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Comparative Study to Evaluate the Efficacy of Hydrogen Sulfide Separately or in Combination with some Antioxidants against Myocardial Dysfunction in Irradiated Rats

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Hydrogen sulfide (H_2S) is an endogenously produced gaseous second messenger capable of mitigating pathological cardiac remodeling by regulating several cellular processes including fibrosis, hypertrophy, apoptosis, and inflammation. This study was dedicated to determine the cardio-protective efficacy of hydrogen sulfide alone or in combination with L-carnitine, co-enzyme Q_{10} or alpha lipoic acid against cardiovascular disease that occurred as a result of γ-irradiation in male albino rats. The obtained results revealed a remarkable change in all studied parameters after exposure to γ-radiation. However, irradiated rats treated by H_2S showed a significant ($p<0.05$) decrease in the activities of serum cardiac enzymes (CK, CK-MB, LDH & AST) and in the concentration of heart fatty acid binding protein (H-FABP) and endothelin-1 as well as the levels of cytokines profile (IL-1β, IL-10 & TNF-α) associated with a remarkable decrease in the level of heart TBARS than those in irradiated rats. On the other hand, H_2S caused a significant ($p<0.05$) increase in the level of serum TNO as well as in the heart GSH content and GPx activity than those of irradiated rats. Additive of L-carnitine or Co-Q_{10} to H_2S produced considerable improvement in all studied parameters, which become more pronounced in case of L-carnitine but less in case of Co-Q_{10} while, the addition of α-lipoic acid did not induce any noticeable changes. It is concluded that the use of L-carnitine increases efficacy of H_2S and could exhibit modulatory effects on γ-radiation-induced cardiovascular disease in rats

Keywords: *Hydrogen sulfide/ Radiation/ Myocardial Dysfunction/ Antioxidants.*

Introduction

Cardiac disease (CD) is the leading cause of morbidity and mortality in the world and accounts for nearly one-third of all deaths worldwide [1]. The controllable nature factors such as life-style, dietary factors and metabolic disorders as well as non-controllable factors such as gender, age and genetic predisposition are multiple contributory risk factors for CD [2]. In addition, there are environmental factors affecting the risk of CD, ionizing radiation being one such factor. Also, it has been known for a long time that high doses of radiation, such as those given during radiotherapy, causing damage to the heart and vasculature and leading elevation the risk of CD. These observations are supported in several experimental animals [3,4].

Hydrogen sulfide (H2S), the third endogenous gaseous transmitter in mammals besides nitric oxide (NO) and carbon monoxide (CO), plays an important role in many organ systems [5]. In the cardiovascular system, H2S is produced in the myocardium, fibroblasts and blood vessels from L-cysteine by the enzyme CSE (cystathionine γ-lyase). Interestingly, H2S executes the physiological functions of vasorelaxation,

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cardioprotection and inhibition of vascular remodeling [6]. It is also brought into context with a variety of cardiovascular diseases such as spontaneous hypertension, hypoxia-induced pulmonary hypertension and high pulmonary blood flow-induced pulmonary hypertension [7].
L-carnitine (β-hydroxy-γ-trimethylaminobutyric acid) is widely distributed in food from animal's sources but there is limited availability in plants. In humans, 75% of carnitine is obtained from the diet. L-carnitine (the biologically active stereoisomer) is absorbed from foods *via* both active and passive transport across enterocyte (intestinal cell) membranes [8]. L-carnitine has been shown to have favorable effects in patients with severe cardiovascular disorders, such as coronary heart disease, chronic heart failure and peripheral vascular disease. So, carnitine has been widely recommended as a supplement in cardiovascular disease [9].

