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Hydrogen Sulfide Generation Protects against Renal Ischemia/Reperfusion-induced Cardiac Hypertrophy and Arrhythmia via Amelioration of Connexin- 43 Expression and Opening of K_{ATP} Channels

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Keywords

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- **Cardiac hypertrophy**
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Abstract

Hydrogen sulfide has been recently studied as a potential treatment for many cardiovascular disorders. Here, we describe its role in I/R- induced cardiac hypertrophy and arrhythmia model. Thirty six male Sprague Dawley rats were used in this study. Rats were randomly divided into 6 groups, G 1(n = 6/group): control negative: received saline, G2, I/R rats, (I/R, n = 6), after right nephrectomy, the rats were subjected to left kidney ischemia for 45 minutes followed by reperfusion:,G3, I/R rats in which rats were treated by NaHS, the donor of H_2S (5 mg/kg/day×14 days) intraperitoneal,G4, I/R rats in which rats were treated by the blocker of H_2S aminooxyacetic acid (10 mg/kg/day×14 days) intraperitoneal,G5, I/R rats in which rats were treated by converting enzyme inhibitor (Captopril) (50 mg/Kg/day ×14 days) and, G6, I/R rats in which rats were treated by NaHS and K_{ATP} channel blocker (Glibenclamide) (20 mg/kg/day ×14 days). NaHS led to a highly significant decrease in the duration of ventricular action potential indicated by QTc. Furthermore, it decreased, significantly, both of mean blood pressure and plasma renin activity. Moreover, H_2S donor and converting enzyme inhibition increased the expression of CX-43. While (I/R + NaHS + K_{ATP} channel block) led to a non-significant decreased in the duration of QTc interval. This showed that hydrogen sulfide generation has a potential therapeutic role in IR-induced cardiac hypertrophy and arrhythmia via amelioration of CX - 43 expression and opening of K_{ATP} channels.

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1. Introduction

Cardiac hypertrophy represents a fundamental adaptive response of heart to the increased working demand (1) and has been hypothesized to be a response to biomechanical stress, such as pressure overloading and volume overloading in which heart initiates a process leading to the increase in cardiac mass and augmentation of the pump function (2). The hypothesis is supported by in-vivo experiments where the expansion of myofibrillar apparatus of cardiomyocytes and collagen deposition occurring during hypertrophy results from the interaction between mechanical stresses (i.e. external load) and the release of autocrine/paracrine growth factors, such as angiotensin II and insulin-like growth factor-I (IGF-I) (3,4). Although initially beneficial, sustained cardiac hypertrophy may incur a temporal deterioration leading to the impairment of the cardiac functions (5, 6). Cardiac hypertrophy, usually, is considered as an effective compensation mechanism, can maintain or even increase cardiac output. However, in the long term, persistent hypertrophy will ultimately result in cardiac dilatation, decreased ejection fraction, and subsequent heart failure (7).

Cardiac gap junctions play a major role in impulse propagation and have also been implicated in arrhythmogenesis. Connexin-43 (CX-43) is the principal connexin in the mammalian ventricles and has been proven to have a close association with arrhythmia (8, 9). In fact, reduced expression of myocardial CX-43 protein, has been related to abnormalities in the intercellular propagation of the electrical impulses (10) and, in turn, to mechanical deficiencies observed in heart under hypertensive conditions (11) and in hypertrophied or congestive heart failure (12). However, only a few “in vitro” studies have postulated an involvement of this protein in the development of the immediate adaptive hypertrophic response. Early modulators of heart hypertrophy, such as cAMP and angiotensin II (10), as well as, 1 to 4 hours of pulsatile stretches (13, 14) have been shown to induce a rapid up-regulation of CX-43 protein associated with a marked increase in impulse propagation velocity. CX-43 quantity in failing myocardium was reduced by 40% in every transmural muscle layer compared with control, corresponding well with previous reports of a 50% reduction of CX-43 protein expression and 40% reduction of CX-43 mRNA in congestive heart failure (15, 16, and 17). However, the significance of CX-43 expression patterns on electrophysiological function and arrhythmogenesis was poorly understood.

Hydrogen sulfide (H2S) is an endogenously produced gaseous molecule with important roles in cellular signaling. This chemical has been implicated in

regulation of inflammatory responses, cardiovascular functions, renal functioning and gastrointestinal system (18, 19). Furthermore, H2S has been shown to exert a potent cytoprotective abilities against tissue injury, including organ fibroses such as myocardial and renal fibrosis (20, 21). Cystathionine-synthase and cystathionine-lyase use L-cysteine as a substrate to produce H2S. The discovery of cystathionine-lyase in the rat heart, as well as, identification of H2S as an important modulator is a breakthrough in the investigation of the role of H2S in heart functions. Increasing evidence has demonstrated that disturbed H2S production is relevant to heart diseases. Plasma H2S levels were significantly lowered in coronary heart disease patients compared with that in angiographically normal subjects (22, 23). The endogenous production of H2S is significantly reduced in many heart diseases, including myocardial ischemia and myocardial infarction- induced heart failure (24). These findings imply that cardiac diseases may impair the endogenous synthesis of H2S, which may further exacerbate the disease state. Meanwhile, these findings are clear evidence which support the involvement of endogenous H2S in maintaining basal physiological functions of heart. However, the role of H2S in the pathogenesis of cardiac hypertrophy, and the relationship between H2S and the dysregulation of cardiac rhythm remains unclear.

We set out therefore to: (i) test the hypothesis that a unilateral renal I/R model can induce cardiac hypertrophy and arrhythmogenesis; and, if confirmed, (ii) assess whether the decrease in CX-43 expression is involved in renal I/R-induced cardiac hypertrophy and arrhythmia. (iii) investigate the possible protective effects of H2S against cardiac hypertrophy and arrhythmia in I/R- induced cardiac hypertrophy and arrhythmia model.

2. Materials and Methods

2.1. Experimental Animals

Adult male Sprague Dawley rats (n=36; weight, 250±40 gram) were obtained from the medical experimental research center (MERC) of faculty of medicine, Mansoura university. Our Local Committee of Animal Care and Use approved I/R protocol (Code number: R.19.04.490). The animals had free access to food and water and were housed in individual cages with a 12-hour light-dark cycle. The animals were adapted to these conditions for at least one week before being used in the experiments and general conditions were monitored throughout the study.

