45%, respectively, compared with the D-galactose groups.

Exposure to γ-irradiation significantly ($P \leq 0.05$) increased the MDA, NO, and GSH levels and SOD activity by 558.24%, 101.73%, 210.06%, and 102.9%, respectively, compared with the normal control value. However, the rat model that received βOHB with γ-irradiation recorded remarkable percentage decrease in MDA, NO, GSH levels and SOD activity by 58.04%, 25.85%, 48.92%, and 33.35%, respectively, compared with γ-irradiation group.

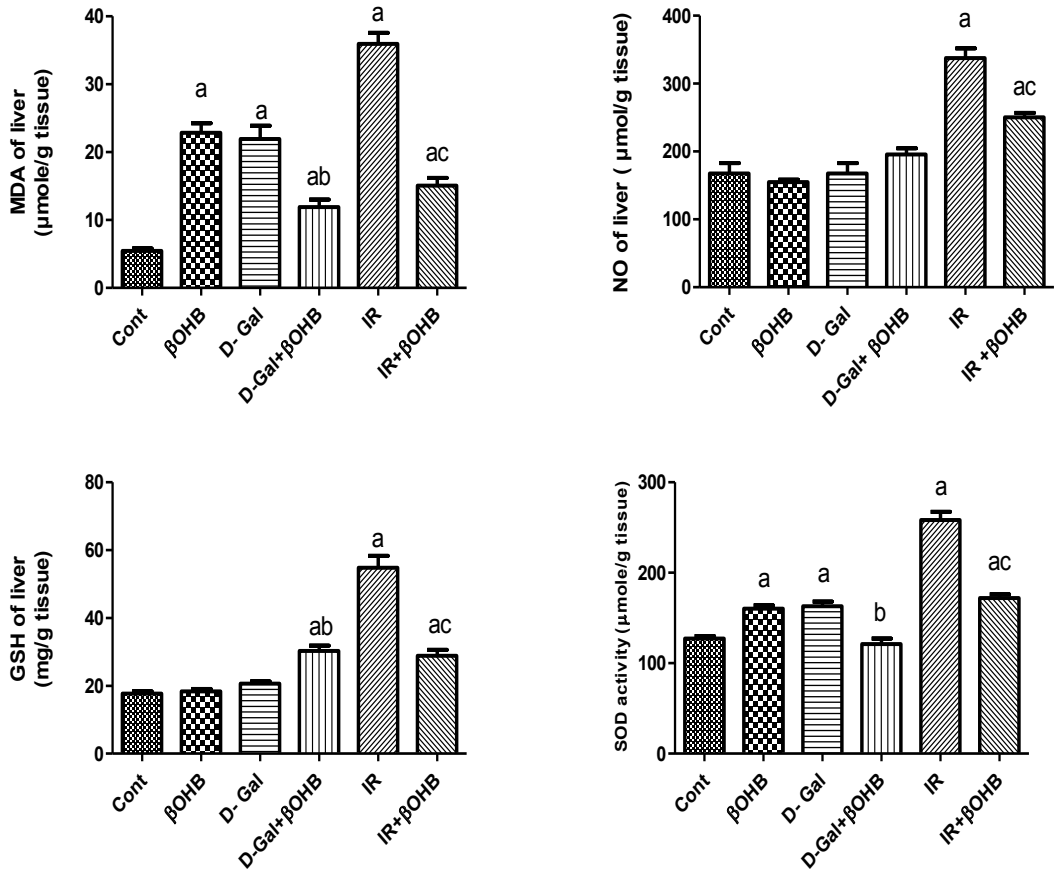

Fig. 2. Effect of DL-β-hydroxybutyrate (72.8 mg/kg) on MDA, NO, GSH levels and SOD activity in senescent liver induced by D-galactose (150 mg/kg b.wt) or γ-irradiation (5 Gy) [Data are presented as mean ± SE (n = 8). a indicates significant changes from the control, b indicates significant changes from D-Gal, c indicates significant changes from IR at P≤ 0.05 using ANOVA followed by Tukey–Kramer as a post-ANOVA test. βOHB: β-hydroxybutyrate , D-Gal: D-galactose, IR: γ-irradiation]

Figure 3 pinpoints that βOHB administration significantly increased (P≤ 0.05) liver copper content by 28.21% relative to the corresponding control group. In addition, the D-galactose group significantly exhibited a 22.29% liver iron increase and 19.61% zinc decrease compared with the control group. Meanwhile, treatment with βOHB for D-galactose-exposed rat significantly increased copper content by 24.7% and showed nonsignificant change (P ≤ 0.05) in liver iron and zinc concentration compared with the D-galactose group.