Coenzyme Q10 (Ubiquinone-10) is a compound which serves as a coenzyme in key enzymatic reactions during the energy production in the cell, may be found in each cell in different amounts, has the lipid structure, and is similar to vitamin. Although it resembles vitamins in structure, it is not classified as a vitamin [10]. Tiano *et al.* [11] suggested that the effect of coenzyme Q10 (CoQ10) plays a positive role in endothelial relaxation. Moreover, Kumar *et al.* [12] found that CoQ10 levels of patients who have chronic cardiac failure are too low in tissue and serum samples. It was noted that CoQ10 administration in patients having coronary artery diseases led to improvement in vasodilatation and decrease in myocardial damage [13]. Also, CoQ10 decreases endothelial cell deaths by minimizing the inflammatory cytokines and can be used as a drug in ischemic cardiac diseases [14].
Alpha-lipoic acid (ALA) is an organo-sulfur compound derived from octanoic acid. It is synthesized in the mitochondria of the liver and other tissues, which plays an essential role in aerobic metabolism [15]. ALA acts as a cofactor for several mitochondrial enzymes by catalyzing the α-ketoacid. Several studies have recorded the therapeutic potential of ALA in a variety of diseases, including diabetes mellitus [16], cardiovascular diseases [17] and cancers [18]. The authors suggested the antioxidant property of ALA due to its ability to scavenge ROS directly, its metal chelating activity and its potential to regenerate other antioxidants such as glutathione and vitamin C &E.
This study was undertaken to clarify the function of H2S which acts as a vasodilator agent and has antioxidant properties in irradiated rats. Also, this investigation was designed to compare between the therapeutic roles of H2S alone or in combination with L-carnitine, CoQ10 and ALA on myocardial dysfunction as a result of exposure to γ-ray.

Material and Methods
Healthy forty two male albino rats (*Rattus rattus*) with an average weight of 120±10g representing 10±1 weeks of age were used in this study. They were obtained from the Animal House of the Nuclear Research Center, Atomic Energy Authority, Egypt. Animals were kept under good ventilation and illumination conditions, received a standard diet, and had free access to water throughout the study. All experimental protocols used in this work received prior approval of the Egyptian Atomic Energy Authority, Nuclear Research Center Animal Care Committee following the Ethics Committee of the Guide for the Care and Use of Laboratory animals published by the US National Institutes of Health (NIH publication No. 85-23, 1996).

Material
Sodium hydrosulfide (NaHS) and α-lipoic acid (ALA) were supplied from (Sigma Chem. Co. USA). L-carnitine and coenzyme Q_{10} (CoQ_{10}) were purchased from Arab Company for Pharmaceuticals & Medicinal Plants (MEPACO-MEDIFOOD) Anshas, Egypt.

Radiation processing
Animals were irradiated by using gamma cell-40 biological irradiator (Cesium-137), located at the National Centre for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Nasr City, Cairo, Egypt. Animals were irradiated at an acute single dose level of 6Gy delivered at a dose rate of 0.46Gy/min. at the time of experimentation.

Animal groups and experimental protocol
The rats were randomly distributed into six groups of 7 each.

Group 1: Rats served as control, injected intraperitoneally (i.p) with normal saline solution (0.9%NaCl).

Group 2 (IR): Rats exposed to 6Gy whole body γ-radiation as a single dose shot.
Group 3: Rats injected i.p with 10mmolNaHS/kg b.wt/day dissolved in normal saline for one month after exposure to whole body γ-irradiation as previously described.

Group 4: Rats injected i.p with NaHS as mentioned before plus 200mgL-carnitin/kg b.wt/day[19] for one month later exposure to whole body γ-irradiation.

Group 5: Rats injected i.p with CoQ_{10} at a dose of 200mg/kg b.wt/day[20] associated with NaHS as mentioned before subsequent γ-radiation exposure for one month.

Group 6: Rats were irradiated and injected i.p with NaHS as described before together with 100mgALA/kg b.wt/day [21] for one month.