2.2. Study groups

Rats were randomly divided into 6 groups G 1 (n = 6/group): control negative: in which rats received saline (Sal group= Sham-operated control) and were subjected

to surgical opening and closing in the abdominal region with gentle external manipulation of the vascular pedicle without occlusion. G2, control positive I/R rats, (I/R, n = 6/group), G3 (n = 6/group), I/R rats: in which rats were treated by NaHS, the donor of H2S, administered intraperitoneally with NaHS saline solution (NaHS as a H2S donor 5 mg/kg/day×14 days intraperitoneal), The first dose of NaHS was administered 24 hours after model was successfully established (25), G4 (n = 6/group), I/R rats in which rats were treated by the blocker of H2S, aminooxyacetic acid (AOAA) 10 mg/kg/day×14 days intraperitoneal purchased from Sigma-Aldrich (25). G5 (n = 6/group), I/R rats in which rats were treated by angiotensin converting enzyme inhibitor (ACEI) (Captopril) 50 mg/Kg/day ×14 days, given with gastric gavage (26). And, G6 (n = 6/group), I/R rats in which rats were treated by NaHS, the donor of H2S and the ATP-sensitive potassium (KATP) channel blocker (Glibenclamide) (20 mg/kg/day×14 days, given with gastric gavage) (26).

2.3. Ischemia reperfusion (I/R) surgery

After right nephrectomy through median abdominal incision to make the solitary kidney model, left renal artery was separated from renal pedicle. In I/R group, the left renal artery was blocked for 45 minutes before reperfusion. After recovery, rats were maintained with food and water ad libitum. Once surgery was done to the animals, a group of them (n = 6) was selected at random to receive sodium hydrogen sulfide (NaHS), an exogenous donor of H2S, was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Another group (n = 6) was selected at random to receive blocker of H2S aminooxyacetic acid (AOAA). Another group (n = 6) was selected at random to receive ACEI (Captopril). Another group (n = 6) was selected at random to receive NaHS and ATP-sensitive potassium (KATP) channel blocker (Glibenclamide). Arterial blood pressure was measured daily with a non-invasive pressure meter (LE 5001). Administration of drugs was stopped one day before the end of the experiment.

2.4. Collection of cardiac samples and determination of heart to body weight (HW/BW) ratios

Fifteen days after ischemia reperfusion surgery, the animals were anesthetized with pentobarbital (0.6 ml/ kg) and the blood collected by heart puncture and allowed to clot for 30 minutes. Serum was separated by centrifugation at 2500 rpm for 15 minutes and used for biochemical estimations. After that, rats were sacrificed by cervical dislocation and the chest of terminated animal was cut open quickly and the heart was immediately removed and washed thoroughly with ice-cold 0.9 % sodium chloride solution (Saline) and dried with

filter paper before weighing to measure heart weight (HW) to find out HW/BW ratio. After removal of the atria and large vessels of the base, the left ventricle was transversely sectioned into 3 parts of 3 mm thickness between apex and base; one part was fixed in 10% formaldehyde, which was left for H& E and Masson's trichrome staining. The other parts were used to assess biochemical parameters.

2.5. Histopathological examination of cardiac tissue by Hematoxylin /Eosin (H&E) and Masson trichrome staining

Portions of isolated hearts were preserved in 10% formalin for 24 hours. Specimens were cut in section of 3–5 μm in thickness to be stained with (H&E) stain in order to examine the morphological changes. Additionally, ventricular sections were stained with Masson's trichrome to examine extracellular matrix (ECM) deposition. The grading system used for assessment of parameters was [–: Absence of change; +: 0–30% area shows changes (Mild); + +: 30–60% area shows changes (Moderate); + + +: 60–100% area shows focal changes (Severe focal); + + ++: 60–100% area shows diffuse changes (Severe diffuse)] (27).

2.6. Quantitative real-time PCR for connexin-43 (CX-43) mRNA assay

RNA isolation and reverse transcription were performed. The obtained single-chain DNA was used for real-time PCR. Amplification was performed in 10 μl of SYBR Green PCR Master Mix containing 30 pmol.l-1 of each primer. For amplification of GJP43 gene and beta-actin (Housekeeping gene for normalization was not reported to be changed in I/R), gene fragments of the following primers were used to determine CX43-mRNA level: GJP43, sense 5′-TCC TTG GTG TCT CTC GCT TT-3′, antisense 5′-GAG CAG CCA TTG AAG TAG GC-3′; and beta-actin, sense 5′-TCA TCA CTA TCG GCA ATG AGC-3′, antisense 5′-GGC CAG GAT AGA GCC ACC A-3′. Sample volume was adjusted to 20 μl with deionized water. Amplification was performed as previously described (28). Cycle threshold (CT) was defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. We calculated the expression of the target gene relative to the housekeeping gene as the difference between CT values of the two genes.

2.7. Electrocardiography (ECG)

Basal ECG (lead II) of each rat was recoded for 30 seconds, fifteen days after ischemia reperfusion, by using computerized data acquisition system (BIOPAC Student Lab 3.7.6). The rats were anaesthetized by Ketamine (0.2 mg/g) and xylazine (10 mg/kg) injected intraperitoneally. The following ECG variables were analyzed: QRS duration, a corrected QT interval (QTc), and RR interval. QTc was calculated using Bazett's formula. The formula is based on

dividing QT interval by the square root of RR-interval in seconds. A corrected QT interval (QTc) that takes into account changes in heart rate is often used as a more objective parameter of depolarization and repolarization of ventricles.

$$QTc = \frac{QT}{\sqrt{\frac{RR}{f}}},$$

Where f is the normalization factor according to the basal RR duration in rats that is 150 ms. As QT interval is inversely correlated to heart rate, correction of QT interval is important to interpret QT interval independent of heart rate. The results were expressed in tables, and data were analyzed statistically.

