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Abscisic Acid Can Protect the Kidney Against Ischemia/Reperfusion Injury Via Antiapoptotic Activity, Downregulation of NOX-4 and Upregulation of Connexin-43.

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Abstract

Renal ischemia/reperfusion injury (IRI) is a common clinical problem involving oxidative stress and gap junction protein defects. Abscisic acid (ABS), a phytohormone regulating physiological functions in plants against various stresses, has been identified in mammalian tissues, too. We aimed to assess the role of synthetic ABS in management of renal IRI in comparison or combined with Metformin and whether it can modulate the gap-junction protein; CX43, and the oxidative stress related factors; NOX4, MDA and GSH; and apoptosis markers; BAX, BCL2 and P53. Rats were assigned to five groups ;1, Saline: 2, I/R+Sal., 3 I/R+Metformin, 4, I/R +ABS and 5, I/R + Metformin + ABS. Serum creatinine and K^+, mRNA for CX43, NOX4 and P53, western blotting for CX43 and P53 in renal tissue, immunohistochmistry for renal BAX and BCL2 with H&E and PAS staining were performed. Administration of Metformin, ABS or their combination led to a significant attenuation of the I/R induced renal injury with significant decrease in serum creatinine, K^+ levels, and in the expression of P53, BAX, NOX-4 and caused a significant increase in CX43 and BCL2 expressions with attenuation of renal histopathological changes e.g. glomerular atrophy, tubular necrosis, tubular dilatation, sloughing of tubular epithelium, loss of brush border, cast formation and the inflammatory infiltration. Moreover, the combined therapy of Metformin and ABS produced more significant improvement. Our results approved a possible protective role of ABS especially when combined with Metformin in I/R renal injury.

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INTRODUCTION

Renal Ischemia/reperfusion injury (IRI) involves reduction of the renal arterial supply with subsequent tissue hypoxia, followed by recovery of blood supply and reoxygenation. IRI aggravates the kidney injury through starting inflammatory processes involving reactive oxygen species (ROS), chemokines and cytokines **[1,2].** Renal IRI causes acute kidney injury; characterized by rapidly deteriorating renal dysfunction and increased death rates **[3, 4].** NAD(P)H oxidase 4 (NOX-4) is the main form of renal NOX family that is involved in renal ROS production associated with diabetic nephropathy and chronic kidney disease and IRI **[5].** Moreover, several studies suggest that the transcription factor P-53 can aggravate apoptosis through interaction with BCL2 family **[6].** P-53 was proved to be involved in renal IRI **[7].** Gap junctions (GJ) are essential for transmitting signals between cells. Previous studies reported the presence of GJs in renal vascular endothelium and smooth muscle cells. GJs are altered in renal IRI, in particular, gap junction Connexin 43 (Cx43) which is essential for the integrity of endothelium **[8].**

Previous studies have reported the protective effects of different drugs against IRI, e.g. doxycycline by decreasing the level inflammatory mediators **[9],** leptin by downregulating the level of tumor necrosis factor alpha (TNF-α) and upregulating the level of nitrite **[10]**, levosimendan via antioxidant activity and nitric oxide-linked mechanisms **[11]**. Moreover, Metformin, a biguanide drug, is known to be an insulin-sensitizer, commonly prescribed for diabetic patients. Metformin was found to attenuate experimental IRI in many studies **[12, 13].**

Abscisic acid (ABS) is a phytohormone regulating fundamental physiological activities in plants. ABS was identified in mammalian plasma and several cell types such as human granulocytes and pancreatic β cells. Synthetic ABS could suppress thioacetamide-induced liver fibrosis in mice through anti-oxidant, anti-inflammatory and anti-apoptotic activities **[14].** Moreover, treatment with ABS exerted a significant improvement in cardiac markers and pro-inflammatory cytokines in a model of diabetic cardiomyopathy with improvement of histopathological changes through antiapoptotic activity as well as through upregulation of myocardial Cx43 mRNA **[15].**

The aim of the present study

We aimed to assess the possible protective role of ABS in management of renal IRI in comparison or combined with Metformin and whether it can modulate the gap-junction protein; CX43, and the oxidative stress-related factors; NOX4, Malonaldialdehyde (MDA) and reduced glutathione (GSH) and apoptosis markers; BAX, BCL2 and P53.