At the end of experimental period, the animals were fasted overnight; water was not restricted and anaesthetized with ether. Blood samples were collected and sera were separated by centrifugation and stored at -4°C till estimation of biochemical parameters. The activities of creatin kinase (CK), creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured by using commercial kinetic kits (Spainreact, Ctra. Santa Coloma, Spain). Rat heart fatty acid binding protein (H-FABP) quantitative test based on a solid phase enzyme-linked immunosorbent assay (ELISA) developed by Life diagnostics Inc. West. Chester PA, USA. The concentration of endothelial-1 (ET-1) and the levels of cytokine profile [Interleukin-1β (IL-1β), interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α)] were determined using commercial ELISA kit specific for rats (Immuno-Biological Laboratories Co., Ltd. USA). Serum rat total nitric oxide (TNO) as a free radical was estimated using a commercial ELISA kit (Assay Designs, Inc.; Germany).

Also, the heart was directly separated, washed in ice-cold saline then homogenized in distilled water (10% W/V) using a homogenizer. The cell debris was removed by centrifugation at 3000 rpm for 10min. The homogenate supernatants were subjected to estimate glutathione (GSH) content, glutathione peroxidase (GPx) activity and thiobarbituric acid reactive substance (TBARS) level were determined by using commercial ELISA kits (Cell BioLabs, Inc., San Diego, USA).

Statistical analysis
Data were statistically analyzed using one way analysis of variance followed by Duncan's multiple range test using a computer program (Costate) (Version 6.303 software, serial number; s/n: 1053500000). Values of $P<0.05$ were considered statistically significant.

Results and Discussion
It is known that the exposure of gamma ionizing radiation causes myocardial dysfunction in both humans and animals [4,22]. Tables (1-4) depict the studied parameters of control and experimental groups. Significant ($p<0.05$) increments occurred in the serum activities of cardiac enzymes (CK, CK-MB, LDH and AST) and the concentrations of rat heart fatty acid binding protein (H-FABP), endothelial-1 (ET-1) as well as the serum levels of cytokine profile (IL-1β, IL-10 and TNF-α) associated with a remarkable elevation in the heart level of thiobarbituric acid reactive substance (TBARS) of the irradiated rats when compared to control group. In contrast, significant ($p<0.05$) depletion occurred in the serum level of total nitric oxide (TNO) associated with decreasing in the heart glutathione (GSH) content and glutathione peroxidase (GPx) activity of the irradiated rats group when compared with their corresponding normal animals group (Table 1-4). These data may be due to the elevation of free radical production; the increment of SH-bond destruction and decreasing in the antioxidant system as well as the damage of DNA associated with cell membrane accompanied with cellular lesions and cell death. So, elevated heart marker enzymes in serum are a reflection of radical-mediated lipid peroxidation of cardiac cell membrane. Several investigations indicated that exposure to radiation increases free radicals activity [22-23]. The production of free radicals is responsible to be the primary cause of the injury effect. These free radicals combined with the cellular lipid and proteins which in turn, initiate lipid peroxidation process and protein carbonylation, resulting in structural changes of

bio-membranes and loss of cardiac integrity and reduction in the metabolic activity of heart. Furthermore, Schaue *et al.* [24] observed that radiation has been shown to induce inflammatory response. Inflammation is also an integral component on the host's response to tissue injury or host invasion and plays a particularly active role after myocardial infraction. These results were in harmony with Ozyurt *et al.* [25] who demonstrated a significant increment in the levels of proinflammatory cytokines (IL-1β and TNF-α) as well as an anti-inflammatory cytokine (IL-10) in plasma following radio-therapy.