2.8. Assay of lipid peroxidation marker malondialdhyde (MDA) and antioxidant reduced glutathione (GSH) activity in cardiac tissues

About 50 -100 mg of cardiac tissues were homogenized in 1-2 ml cold buffer (50 mM potassium phosphate, PH 7.5,1 mM EDTA) using mortar and pestle then centrifuged at 4,000 rpm for 15 minutes at 4°C. The supernatant was kept at - 20 ° C until used for analysis of oxidants and antioxidants. MDA and GSH in the supernatant of cardiac homogenates were measured using a colorimetric method according to the manufacturer's instructions (Bio-Diagnostics, Dokki, Giza, Egypt).

2.9. ELISA for determination of cardiac marker creatine kinase- MB isoenzyme (CK-MB)

It is a sandwich enzyme immunoassay for in vitro quantitative measurement of CK-MB (Cardiac marker) in *rat serum*. It was purchased from Sigma- Aldrich co. Egypt.

2.10. Arterial blood pressure measurement

Arterial blood pressure was measured by pressure meter (LE 5001). This pressure meter is a microprocessor based instrument designed to perform non-invasive blood pressure determination from rats' tails.

2.11. Determination of plasma renin activity (PRA)

PRA indicates the overall activity of renin angiotensin aldosterone system. Furthermore to generate angiotensin I (A-I), PRA measurements are valid to monitor the renin angiotensin aldosterone system during pharmacological interventions. PRA-RIA kits were used for the quantitative determination of PRA by radioimmunoessay of the generated A-I (29, 30).

2.12. Assessment of renal functions

Determination of blood urea nitrogen

Urea in the sample was hydrolyzed enzymatically into ammonia and carbon dioxide. Ammonia ions formed react with salicylate and hypochlorite (NaClO) in the presence of the catalyst nitroprusside, to form green indophenols. The intensity of the color formed is proportional to the urea concentration in the sample (31).

Determination of serum creatinine

The collected blood samples were allowed to clot for 30 minutes, serum was separated by centrifugation at 2500 rpm for 15 minutes and stored at - 20°C for analysis of serum creatinine. Serum creatinine level was estimated according to the manufacturer's instructions (Bio-Diagnostic Dokki, Giza, Egypt) (32).

2.13. Statistical data and analysis

Data are presented as means +/- standard deviation (SD) *: (P<0.05), **: (P<0.01), ***: (P<0.001) by using SPSS (SPSS, Sigma Plot Software, Inc, Chicago, IL) program statistical package for social science version 17. One-way analyses of variance (ANOVA) were used to compare each molecular variable between control negative (Saline), control positive (I/R), I/R + H_2S donor NaHS, I/R + H2S blocker aminooxyacetic acid (AOAA), I/R + ACEI (Captopril) and I/R + NaHS + K_{ATP} channel blocker (Glibenclamide) groups. When a significant interaction was detected, post-hoc t-tests were used to compare the two groups at different time points. Pearson correlation analyses were used to study the relationships between relative heart weights, mean blood pressure, plasma renin activity, serum creatinine, blood urea nitrogen and corrected QT interval in I/R group. Results were considered significant when (P <0.05).

3. Results

3.1. I/R- induced cardiac hypertrophy

As shown in table (1), heart weight and its relative weight increased significantly in I/R group (P < 0.05) in proportional to control sal group. While heart weight and its relative weight decreased significantly in (I/R + H2S donor NaHS) group in proportional to I/R group. Moreover, heart weight decreased, non-significantly in I/R + H_2S blocker aminooxyacetic acid (AOAA) group in proportional to I/R group. In addition, heart weight and its relative weight decreased significantly in (I/R + ACEI) group (P < 0.05) in proportional to I/R group. Also, heart weight and its relative weight decreased significantly (P < 0.05) in (I/R + NaHS + glibenclamide) group in proportional to I/R group.

Table (1): Body weight (g), heart weight (g) and relative heart weight (g/g body weight) (HW/BW) in different studied groups

	Control Sal.	**I/R**	**I/R + H_2S donor (NaHS)**	**I/R + H_2S blocker (AOAA)**	**I/R + ACEI**	**I/R + NaHS + K_{ATP} channel blocker Glibenclamide**
Body weight (g)	250±21.5	240±29.5	252±27.5	233±21	230±27	225±27
Heart weight (g)	0.649±0.1	1.13±0.1#	0.75±0.2#*	0.93±0.2#	0.8±0.2#*	0.7±0.1#*
HW/BW	0.002	0.004#	0.002*	0.003#*	0.003#*	0.003#*

Test used: ANOVA followed by posthoctukey for multiple comparisons. Values are expressed as means ± S.D (n = 6). #: (P <0.05) significant vs control group (G1). *: (P <0.05) significant vs I/R group (G2). Sal.: saline.

3.2. Effects of I/R, H2S, angiotensin converting enzyme inhibitor (Captopril) and K_{ATP} channel blocker (Glibenclamide) on mean blood pressure (MBP), plasma renin activity (PRA), cardiac MDA, cardiac GSH and creatine kinase- MB isoenzyme (CK-MB)

As shown in table (2), I/R led to a significant increase in MBP after 15 days of reperfusion in proportional to control group. Also, I/R led to a significant increase ($P< 0.05$) in PRA in proportional to control group. I/R led to a significant increase in MDA ($P < 0.05$) in proportional to control group. I/R led to a significant decrease in GSH ($P < 0.05$) in proportional to control group and a non-significant increase in CK-MB, in proportional to control group. In I/R + H_2S donor group, H_2S donor NaHS led to a significant decrease in MBP in proportional to I/R group. Also, NaHS led to a significant decrease ($P < 0.05$) in PRA in proportional to IR group. NaHS led to a significant decrease in MDA in proportional to I/R group. NaHS led to a significant increase ($P<0.05$) in GSH in proportional to I/R group and a non-significant decrease in CK-MB in proportional to I/R group. In I/R + H_2S blocker aminooxyacetic acid (AOAA) group, AOAA led to a non-significant decrease in MBP in proportional to I/R group. Also, AOAA led to a significant decrease in PRA in proportional to I/R group. AOAA led to a significant decrease in MDA in proportional to I/R group. AOAA led to a non-significant increase in GSH in proportional to I/R group and a non-significant increase in CK-MB in proportional to I/R group. In (I/R + ACEI captopril) group, captopril led to a significant decrease in MBP in proportional to IR group. Also, captopril led to a non-significant increase in PRA in proportional to IR group and a significant decrease in MDA in proportional to IR group. Moreover, captopril led to a significant increase in GSH in proportional to IR group and a non-significant decrease in CK-MB in proportional to IR group. In (I/R + NaHS + glibenclamide) group, there is a significant decrease in MBP ($P < 0.05$) in proportional to IR group. Also, there is a significant decrease in PRA in proportional to IR group ($P < 0.05$). Also, there is a significant decrease in MDA in proportional to IR group ($P < 0.05$). Moreover, glibenclamide led to a significant increase in GSH in proportional to IR group and a non-significant decrease in CK-MB in proportional to IR group.