1. Materials and Methods

2.1. Experimental Animals

Thirty Sprague Dawley rats were used in the present study. Free access to water and food was available to the animals. They were kept in cages, through the normal day-night cycle, with firm conditions of temperature, humidity and pathogen free circumstances. The rats were kept in these conditions for one week to acclimatize before the start of the experiment.

2.2. Study Groups

The animals were randomly divided into five groups (n = 6/group):1, Saline control, 2, I/R control (I/R+ Saline) given saline, and then ischemia/reperfusion was performed at the end of fourth week, 3, I/R +Metformin group: same as group 2 but the rats received additional Metformin hydrochloride (Cidophage, CID, Egypt, 300 mg/kg/day) **[3]** by oral gavage daily for four weeks before induction of I/R, 4, I/R +ABS group: same as group 2, but the rats received additional ABS (1mg/kg/day) **[16]** by oral gavage daily for four weeks before induction of I/R, 5, I/R + Metformin + ABS group: same as group 2, but the rats received additional oral Metformin (300 mg/kg/day) and oral ABS (1mg/kg/day) daily for four weeks respectively, before induction of the I/R. ABS was obtained from Sigma-Aldrich (A1049) and was freshly prepared by dissolving in dimethyl sulfoxide. For all groups, right nephrectomy was performed to make the single kidney model. Serum and renal tissue samples were obtained seven days (168 hours) after surgery **[17].**

2.3. I/R surgery

After the right nephrectomy, the left renal artery was dissected from the renal pedicle. In IR group, the left renal artery was occluded for 45 minutes prior to reperfusion **[3].**

2.4. Collection of renal samples

After reperfusion, the rats were anesthetized by pentobarbital [0.6 ml/ kg] and the blood was obtained through heart puncture and left to clot for half an hour. Sera were separated through centrifugation at 2500 rpm for 15 min and utilized for biochemical assessment. After that, the animals were terminated by cervical dislocation then the abdomen was cut open quickly and the kidneys were immediately removed and washed thoroughly with ice-cold saline and dried with filter paper.

2.5. Evaluation of kidney functions (serum creatinine and serum K^+ assessment)

The collected blood samples were left to clot at room temperature, then centrifugation was performed (Hettich universal 32A, Germany) and subsequently separation of serum. Storage of serum samples was done in aliquots at -20ºC till analysis of serum creatinine, and K^+ level. The serum creatinine level was estimated according to the manufacturer's instructions (Bio-Diagnostic Dokki, Giza, Egypt), while serum K^+ was measured using Erba CHEM-7 device (ERBA Diagnostics, India) **[18].**

2.6. Measurement of oxidative stress biomarkers

An average of 100 mg of renal tissues was homogenized in 2 ml cold buffer (50 mM potassium phosphate, PH 7.5,1 mM EDTA) utilizing mortar and pestle then centrifugation for 15 min, through 4,000 rpm at 4°C was done. The supernatant was preserved at - 20 ° C till being used for analysis of oxidants and antioxidant markers. Malondialdhyde (MDA) and reduced glutathione (GSH) level in the supernatant of renal homogenates was estimated using a colorimetric method following the manufacturer's instructions (Bio-Diagnostics, Dokki, Giza, Egypt), and by Erba CHEM-7 device **[19, 20].**

2.7. Histopathological examination of kidney tissue

A part from each kidney was fixed in 10% formaldehyde then kept in paraffin **[21]** for histopathological investigation. Next, the paraffin

blocks were dissected into 7-10 µm thick sections. These sections were subjected to staining by hematoxylin and eosin (H&E) for assessment of histopathological alteration, and by PAS stain for glycogen detection. Photomicrography was done using Olympus Microscope with SC100 camera.