Rats submitted to γ-irradiation (5 Gy) exhibited a significant decrease in copper (Cu) and zinc (Zn) by 34.15% and 20.55%, respectively, and a non-significant decrease in iron content compared with the normal control value. Meanwhile, a significant recovery exists in copper concentration in γ-irradiation co-treated with the βOHB group compared with the γ-irradiation group. However, liver iron and zinc significantly decreased (P ≤ 0.05) by 20.26% and 18.84%, respectively, compared with the normal control value.

Discussion

The present study focuses on the potential inhibitory effect of DL-β-hydroxybutyrate (βOHB) against hepatic cellular senescence induced by D-galactose or γ-irradiation in rats based on antioxidant and certain trace element estimation in the liver following our previous study by Habieb et al. (2021), targeting the estimation of certain antioxidants, oxidative stress biomarkers and some essential elements in senescent liver induced by D-galactose or γ-irradiation.

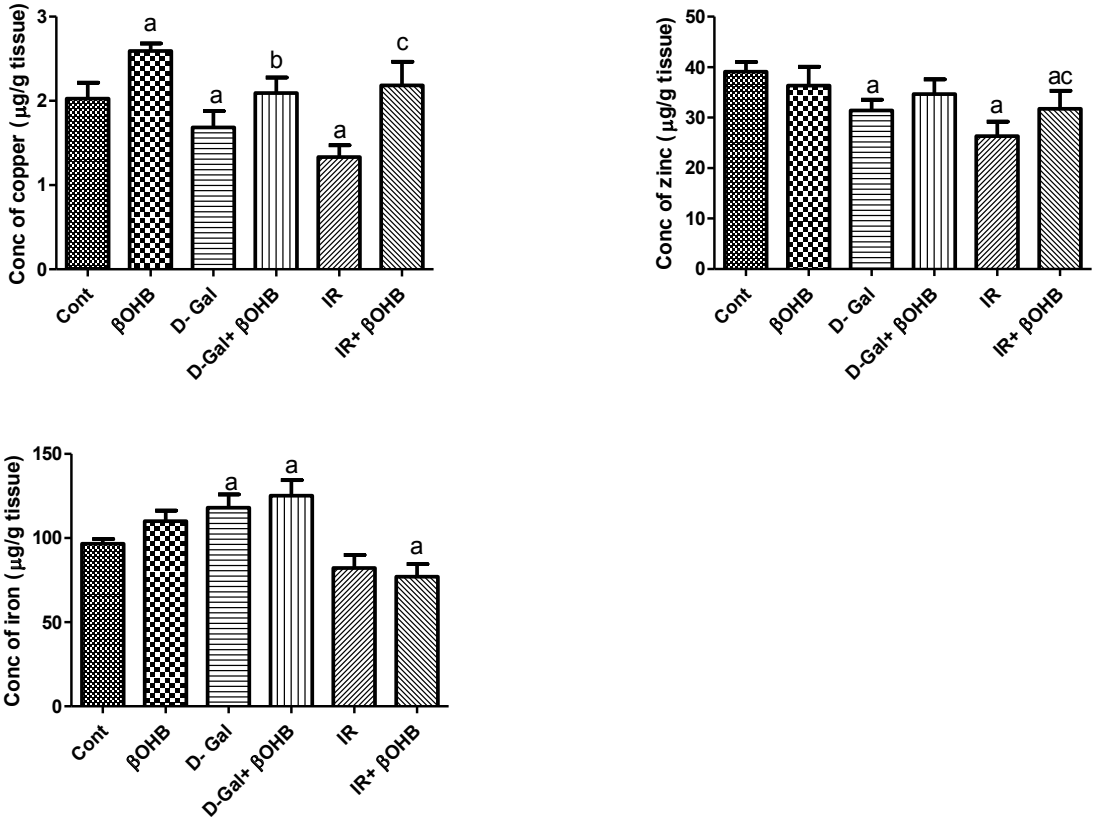

Fig. 3. Effect of DL-β-hydroxybutyrate (72.8 mg/kg) on hepatic copper, zinc and iron contents in senescent liver induced by D-galactose (150 mg/kg b.wt) or γ-irradiation (5 Gy) [Data are presented as mean ± SE (n = 8). a indicates significant changes from the control, b indicates significant changes from D-Gal, c indicates significant changes from IR at P≤ 0.05 using ANOVA followed by Tukey–Kramer as a post-ANOVA test. βOHB: β-hydroxybutyrate, D-Gal: D-galactose, IR: γ-irradiation]

The results of the current study clearly showed that a rise in the WBC counts exist, followed by a substantial decrease in the G6PD activity which may be attributed to induced synthesis of advanced glycation end-products that may induce elevation of WBC's by D-galactose (Hofmann et al., 1999; Yang et al., 2007). Moreover, D-galactose interacts with the amino protein group creating derivatives called advanced glycation end-products (AGEs). These AGEs have been shown to induce some angiogenic and inflammatory cytokine expression, including vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF-α), and interleukin 6 (IL-6). Moreover, this process could be the reason for leukocyte recruitment from bone marrow storage pool and activation (Costa et al., 2007).

The current research has shown an increase in platelets upon βOHB administration, which reflected enhancement of the differentiation process by βOHB *per se*. Platelet count elevation could be associated with increased liver production of thrombopoietin, which in turn may be responsible for the proliferation of megakaryocytes and their conversion to platelets (Subenthiran et al., 2013; Lee & Bergmeier, 2017).