Table (2): Effects of I/R, H_2S donor NaHS, H_2S blocker aminooxyacetic acid (AOAA), angiotensin converting enzyme inhibitor (Captopril) and K_{ATP} channel blocker (Glibenclamide) on mean blood pressure (MBP), plasma renin activity (PRA), cardiac MDA, cardiac GSH and creatine kinase- MB isoenzyme.

	Control Sal.	**I/R**	**I/R + H_2S donor (NaHS)**	**I/R + H_2S blocker (AOAA)**	**I/R + ACEI (Captopril)**	**I/R + NaHS + K_{ATP} channel blocker (Glibenclamide)**
MBP (mmHg)	92 ± 2.5	156 ±19.5*	133 ±17.5*#	154 ±15.5*	106 ±19.5*#	131 ±15.5*#
PRA (ng/ ml./ h.)	4.6± 1.3	29± 4.5*	17± 3.5*#	14± 4.5*#	31± 3.1*	15± 4.9*#
MDA (nmol/g. cardiac tissue)	26.7 ±3.77	59 ±4.58*	28 ±3.63#	42 ±5.83*#	31 ±3.63*#	47 ±3.41*#
GSH (nmol/g. cardiac tissue)	7.96 ± 1.3	3.55 ±1.5*	21 ±2.31*#	6 ±2.17	19 ±2.34*#	18 ±3.11*#
CK-MB (IU/L)	19.66± 3.66	22.41± 4.53	20.± 6.42	24.32± 4.83	21.5± 5.71	21.66± 2.91

Test used: ANOVA followed by posthoctukey for multiple comparisons. Values are expressed as means ± S.D. (n = 6). *: (P <0.05) significant vs control (Sal) group. #: (P <0.05) significant vs I/R group. Sal: saline.

3.3. Cardiac electrical profile "In vivo"

ECG record showed that QT interval was (108 ±17) (m. sec.) in I/R group with a significant increase in proportional to control (Sal) group (88 ±25) (m. sec.) (Figure 1 A &1 B). In (I/R + H_2S donor NaHS) group, QT was (88 ±15) (m. sec.) with a significant decrease in proportional to I/R group (Figure 1C). In (I/R + ACEI) group, QT was (84 ±17) (m. sec.) (Figure 1 E) with a significant decrease in proportional to I/R group. In (I/R + H2S donor + Glibenclamide) group, QT was (100 ±15) (m. sec.) (Figure 1F) with a non-significant decrease in proportional to I/R group. ECG record showed that there was an increase in ventrcilular action potential duration (Indicated by prolonged QTc) in I/R group, it increased significantly from (179±18) (m. sec) to (220±11) (m. sec.) (Figure 1B). While NaHS, captopril and K_{ATP} channel blocker (Glibenclamide) decreased it. QTc decreased from (220±11) (m. sec.) to (179±16) (m. sec.) (P < 0.001), (171±5) (m. sec.) (P < 0.001) and (204±34) (m. sec.) respectively (Figure 1 C, 1 E &1 F).