The significant elevation in the oxidation of lipid and the depletions in the heart glutathione (GSH) content and glutathione peroxidase (GPx) activity were pronounced in the irradiated rats group compared to control rats group. These results could be due to oxidation of sulphhydryl group of GSH as a result of decrease in glutathione reductase, the enzyme which reduces the oxidized glutathione (GSSG) into a reduced form (GSH) in presence of NADPH [26]. In addition, the significant elevation in lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS) content, is due to the peroxidation of the unsaturated fatty acids resulting from free radical propagation consequent to the suppression in bio-oxidase activities [3,4]. The polyunsaturated fatty acids present in the membranes phospholipids are particularly sensitive to attack by hydroxyl radicals and other oxidants, resulting destroy cells by damaging membranes, lipid peroxidation (LPO) can result in the formation of reactive products that themselves can damage proteins and DNA [27]. These results are in parallel with those obtained by Abd El-Rahman *et al.* [28]and Meky *et al.* (23) who showed the significant elevation in the serum CPK, LDH, AST activities and heart TBARS level accompanied with reduction in the GSH content and activity of GPx in cardiac tissue of γ-irradiated rats.

The elevation in the level of endothelin-1 has been reported in humans [29] and animals [30] after exposure to ionizing radiation. In this work, a significant ($p<0.05$) reduction in TNO level concomitantly with a significant elevation in ET-1 level were recorded in irradiated rats. These results may be attributed to both decreased production and increased consumption, with possible endothelial dysfunction and vascular impairment [31]. Also, reactive oxygen species (ROS) can react with NO, forming peroxynitrite (ONOO-) and thus decrease the bioavailability of NO resulting in endothelial dysfunction [32].

In mammalian and human tissues, the bulk of endogenous H2S synthesis appears to be from the pyridoxal-5-phosphate (PLP)-dependent enzymes cystathionine-γ -lyase (CSE) and cystathionine-β-synthase (CBS) *via* the amino acids cysteine, homocysteine and cystathionine [33]. H2S is a highly lipophilic molecule able to freely penetrate the membranes of cells of all types by diffusion without the requirement for specialized membrane transporters [34].

Table (1): Comparison between treatment by H_2S alone or associated with L-carnitine, CoQ_{10} and α-Lipoic acid on serum cardiac enzymes activity of irradiated rats (Mean±SD).

Groups / Parameters	Control	Radiation (IR)	IR H_2S	H_2S + L-Carnitine	H_2S + CoQ_{10}	H_2S + α-Lipoic acid
CK (U/L)	89.61± 3.36 ^{E}	202.21± 6.02 ^{A}	138.23± 6.06 ^{B}	107.66± 6.88 ^{D}	119.60± 5.78 ^{C}	138.24± 8.17 ^{B}
CK-MB (U/L)	11.42± 0.55 ^{E}	28.24± 1.48 ^{A}	19.81± 1.3 ^{B}	15.01± 1.58 ^{D}	17.49± 1.14 ^{C}	20.22± 1.92 ^{B}
LDH (U/L)	229.20± 4.55^{E}	426.60± 91.20^{A}	341.61± 9.29 ^{BC}	268.23± 7.46^{DE}	297.88± 9.55^{CD}	354.03± 13.54^{B}
AST (U/L)	117.41± 2.70 ^{E}	252.63±6. 65 ^{A}	151.82±9.8 8 ^{B}	129.65±4. 22 ^{D}	141.87±7.53 ^{C}	156.82±8. 73 ^{B}

- $^{A, B, C, D, E}$ Means with different superscript within a row are significantly different at ($p<0.05$).

Table (2): Comparison between treatment by H_2S alone or associated with L-carnitine, COQ_{10} and α-Lipoic acid on serum H-FABP, ENT-1 and TNO levels of irradiated rats (Mean±SD).