A significant decline in RBC's, WBC's, platelets, and hemoglobin content in irradiated rats could be due to erythroblast destruction in bone marrow (Harish Kumar et al., 2011). In addition, compromised synthesis of kidney erythropoietin hormone, which is essential for erythropoiesis, could be attributed to elevated breakdown of RBC's by γ-irradiation (Hassan et al., 2016). The decline in hemoglobin levels may be related to erythrocytopenia (Malhotra & Srivastava, 1978; Chew & Park, 2004). Free radicals are known to be responsible for RBC's

and WBC's decline as they include enormous content of polyunsaturated fatty acids (PUFAs) embedded in phospholipid membrane and to oxygen transfer linked to hemoglobin molecules, which are efficient promoters of reactive oxygen species (Ebrahimzadeh et al., 2009). Exposure to γ-irradiation significantly increased MCV. This result coincides with that of Abdelhalim et al. (2015) who suggested that alteration in morphology and deformation of RBC's could be the trigger of RBC size increase by γ-irradiation.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.4), which catalysis glucose-6-phosphate oxidation to 6-phosphogluconolactone and finally to ribose-5-phosphate, is the main rate-limiting enzyme of the hexose monophosphate shunt. This pathway results in synthesis of reducing equivalent in the form of NADPH to cover cellular requirements for NADPH mediated fatty acids biosynthesis and maintenance of the cellular redox balance (Maurya et al., 2016).

γ-irradiation has a more pronounced effect on G6PD decline activity compared with the decrease induced by D-galactose. Inhibition of G6PD can then render the exposed RBC more vulnerable to oxidative damage (Demirdag et al., 2015). These changes can result from the vulnerability to oxidation induced by D-galactose or γ-irradiation of reduced sulfhydryl groups (-SH) essential for its activity (Giblin et al., 1979). Moreover, D-galactose or γ-irradiation alteration of the redox state of the RBC's subsequently decreases NADPH formation, which in turn inactivates the G6PD activity (Pari & Venkateswaran, 2003). Increased RBC's exposure to ionizing radiation resulted in a substantial decrease in G6PD activity, thereby decreasing survival time (Agarwal et al., 2007). These findings were consistent with those of Van Heyningen et al. (1954) who revealed X-irradiation affected reduced sulphahydryl (-SH) based enzymes than those enzymes that do not have free -SH groups for its activity. Thus, these findings suggest a general improvement in hematological parameters by βOHB via modulation of G6PD activity and decreasing oxidative stress.