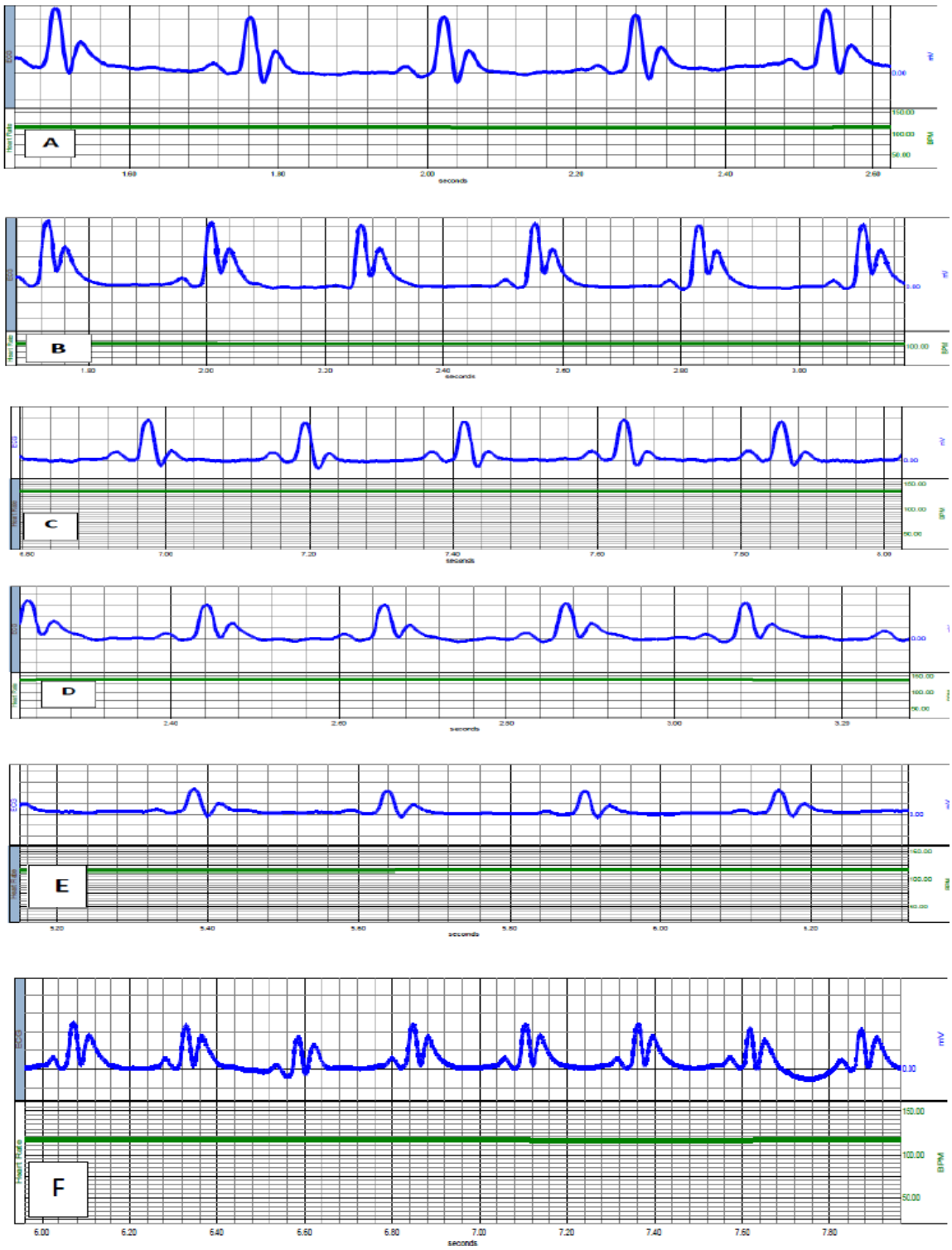

Figure (1): Representative traces of electrocardiograms. **A)** ECG of sal group. **B)** ECG of I/R group. **C)** ECG of I/R + H_2S donor group NaHS. **D)** ECG of I/R + H_2S blocker aminooxyacetic acid (AOAA). **E)** ECG of I/R + angiotensin converting enzyme inhibitor (Captopril). **F)** ECG of I/R + NaHS + K_{ATP} channel blocker (Glibenclamide).

3.4. Assessment of kidney functions

As shown in table (3), I/R increased both of serum creatinine level and blood urea nitrogen (BUN) level significantly ($P < 0.05$) in proportional to control. While H_2S donor (NaHS) decreased both of serum creatinine level and BUN level significantly ($P < 0.05$) in proportional to I/R group. In addition, H_2S blocker aminooxyacetic acid (AOAA) increased both of serum creatinine level and BUN non-significantly in proportional to I/R group. While ACEI (Captopril) decreased both of serum creatinine level and BUN significantly ($P< 0.05$) in proportional to I/R group. Also, H_2S and glibenclamide decreased both of serum creatinine level and BUN significantly ($P<0.05$) in proportional to I/R group.

Table (3): Effects of I/R, H_2S donor NaHS, H_2S blocker aminooxyacetic acid (AOAA), angiotensin converting enzyme inhibitor (Captopril) and K_{ATP} channel blocker (Glibenclamide) on serum creatinine and blood urea nitrogen (BUN) level.

	Control Sal.	**I/R**	**I/R + H_2S donor (NaHS)**	**I/R + H_2S blocker (AOAA)**	**I/R + ACEI Captopril**	**I/R + NaHS + K_{ATP} channel blocker Glibenclamide**
Serum creatinine (mg %)	0.58± 0.08	1.29 ±0.3#	0.9 ±0.13*#	1.33 ±0.17#	0.83 ±0.23*#	0.93 ±0.3*#
BUN) (mg %)	17.17± 6.1	42.76± 5.8#	33.43± 5.6*#	43.88± 5.#	21.66± 7.*#	20.56± 5.*#

Test used: ANOVA followed by posthoctukey for multiple comparisons. #: ($P < 0.05$) vs control. *: ($P < 0.05$) vs I/R. n = (6) in all groups. Sal.: saline.

3.5. Effects of I/R, H_2S, ACEI (Captopril) and K_{ATP} channel blocker (Glibenclamide) on morphology of cardiac muscle fibers

Ventricular slices from sal group (Figure 2A& Table 4) showed a normal appearance for cardiac muscle fibers, while those obtained from I/R group (Figure 2B& Table 4) showed degeneration of myocardial muscle with hypertrophy of ventricular muscle as compared to sal. group with increased cardiomyocytes width (++++) (H&E × 100). The number of abnormal fibers was reduced in the cardiac tissues obtained from I/R + H_2S donor NaHS, it showed mild hypertrophy of the ventricular muscle, cardiomyocytes width (+) (Figure 2C& Table 4). I/R + ACEI (Captopril) showed mild hypertrophy of the ventricular muscle, myocytes width (+) (Figure 2E& Table 4). Also, I/R + NaHS + K_{ATP} channel blocker (Glibenclamide) group showed mild hypertrophy of the ventricular muscle, cardiomyocytes width (+) (Figure 2F& Table 4).