2.8 Histopathological score

Histopathological score for the kidney changes in IR model was calculated through EGTI (Endothelial, glomerular, tubular and interstitial) semiquantitative histopathological scoring system **[22]** using random non-overlapping fields from the rats of each group.

2.9. Immunohistochemical study of apoptotic marker BAX and anti-apoptotic marker BCl2 in kidney tissues

Immunohistochemistry was performed on 3µm thick sections, according to the method described by **Elsayed et al**. **[23].** Concisely, deparaffinization of the sections was completed, then treatment with H2O2 (0.3 %)/ methanol at room temperature for 10 min to stop the activity of peroxidase. Antigen retrieval has been achieved by heating the kidney sections for 10 minutes in 10 mM citrate buffer at 95-100C (PH 6) then allowed to cool for 60 min at room temperature. Renal sections were kept with the primary rabbit polyclonal antibody for BAX as an apoptotic marker and primary mouse monoclonal for Bcl-2 as an anti-apoptotic marker (Ab-clonal; A12009 and Santa cruz; sc-7382, respectively) overnight at 4ºC (1:100 and 1: 50 dilutions, respectively. Cosequently, the slides were kept in Diaminobenzidine (DAB) to assess peroxidase action. The slides were kept overnight in phosphate buffered saline (PBS) at 4ºC. The slides were incubated with mouse/rabbit polydetector plus (BSB 0257, Bio SB) for half an hour, then they were washed by PBS to assess the binding of antibody. Lastly, adding DAB to the sections for four minutes then counterstaining by hematoxylin was done. For the negative control, PBS was used to replace the primary antibody. The sections were then washed, dehydrated and examined. Dark brown areas in cytoplasm or nucleus reveal positive staining while the background is blue **[24].**

2.10 Morphometric analysis of apoptotic marker BAX and anti-apoptotic marker BCl2 in kidney tissues

The immunohistochemical results were evaluated by quantifying the percentage of the BAX and Bcl2 positive area (in x400) (dark brown areas) in non-overlapping fields, using ImageJ software version (1.52a) and Fiji Image j software **[25].**

2.11. Detection of mRNA expression of Connexin-43, NOX-4 and P53

Tissue samples were homogenized by liquid nitrogen, then RNA was extracted by QIAzol reagent (Qiagen, Germany), following the manufacturer's protocol. RNA concentration was estimated by Thermo Scientific NanoDrop 2000 (USA). Reverse transcription of 1ug of RNA was performed utilizing Bioline cDNA synthesis kit (Bioline, USA). cDNA templates were amplified by a RT PCR device (Pikoreal 96, ThermoScientific) through the method described in the previous work of **Elsayed et al. [23]**. **Table (1)** provides the gene sequences of the applied primer pairs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was applied as a house keeping gene. Primers sets were prepared by Vivantis (Vivantis Technologies, Malaysia). Relative gene expression levels were calculated according to the method

described by **Livak and Schmittgen [26],** followed by running of products of PCR on agarose gels (3%), visualization, and consequently, photographing of the gels by Bio-Rad gel documentation system (Bio-Rad, USA) **(Figure 5).**

2.12. Detection of Connexin-43 and NOX-4 protein by Western blotting

The processing of the proteins followed the methods, described by **Elsayed et al. [23].** The nitrocellulose membranes were kept at 4ºC overnight with the primary rabbit polyclonal antibody for Phospho-Connexin 43 (Ser367), mouse monoclonal antibodies for NOX-4, and β-actin (*PA5-36839* Invitrogen, sc-518092 and sc-47778, Santa Cruz, USA; respectively) in blocking buffer. Secondary anti-mouse antibody (sc-516102, Santa Cruz) or anti-rabbit antibody (sc-2357, Santa Cruz) solutions was added, at room temperature, for 60 min. The chemiluminescence activity was photographed by a CCD camera. Image analysis was performed to assess the bands of the blotted proteins versus β-actin (the control protein) after protein normalization.