Oxygen-derived free radicals enhance membrane lipid peroxidation, enzyme inactivation, DNA fragmentation, and apoptosis activation resulting in disturbance in the living cells (Speakman & Selman, 2011). MDA is the end product of lipid peroxidation, which is considered a significant biomarker. There are many enzymes in the body such as SOD and glutathione peroxidase which are responsible for scavenging superoxide anion and hydrogen peroxide to prevent ROS-induced damage. Thus, it has been important to determine the oxidative stress status including MDA, NO, GSH levels and SOD activity. Our data recorded that βOHB along with D-galactose or γ-irradiation significantly reduced the oxidative stress in the injured liver by restoring GSH levels and SOD activity in the liver, decreasing MDA level and modulating the trace element alterations, supporting the βOHB role against oxidative stress.

βOHB *per se* dramatically increased MDA levels in the liver. However, this finding was not predicted. Thus, this is likely to be because the daily injection of βOHB could cause recurrent bouts of oxidative stress to the liver and this finding requires further mechanistic analysis (Lu et al., 2018). The unusually high level of MDA caused by βOHB alone correlates with Milder et al. (2010), which could be attributable to the acute increase of hydrogen peroxide (H_2O_2) and 4-hydoxyneonal, an electrophilic lipid peroxidation end-product known to activate nuclear factor erythroid 2-related factor 2 (Nrf-2) detoxification pathway. Moreover, Nrf-2 can upregulate GSH biosynthesis and is a primary response to cellular stress. In addition, another possible explanation for this increase in MDA by βOHB per se may be due to lipid peroxidation mediated by kupffer cells of liver as NADPH oxidase (NOX) enzyme is upregulated by βOHB which acts as a mediator of the development of reactive oxygen species (Baffy, 2009; Kanikarla-Marie & Jain, 2015). The results concur well with the histological alteration in our previous findings (Habieb et al., 2021) and coincide with those of Anilkumar et al. (2008) who reported that high doses of ketone were reported to be associated with increased oxidative stress in cell models. Moreover, Baffy (2009) correlated elevation of oxidative stress on macrophage recruitment along with liver resident Kupffer cells, which subsequently raises the levels of hepatic MDA. Furthermore, the findings of the present study are in line with the results reported by De Almeida et al. (2010), who showed an increase in protein oxidation and lipid peroxidation in animal model rat livers subjected to acetone-induced ketosis via a free radical dependent mechanism.

Liver SOD activity was preserved in βOHB group. These data indicate that βOHB initially generates mild oxidative stress, which through redox signaling can systematically activate the Nrf-2 pathway, leading to an adaptive redox response to oxidative stress (Lu et al., 2018). Unlike other research carried out in this area, the authors did not find a significant decline in SOD activity along with D-galactose administration or γ-irradiation exposure. However, the cellular antioxidant SOD activity was significantly elevated by D-galactose or γ-irradiation exposure. The reason for this rather contradictory result is still not entirely clear, but this outcome can be justified in part by stimulation of MDA at sublethal concentration which induces adaptive response and enhance hepatocyte tolerance primarily through induction of SOD via transcriptional activation Nrf-2 signaling pathway, thereby attempts to protect hepatocyte against forthcoming oxidative stress (Pahl & Baeuerle, 1994). The previous findings can be elucidated by the existence of a better equilibrium between endogenous antioxidants and ROS generation in liver young tissue, which is very essential for the cellular defiance against oxidative stress. The findings may spotlight the noteworthiness of the animal starting age in relation to D-Gal-mediated dwindling of antioxidants due to oxidative stress (Xu et al., 2009). The previous findings contradicted those of Liu et al. (Liu et al., 2013b), who suggested a significant decrease in SOD activity which is an distinctive of various ages at the start of the D-Gal treatment.