Groups / Parameters	Control	Radiation (IR)	IR H_2S	IR H_2S + L-Carnitine	IR H_2S + CoQ_{10}	IR H_2S + α-Lipoic acid
H-FABP (pg/ml)	8.68 ± 0.11 ^{E}	23.47 ± 0.48 ^{A}	16.21 ± 0.49 ^{B}	11.18 ± 0.84 ^{D}	14.17 ± 0.81 ^{C}	16.38 ± 1.10 ^{B}
ET-1 (pg/ml)	0.396 ± 0.005 ^{E}	1.10 ± 0.061 ^{A}	0.679 ± 0.009 ^{B}	0.511 ± 0.025 ^{D}	0.588 ± 0.019 ^{C}	0.670 ± 0.019 ^{B}
TNO (μmol/L)	55.72 ± 2.08 ^{A}	20.62 ± 1.23 ^{D}	48.68 ± 1.58 ^{C}	55.58 ± 1.56 ^{A}	52.56 ± 0.99 ^{B}	48.54 ± 1.44 ^{C}

- $^{A, B, C, D, E}$ Means with different superscript within a row are significantly different at ($p<0.05$).

Table (3): Comparison between treatment by H_2S alone or associated with L-carnitine, CoQ_{10} and α-Lipoic acid on serum inflammatory cytokine (IL-1β, IL-10 and TNF-α) levels of irradiated rats (Mean±SD).

Groups / Parameters	Control	Radiation (IR)	IR H_2S	IR H_2S + L-Carnitine	IR H_2S + CoQ_{10}	IR H_2S + α-Lipoic acid
IL-1β (pg/ml)	3.50 ± 0.05 ^{F}	11.37 ± 0.52 ^{A}	5.25 ± 0.19 ^{C}	4.01 ± 0.11 ^{E}	4.47 ± 0.03 ^{D}	5.87 ± 0.16 ^{B}
IL-10 (pg/ml)	23.68 ± 1.39 ^{E}	106.59 ± 5.27 ^{A}	40.38 ± 1.69 ^{C}	31.74 ± 1.40 ^{D}	40.07 ± 1.42 ^{C}	43.93 ± 1.92 ^{B}
TNF-α (pg/ml)	5.17 ± 0.01^{F}	19.42 ± 0.97 ^{A}	9.07 ± 0.32 ^{C}	6.23 ± 0.37 ^{E}	7.70 ± 0.56 ^{D}	10.05 ± 0.71 ^{B}

- $^{A, B, C, D, E}$ Means with different superscript within a row are significantly different at ($p<0.05$).

The treatment of irradiated rats with H2S led to a considerable correction in the obtained data of all studied parameters (Tables 1-4). These data may be attributed to the antioxidant effects of H2S. H2S salt donors reduced the formation of nitrosatively and oxidatively modified cellular proteins, DNA and lipids in animal models of myocardial and hepatic ischaemia/reperfusion [35,36]. Also, NaSH is reported to degrade lipid peroxides (37), inhibit the expression and activity of NADPH oxidase [38] and up-regulate thioredoxin-1 expression in vascular endothelial cells [39]. Increased hepatic GSH synthesis and decreased lipid peroxidation are also observed with Na2S treatment in a murine hepatic ischaemia/reperfusion injury model [40].

Serum heart FABP, a protein emitted fastly from cardiomyocytes in response to injury and marked as early as 90-120 minutes after injury [41]. In the current work, H-FABP was significantly ($p<0.05$) lowered in hydrogen sulphide treated irradiated rats. This result is in correspondence with that observed by Sodha *et al.* [42] who reported the therapeutic usefulness of sulfide administration as elevate levels of FABP after acute coronary syndrome are prognostic for an increased risk of death and congestive heart failure in patients.

In a mouse example of pressure excess-induced heart failure, H2S can activate endothelial nitric oxide synthase (eNOS) through phosphorylation and elevate NO bioavailability [43]. Moreover, Patel *et al.* [44] suggested that H2S is an endothelium-derived factor that mediates the vasodilator effects of ET-1 in the cerebral circulation *via* activation of ATP-sensitive K+ (KATP) and conductance Ca2+ activated K+ (BKCa) channels in vascular smooth muscle and therefore, it causes smooth muscle relaxation vasodilatation. In addition, H2S has a leading role in the inflammatory process [45]. Zanardo et al

(2006) reported that H2S donor, Na2S and NaHS were capable of suppressing leukocyte adherence and producing inflammatory pathology *via* activation of KATP channels [46].