2.13. Statistical Analysis

Normally distributed data were expressed in the form of mean ± standard error (SE) and One-Way ANOVA test with post-hoc analysis were utilized to compare normally distributed data. The data that aren`t normally distributed were tabulated as median and interquartile range) and Kruskal-Wallis H test was used to compare them using SPSS version 17. Data were considered statistically significant when p value ≤ 0.050.

2.14. Ethical statement:

All experimental procedures were approved by institutional review board (IRB) at Faculty of Medicine, Mansoura university (R.20.08.991).

3. Results

3.1. Effect of Metformin and ABS on renal function in response to I/R

I/R led to a significant elevation in serum creatinine in proportional to control. While it decreased, significantly, in IR+Met, IR+ABS and IR+Met+ABS groups when compared to I/R group. There was no significant difference among IR+Met, IR+ABS and IR+Met+ABS groups. However, IR+Met, IR+ABS and IR+Met+ABS groups showed no significant difference from the negative control. In addition, I/R caused a significant increase in serum K^+ level, proportional to the control. While it decreased, insignificantly, in IR+Met, IR+ABS and IR+Met+ABS groups as proportional to I/R group with insignificant difference from the control group. There was no significant difference among IR+Met, IR+ABS and IR+Met+ABS groups **(Table 2).**

3.2. Effect of Metformin and ABS on renal oxidative stress markers in response to I/R

Moreover, I/R led to a significant elevation in Renal MDA in proportional to control. While it decreased, significantly, in IR+Met, IR+ABS and IR+Met+ABS groups when compared to I/R group. In addition, there was no significant difference among IR+Met, IR+ABS and IR+Met+ABS groups. However, IR+Met, IR+ABS and IR+Met+ABS groups showed no significant difference from the negative control.

Moreover, I/R led to a significant reduction in Renal GSH in proportional to control. While it increased, significantly, in IR+Met, IR+ABS and IR+Met+ABS groups when compared to I/R group. In addition, there was no significant difference among IR+Met, IR+ABS and IR+Met+ABS groups. However, IR+Met, IR+ABS

and IR+Met+ABS groups showed no significant difference from the negative control **(Table 2).**

Table (1): The sequence of rat primers applied in qRT-PCR analysis

Gene	Sequence	Product size
Connexin-43	Forward primer: ATGGCTGCTCCTCACCAACG Reverse primer: GGTCGTTGGTCCACGATGGC	277 bp
NOX-4	Forward primer: TGTTGGGCCTAGGATTGTGT Reverse primer: CTTCTGTGATCCGCGAAGGT	119 bp
Tumor protein p53 (Tp53)	Forward primer: CCCCTGAAGACTGGATAACTGTC Reverse primer: ATTAGGTGACCCTGTCGCTG	146 bp
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward primer: AGGTCGGTGTGAACGGATTTG Reverse primer: TGTAGACCATGTAGTTGAGGTCA	123 bp

Table (2): effect of I/R, I/R +Metformin and I/R + ABS and I/R + ABS +Metformin on serum creatinine level, serum K^+ level, malondialdhyde (MDA) and reduced glutathione (GSH)

	Sal. group	I/R group	I/R + Metformin group	I/R + ABS group	I/R + ABS +Metformin group	F value	P value
Creatinine (mg %)	0.56 ± 0.06 A	1.21 ± 0.29 B	0.77 ± 0.14 A	0.66 ± 0.1 A	0.5 ± 0.13 A	17.648	**<0.0005**
K^+ (mmol/L.)	4.6±0.1 A	5.8±0.9 B	4.7±0.7 AB	5.1±1.1 AB	4.7±0.1 AB	2.962	**0.039**
MDA (nmol/g. renal tissue)	15.7±0.47 A	27.6±6.4 B	19.9±1.6 A	18.5±1.4 A	16.9±4.8 A	9.566	**<0.0005**
GSH (nmol/g. renal tissue)	119.83± 17.06 A	66.83±13.6 B	100.2± 9.6 A	112.1±11.6 A	103.71± 29.8 A	7.772	**<0.0005**

Values are expressed as mean ± S.D. (n = 6).