Although D-Gal was expected to decrease GSH levels and increase NO concentration, there was non-significant alteration in GSH or NO levels. The fact that D-Gal treated rats was followed by an unchanged GSH level, leads to a possible explanation that either the response of GSH to increased lipid peroxidation is circumscribed or deprivation of change in GSH level is a reason for elevated oxidative stress levels. Absence of appropriate alterations in GSH levels in conditions of increased ROS may impair the capacity of antioxidant system, leading to increased oxidative stress (Hadzi-Petrushev et al., 2015). In addition, oxidative stress stimulation at a sublethal concentration, which induces adaptive response and increases cell tolerance primarily via GSH induction through transcriptional Nrf-2 signaling pathway activation, protects the hepatocytes from potential oxidative stress (Pahl & Baeuerle, 1994). Furthermore, Wei & Lee (2002) indicated that absence of significant D-Gal related changes in GSH or NO in group of young rats could be observed in the light of mandatory attention that must be given to the developmental changes and the influence of age on antioxidant enzyme. The results may highlight the importance of the start age of the animal in relation to D-Gal induced antioxidant depletion by oxidative stress (Xu et al., 2009). The previous results were in contradiction with the findings of Liu et al. (2013a) who indicated that a significant decrease in GSH levels exists, which is a characteristic of various ages at the start of D-Gal treatment.

Non-significant increases in NO levels were observed in the D-Gal-treated group in the present study, which could be interpreted as a result of enhanced NO oxidation by ROS. Consequently, nitrotyrosine generation via NO and tyrosyl radical combination or by peroxynitrite formation via NO and superoxide ion interaction, reduces the availability of NO levels (Reiter et al., 2000; Ferrer-Sueta et al., 2018).

Copper (Cu) is a trace elements known to trigger premature senescence. Cu is considered a cofactor of many enzymes, but free ionic copper is cytotoxic because it mediates the production of the extremely reactive hydroxyl radical through the Fenton reaction.Therefore, it is believed to cause oxidative stress associated tissue injury.

After γ-irradiation, the reduction of the hepatic copper may be related to the excess of its consumption by cuproenzymes which reduces the oxygen to water or hydrogen peroxide (Kotb et al., 1990) or due to de novo syntheses of Cu-SODs and catalase that inhibit the production of superoxide ion, H_2O_2, and hydroxyl radicals (Fee & Valentine, 1977). Nada et al. (2011) suggested that the decrease in copper levels is linked to the radiolytic loss of essential metalloelements cofactors which account for the 20% loss of both Cu-dependent and Zn-dependent SODs in rats following irradiation.

Accumulation of iron (Fe) in several tissues is recognized as a characteristic of aging and is associated with the pathology of multiple age-related diseases (Zecca et al., 2001; Xu et al., 2008). D-galactose induced a significant increase of iron in the liver. These findings are in complete agreement with those of Beregovskaia

et al. (1988) and Nada et al. (2008) who revealed that Fe level elevation could be correlated with bone marrow inability to utilize the Fe available in the diet and released from damaged red cells due to oxidative stress caused by D-Gal. The increased level of iron may be due to structural and conformational modification of ferritin induced by oxidative stress (García-Fernández et al., 2005) and transferrin (Trinder et al., 2000). Fe overload is associated with liver damage, characterized by a large accumulation of iron in hepatic parenchymal cells, resulting in fibrosis and ultimately hepatic cirrhosis (Pietrangelo, 2016). Increased iron levels by D-Gal agree with Xu et al. (2012) who suggested that Fe accumulation is generally accepted as an essential characteristic of the oxidative stress associated with aging process, particularly in postmitotic tissue. Consequently, both heme-Fe and heme biosynthesis decrease significantly with the oxidative stress (Mancuso et al., 2013). These alterations may be a result of iron homeostasis deregulation at the cellular level. However, the mechanism needs to be further investigated. This was confirmed by the previous result of Killilea et al. (2003) who stated that the total iron content in fibroblasts, the total iron content was shown to increase exponentially during cellular senescence, resulting in 10 fold higher levels of Fe compared to young cells. Moreover, a low-dose exposure to H_2O_2 leads to early fibroblast cell cycle arrest and enhances iron accumulation. This accumulation may also associate to the amplified amounts of oxidative stress and the decline of cellular function.