Considerable corrections were reported in all studied parameters after the irradiated rats treated with H2S alone or in the presence of L-carnitine or CoQ10 but not returned to normal values (Tables 1-4). The best correction in all studied parameters was recorded in the irradiated rats group which was treated with both H2S and L-carnitine. These data may be attributed to the antioxidant effects of L-carnitine which acts as an anti-inflammatory agent, improves immune system, reduces the endothelial damage caused by ionizing radiation, decreases the production of procytokines such as (IL-1β, IL-6 and TNF-α), decreases the production of LDL and stabilizes the fluidity of cell membranes. These results are in parallel with those obtained by Xue *et al.* [9] Meky *et al.* [23] and Furat *et al.* [47]. Furthermore, it is known that healthy cell membranes are important for transporting nutrients and other materials into cells and removing wastes from them. These results provide evidence that L-carnitine can maintain the fluidity of the cell in general. This maintenance of membrane flexibility could be the reason that carnitine reduces heart muscle damage during a heart attack and hence, minimizes the leakage of cardiac enzymes into blood vessels [48].

Supplementation of irradiated rats with CoQ10 in the presence of H2S led to amelioration effects on all studied parameters compared to the irradiate rats group which treated with H2S alone but these effects are less than those treated with L-carnitine in the presence of H2S (Tables 1-4). These results may be attributed to the alteration in the *de Novo* lipogeneses, the correction in the antioxidant/oxidative status of cardiac tissues. CoQ10 can mend vascular function and decrease the atherosclerosis (13,14). Also, CoQ10 has remarkable role in barring the initiation and/or propagation of lipid peroxidation in plasma lipoproteins and membrane proteins. Several authors demonstrated that chronic treatment with CoQ10 in rats protected against cardiac injury due to oxidative stress created by hydrogen peroxide (H2O2) in the heart. CoQ10 can inhibit lipid peroxidation in mitochondria, protein oxidation and DNA oxidation [49]. They explained the antioxidant action of CoQ10 to ubiquinone form which can be recycled to the antioxidant active as well as reduced ubiquinol form *via* the mitochondrial Q cycle.

Numerical changes, albeit, not significant, occurred in all studied parameters of the irradiated animals group which were treated with both H2S and ALA compared to the irradiated rats treated with H2S alone (Tables 1-4). These data may be due to the pharmadynamics and pharmakinetics properties of ALA which produces H2S in its catabolism. These data are in harmony with those obtained by Bilska-Wilkosz *et al.* [50]. The last authors indicated that H2S is formed from ALA in the presence of environmental light. They proposed that H2S is the first product of non-enzymatic light-based on breakdown of ALA that is, probably, next oxidized to sulfane sulfur-containing compound(s). Bilska-Wilkosz *et al* also showed that dihydrolipoic acid (DHLA) acts as a reducing agent that liberate H2S from sulfane/sulfur-compounds and this mechanism occurs *in vivo* processes as the pharmacological action of ALA.

Conclusion

The best obtained data was presented in the irradiated rats group which treated with L-carnitine and H_2S. Moreover, supplementation of COQ_{10} associated with H_2S recorded a moderate correction in all studied parameters of irradiated rats group. Finally, this investigation also pointed to the benefit-less treatment of irradiated rats group with ALA in the presence of H_2S. So, this work can practically help to encourage the clinical use of H_2S in the presence of L-carnitine or COQ_{10} treatment for the hazard effects of ionizing radiation on cardio-vascular system.

We can recommend making further studies to determine more clearly and provide explanations for the co-administration of H_2S and L-carnitine or COQ_{10} on the myocardial dysfunction as a result of exposure to ionizing radiation dependent on the time of treatment and the grade of exposure.

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