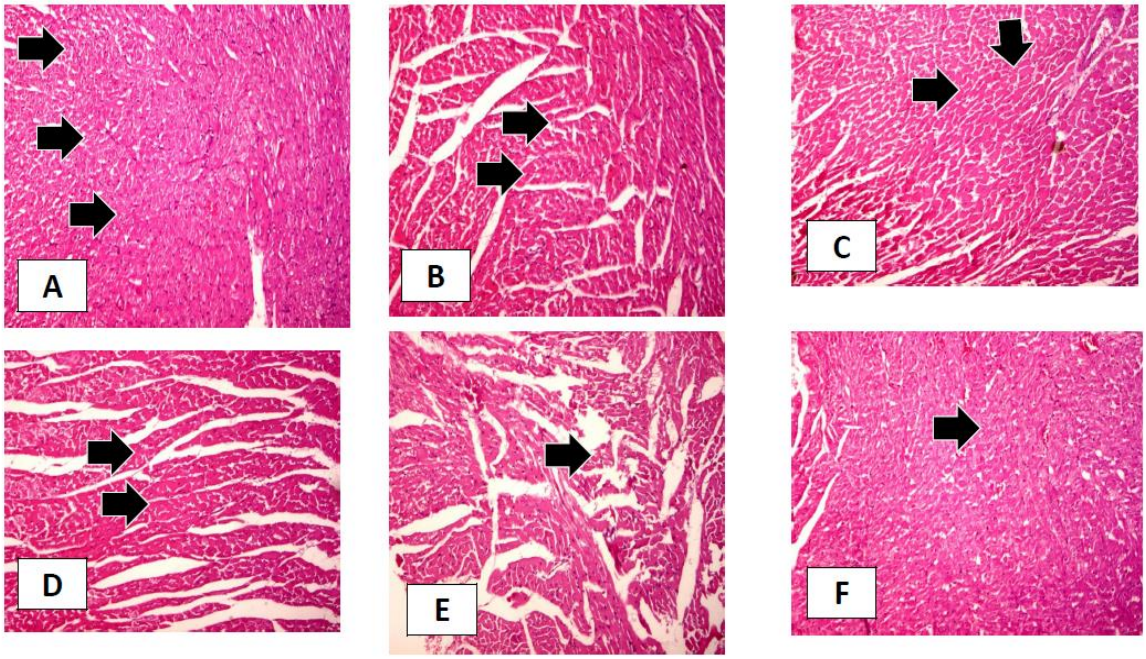

Figure (2): graphical representation of cardiomyocytes obtained using hematoxylin and eosin stained ventricular slices **A)** H&E staining of sal group (n=6): showed a normal appearance for cardiac muscle fibers **B)** H&E staining of I/R group (n=6): hypertrophy and increased cardiomyocytes width (Arrow head) **C)** H&E staining of I/R + H_2S (n=6): decreased cardiomyocytes width (Arrow head) **D)** H&E staining of I/R +H2S blocker group aminooxyacetic acid (AOAA) (n=6): decreased cardiomyocytes width **E)** H&E staining of I/R + angiotensin converting enzyme inhibitor (Captopril) group (n=6): decreased cardiomyocytes width **F)** H&E staining of I/R + NaHS + Glibenclamide (n=6): decreased cardiomyocytes width (Arrow head).

3.6. H_2S donor (NaHS), ACEI (Captopril) and K_{ATP} channel blocker (Glibenclamide) administration attenuated I/R-induced heart fibrosis

Ventricular slices from sal group (Figure 3A& Table 4) showed a normal appearance for cardiac muscle fibers with no evidence of fibrosis, while those obtained from I/R group (Figure 3B & Table 4) showed marked fibrosis, fibrosis (+++), between the hypertrophied fibers (Masson trichrome × 100). The number of abnormal fibers was significantly reduced in cardiac tissues obtained from I/R + NaHS, ACEI (Captopril) and glibenclamide groups which showed mild fibrosis (Figure 3C, 3E & 3F & Table 4).

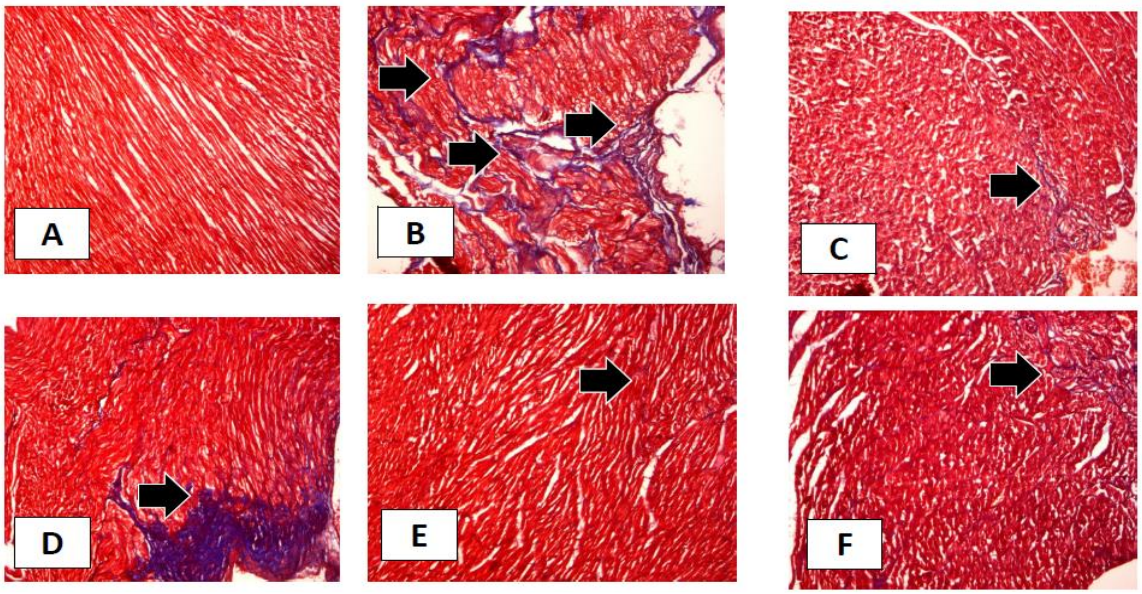

Figure (3): graphical representation of ventricular slices stained with Masson trichrome stain. **A)** Masson trichrome staining of sal. group (n=6): showed a normal appearance for cardiac muscle fibers with no evidence of fibrosis (Masson trichrome × 100) **B)** Masson trichrome staining of I/R group (n=6): Masson's trichrome stain shows marked fibrous connective tissue proliferation, fibrosis (+++), between the hypertrophied fibers with increased cardiomyocytes width (Arrow head) (Masson trichrome × 100) **C)** Masson trichrome staining of I/R + H_2S (n=6): Masson's trichrome stain showed mild degree of fibrosis, fibrosis (+), and decreased cardiomyocytes width (Arrow head) (Masson trichrome × 100) **D)** Masson trichrome staining of I/R+ H_2S blocker aminooxyacetic acid (AOAA) (n=6): decreased cardiomyocytes width and fibrosis (++) (Masson trichrome × 100) **E)** Masson trichrome staining of I/R + angiotensin converting enzyme inhibitor (Captopril) group (n=6): decreased cardiomyocytes width (Arrow head) and fibrosis (+) **F)** Masson trichrome staining of I/R + NaHS + Glibenclamide (n=6): Masson's trichrome stain shows mild fibrosis, fibrosis (+), (Arrow head) and decreased cardiomyocytes width (Masson trichrome × 100).

Table (4): Semi quantitative scoring of myocardial lesions within different studied groups

	Control Sal.	**I/R**	**I/R + H_2S donor (NaHS)**	**I/R + H_2S blocker (AOAA)**	**I/R + ACEI Captopril**	**I/R + NaHS + K_{ATP} channel blocker Glibenclamide**
Normal fibers histology	+++	+	++	++	+	+
Myocardial degeneration	-	+++	+	++	+	+
Myolysis	-	+++	+	++	+	+
Fibrosis	-	+++	+	++	+	+
Myocyte hypertrophy	-	++++	+	++	+	+

(-) indicates no detectable lesions; (+) indicates mild lesions; (++) indicates moderate lesions; (+++) indicates severe focal lesions; (++++) indicates severe diffuse lesions.