Zn is an important cofactor that enhances the activity of several proteins, such as metallothionein and Cu/Zn SOD that play a role in combating oxidative stress. Zn is also a cofactor for proteins involved in mediating DNA damage response and repair, such as the p53 tumor suppressor protein (Theocharis et al., 2004). Suboptimal Zn intake can therefore encourage oxidative stress generation and single- or double-strand DNA breaks comparable to DNA damage caused by radiation (Blount et al., 1997). Thus, precise regulation of cytosolic Zn buffering is important to preserve the redox state of the cell because both increased and decreased Zn levels can induce oxidative stress (Maret, 2006).

The results of the current study showed a significant decrease of Zn in the liver by D-Gal or γ-irradiation which could be due to molecular mechanism disruption that allows optimum Zn levels to be preserved within the cells (Colvin et al., 2010). In addition, increased Zn efflux transporter ZnT-1 levels in the hepatic cell membrane may lead to excess Zn efflux from the hepatocytes, resulting in lower levels of zinc in the hepatic cell cytosol (Sekler et al., 2007). Moreover, lower Zn levels in the liver may be mediated by the incorporation of zinc in superoxide dismutase with antioxidant function in an attempt to preserve proteostasis and cellular function (Andrews, 2000).

Oxidative damage caused by either D-Gal or γ-irradiation may be due to depletion in Zn levels as various evidences point to Zn as a positive regulator of autophagy. This was confirmed by the fact that high doses of Zn in culture medium (20–200 μM) were shown to induce autophagy in MCF-7 breast cancer cells (Hwang et al., 2010), astrocytes (Park et al., 2011), and also in human hepatoma cells (Liuzzi & Yoo, 2013). In addition, Zn depletion caused by TPEN or Chelex-100 cell-permeable Zn chelators has been able to suppress autophagy (Liuzzi et al., 2014). Moreover, a possible function of Zn was suggested as a modulator of inflammation associated with the senescence-associated secretory phenotype (Malavolta et al., 2015).

Zn deficiency coincides with our previous study results (Habieb et al., 2021) which confirm relationship between Zn and hepatic cellular senescence induction. This was explained by the study of Rudolf & Rudolf (2015) who found that colon cancer cells cultivated in a low Zn levels for 6 weeks resulted in morphological changes and typical markers of senescence, while Zn supplementation induced increased ROS formation, causing senescence in vascular smooth muscle cells. Zn also declined the antioxidative ability of the cell by downregulating catalase expression (Patrushev et al., 2012). Additionally, Carrı et al. (2003) stated that an imbalanced intracellular Zn homeostasis mediates both oxidative damage and neuronal cell death in neurodegenerative diseases. Therefore, oxidative damage of liver may be stimulated by unbuffered controlling mechanisms of intracellular/ cytosolic zinc.

As for Zn concentrations in liver tissue, irradiation caused a decrease in liver Zn levels. Similar findings were obtained by Ali et al.

(2012) who suggested that hepatocellular damage and oxidative stress caused by administration of anserine and/or Zn before or after radiation exposure are found to provide safety against γ-irradiation in rats. The results obtained do not coincide with those of Yukawa et al. (1980) and Smythe et al. (1982) who observed that γ-irradiation caused an increase in Zn levels in various organs in the entire body. Consequently, Okada (1970) suggested that the spleen, lymph nodes, and bone marrow of the lymphoid organs are highly radiosensitive. It was clarified that Zn, released from these damaged tissues, could accumulate in the liver, increasing its levels.

Reduced levels of Zn by both D-Gal and γ-irradiation may contribute to the zinc ability to mitigate oxidative damage and promote the process of repair and recovery mediated by its role as important cofactors for various antioxidant enzymes such as Cu and Zn superoxide dismutase. These antioxidant enzymes are necessary to limit lipid peroxidation, help repair the modification of the oxidative base, and help restore the functionality of misfolded proteins that accompany oxidative stress induced by D-Gal or γ-irradiation (Marreiro et al., 2017).

Conclusion

Although βOHB *per se* induced mild oxidative stress, its administration along with D-galactose or γ-irradiation has the potential to minimize hematological and hepatic injury associated with hepatic cellular senescence. This effect can be mediated partially by enhancing G6PD activity and modulation of altered trace elements. These data indicate that βOHB has promising application as an anti-aging medication via partial modulation of antioxidant and trace elements.

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