3.7. Effects of I/R, I/R +H2S donor (NaHS), I/R + angiotensin converting enzyme inhibitor (Captopril) and I/R + NaHS + K_{ATP} channel blocker (Glibenclamide) on CX- 43 expression in cardiac tissues

As shown in figure (4), cardiac tissues from sal group showed a normal expression of CX- 43, while those obtained from I/R group showed a significant decrease in its expression in proportional to control sal group. Moreover, the expression of CX- 43 increased significantly in the cardiac tissues obtained from H_2S donor (NaHS) + I/R in proportional to I/R group. In addition, angiotensin converting enzyme inhibitor (Captopril) + I/R, as well as, (NaHS + K_{ATP} channel blocker Glibenclamide + I/R) increased its expression significantly in proportional to I/R group.

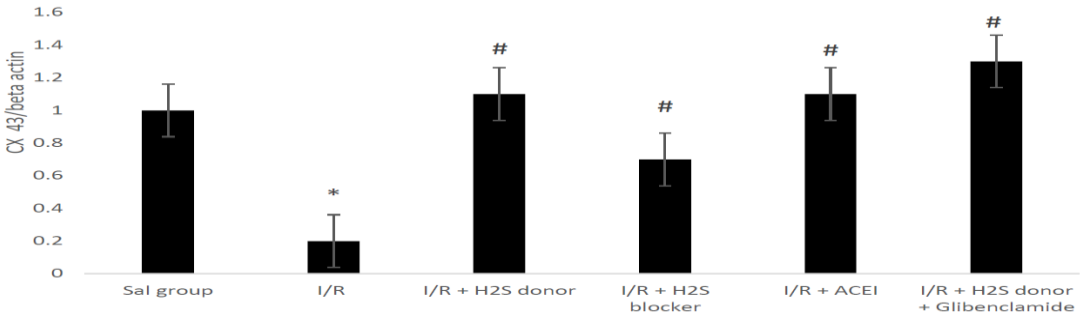

Figure (4): Expression levels of CX-43 in the myocardia of all studied groups. Data are expressed as mean ± SD, test used: One way ANOVA, followed by post-hoc tukey. (Normalized by beta actin, products of RT-PCR for CX-43). P: significance <0.05. *: significant as compared with sal. group. #: significant as compared with I/R group. Sal.: saline.

3.8. Correlations between relative heart weights (HW/BW), mean blood pressure (MBP), plasma renin activity (PRA), serum creatinine, blood urea nitrogen (BUN) and QTc in I/R group

I/R showed significant positive correlations between HW/BW, MBP, PRA, serum creatinine, BUN and corrected QT (QTc) (Table 5).

Table (5): Correlations between relative heart weights (HW/BW), mean blood pressure (MBP), plasma renin activity (PRA), serum creatinine, blood urea nitrogen (BUN) and QTc in I/R group

Parameters		HW/BW	MBP	PRA	Creatinine	BUN	QTc
HW/BW	r		0.710	0.624	0.849	0.859	0.901
	p		0.000	0.001	0.000	0.000	0.000
MBP	r			0.82	0.706	0.516	0.630
	p			0.000	0.000	0.010	0.001
PRA	r				0.381	0.998	0.375
	p				0.066	0.000	0.071
Serum creatinine	r					0.997	0.958
	p					0.000	0.000
BUN	r						0.299
	p						0.155

HW/BW= heart weight/ body weight, MBP = mean blood pressure, PRA = plasma renin activity, BUN= blood urea nitrogen, QTc =corrected QT, QTc indicates QT interval divided by the square root of RR-interval in seconds. r: Pearson's correlation coefficient. P: probability. ($P < 0.05$) is considered significant. ($P < 0.001$) is considered highly significant.

4. Discussion

The correlation of cardiac dysrhythmias symptomatology with changes in gap junctions in cardiac muscle had formed the bases for IR-induced cardiac hypertrophy and arrhythmias. Since then, the general hypothesis of therapeutic effect of hydrogen sulfide generation in cardiac diseases was expanded to arrhythmias and cardiac hypertrophy where it ameliorates CX-43. The main findings in the present study are: (a) I/R arrhythmias are associated with decreased expression of CX-43 protein (b) I/R increased duration of QTc interval while H2S donor (NaHS) normalized it (c) the cardioprotective effect of H2S against arrhythmias, depends on both of KATP channels opening and amelioration of CX-43 expression

To test the hypothesis that the primary kidney acute I/R injury can culminate with a cardiorenal syndrome with several degrees of cardiac structural and electrical dysfunctions (33, 34), we examined morphological, molecular and functional parameters that would reflect an increase in cardiac mass after I/R. Heart weight/body weight (HW/BW) ratios are useful indirect indicators of cardiac hypertrophy, and these parameters increased after 15 days of reperfusion. Other parameters related to cardiac hypertrophy were also measured, including: cardiomyocyte width, CX-43 expression, cardiac MDA, cardiac GSH and CK-MB. The data strongly suggest cardiac hypertrophy developed by 15 days of renal reperfusion. Cardiac electrical profiles are also in line with the morpho-structural alterations described above i.e. longer QTc.

In the present study, a significant elevation of heart weight in I/R group, indicating that I/R resulted in cardiac hypertrophy. HW/BW ratio increased significantly in I/R group. In the present study, I/R is able to modulate cardiac tissue structure, differently regulating collagen content in distinct sections of the heart tissue in rats subject to renal I/R. Myocardial hypertrophy could be explained by the inflammatory mediators which migrate through blood stream to affect heart and induce oxidative stress in cardiac tissue and vessels which culminate with cardiomyocytes hypertrophy and elevated blood pressure. This happened after an intense inflammatory response in the left kidney reperfusion with its impact in cardiac and vascular tissues. This was confirmed by increasing the levels of cardiac MDA, CK-MB, arterial blood pressure and decreasing cardiac GSH.

In the present study, NaHS reduced cardiac hypertrophy. In agreement with the previous findings that H2S could improve cardiac functions and reduce myocardial hypertrophy by reducing NOX4 expression and ROS production in mitochondria (33, 34, 35, and 36). H2S has been reported as a

strong antioxidant and widely proposed to protect the cardiac system through its antioxidant role. The robust antioxidant actions of H2S are associated with direct scavenging of ROS and increased expressions and functions of antioxidant enzymes. In addition, H2S therapy mitigates pathological left ventricular remodeling and reduces myocardial hypertrophy, oxidative stress and apoptosis (37&38). Endogenous H2S in rats could maintain basal arterial blood pressure balance, attenuate elevated arterial blood pressure, and lessen vascular structural remodeling in hypertensive rats (39, 40, 41, and 42).

Here, NaHS led to a highly significant decrease in the duration of ventricular action potential indicated by QTc. This is in agreement with the previous finding that reperfusion with NaHS after ischemia attenuated arrhythmias in the isolated Langendorff-perfused heart and improved cardiac function during I/R (13). Also, preconditioning with 100 μM NaHS attenuated arrhythmias in the isolated heart during I/R and these effects may be mediated by protein kinase C and sarcolemmal KATP channels (13). These effects were blocked by KATP channel blocker (Glibenclamide), indicating that the cardioprotective effect of H2S against arrhythmias during reperfusion, at least partially, depends on opening of KATP channels**.**

Intercellular CX-43 channels are essential for direct communication between cardiomyocytes, ensuring action potential and molecular signal propagation resulting in synchronized heart function. Gap junctional intercellular communication (GJIC) is important in physiological processes such as homeostasis, growth regulation, coordination of cellular responses to stimuli, and apoptosis. In addition, cardiac gap junctions play a major role in impulse propagation. In contrast, aberrant connexin expression and defective GJIC are involved in many diseases such as cardiac arrhythmia and neurodegeneration. In the present study, myocardial CX-43 expression was lesser in I/R versus control sal rats. This deterioration of CX-43 level affects the intercellular communication which may be behind the prolongation of the QRS and QTc. Here, H2S ameliorated the expression of CX-43 in cardiac tissue, which indicated that endogenous H2S may play an important role in regulating heart functions and arrhythmia. This is in agreement with the previous finding that the lowered H2S production may cause overstimulation of the β-adrenergic function which was closely linked with the incidence of ventricular arrhythmias (42, 43, and 44). Exogenous application of H2S negatively modulated β -adrenergic function by inhibiting adenylyl cyclase activity and, finally, protected heart against cardiac arrhythmias.

The longer QRS and QT seen after 15 days of reperfusion are consistent with previous studies which examined the association of electrocardiographic changes with cardiac hypertrophy (45, 46). The longer QT interval has special relevance that, together with the "in vivo" electrophysiology alterations, is considered a predictor of arrhythmias (47, 48). The most consistent electrical abnormality that has been described in association with cardiac hypertrophy is extending action potential duration. In rats, among the different K^+ currents, the transient outward current, a major repolarizing current, is the major determinant of action potential duration (49). In rats, previous studies demonstrated specific alterations in the transient outward current (48). In experimental cardiac hypertrophy, a number of electrophysiological abnormalities have been reported, including myocardial areas of both short and long action potential duration. Such heterogeneous repolarization occurs mainly in fibrotic areas (50, 51).

In the present study, converting enzyme inhibition normalized QTc interval in renal I/R-induced arrhythmia. This could be explained by its inhibitory effect on cardiac angiotensin-converting enzyme activity. In agreement with the previous finding that ACEI reverses left ventricle hypertrophy by reducing the load and by a direct trophic effect on cardiomyocyte proliferation (52,53, 54 &55), through its lowering effect on angiotensin II formation and its inhibition of bradykinin degradation.

Here, in the present study, the histological findings showed marked myocardial necrosis associated with nuclear lysis and marked increase in cardiomyocyte width, together with large patches of strong fibrous tissue reactivity between the hypertrophied fibers in the heart sections of I/R rats. These alterations could be highly attributed to I/R-induced oxidative stress and inflammation that leads to DNA damage and apoptosis (56). Cardiac fibrosis occurs as a consequence of inflammation and cell injury as confirmed by various studies (57&58). However, in the present study, NaHS improved cardiac architecture, and reduced cardiac fibrosis. So, H2S is able to protect the heart against I/R -induced cellular injury and fibrosis probably through its ability to reduce I/R- induced inflammation and apoptosis together with improving CX-43 expression.

In addition, since the preservation of cardiac-renal axis integrity is vital in all physiological scenarios, it could be hypothesized that prevention or attenuation of renal lesions after I/R injury contributes to prevent I/R-induced cardiac hypertrophy. This hypothesis is supported by several studies showing that knockout mice seem to be protected against renal metabolic and cardiovascular injuries (59). Also, in the

present study, NaHS decreased serum creatinine and BUN level significantly ($P<0.05$) in proportional to I/R group. We assessed serum creatinine and BUN level to answer the question of whether there is a correlation between renoprotective effect of H2S and its antiarrhythmic effect? Hydrogen sulfide protects against cardiac hypertrophy and arrhythmia in rats model of renal ischemia/reperfusion via its cardioprotective, as well as, its renoprotective effect.

5. Conclusion

I/R involves the damage of the myocardium through several mechanisms, namely fibrosis and inflammatory induced hypertrophy of cardiomyocytes. The decreased CX-43 expression is a key for the development of arrhythmias in renal I/R -induced cardiac hypertrophy. Hydrogen sulfide protects against renal I/R -induced cardiac hypertrophy and arrhythmia through amelioration of CX-43 expression and opening of K_{ATP} channels. Based on these findings, H_2S replacement therapy may be a crucial cardioprotective and antiarrhythmic intervention for those patients whose plasma H_2S level is reduced. Clinical trials may also be considered to validate the results in humans, following further pre-clinical studies.

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Author Contributions

Mohamed Adel: the idea, the induction of I/R model, ECG record, biochemical analysis, data analysis and paper writing. **Dalia Shaheen**: biochemical analysis and paper writing **Rehan Monir**: biochemical analysis and paper writing. **Ahmed El Adl**: histopathological examination

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