3.3. Effects of Metformin and ABS on renal histopathological changes induced by IR

H & E of the negative control showed normal kidney cortical architecture; normal glomeruli, proximal and distal convoluted tubules, while IR group, showed distortion of kidney architecture and massive inflammatory cellular infiltration, atrophied segmented glomeruli with enlarged Bowman`s spaces and tubular necrosis, vacuolation and dilatation, with sloughing of tubular epithelial cells and cast formation. Metformin only and abscisic only groups showed moderate inflammation with mild distortion of kidney tissue. However, combined treatment showed mild inflammatory reaction with mild affection of renal tubules **(Figure 1).** PAS staining showed normal tubular epithelial brush border in the negative control group while the IR group showed loss of tubular epithelial brush border and the basement membranes of both the Bowman`s capsules and renal tubules appear interrupted at some areas. Metformin only and abscisic only groups showed moderate affection of the tubular epithelial brush border with partial restoration of basement membranes. Combined treatment showed mild affection of tubular epithelial brush

border with strong restoration of basement membranes **(Figure 2).**

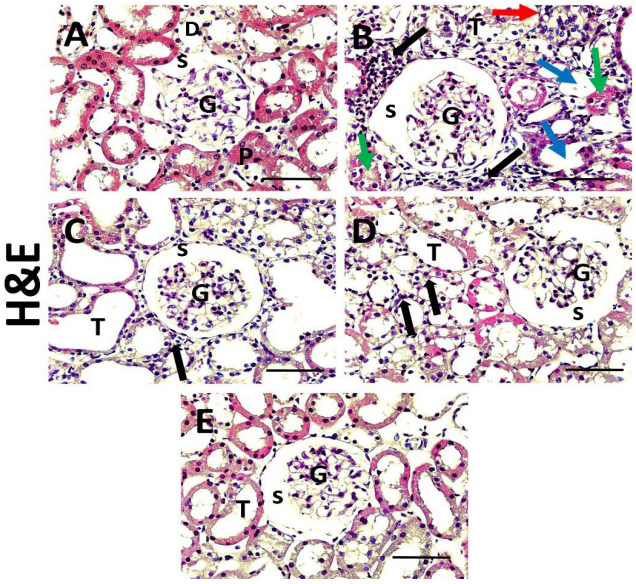

Figure (1): Representative photographs of kidney histopathology; haematoxylin & eosin (×400) (A-E) in kidney tissues of negative control, IR control, IR+Met, IR+ABS and IR+Met+ABS group (A-E; respectively). Scale bar = 50um. H & E of the negative control group show normal kidney cortical architecture (A); normal glomerular size (G), and Bowman`s space (S), normal proximal convoluted tubules (P) and normal distal convoluted tubules (D), while IR group (B), showed distortion of kidney architecture, atrophied segmented glomeruli (G), wider Bowman`s spaces (S), with the tubules showing shedding of tubular epithelium (T), dilated tubules (blue arrows), vacuolated tubular cytoplasm (red arrow) inflammatory cellular infiltration (black arrows) and casts (green arrows). Moreover, both IR+Met and IR+ABS show moderate inflammation (arrows) with moderate atrophy of glomeruli (G) and moderate dilatation of Bowman`s space (S) with mild shedding of tubular epithelium (T). However, IR+Met+ABS showed average size glomeruli (G), average Bowman`s space (S), mild inflammatory reaction (black arrows) with mild affection of renal tubules (T).

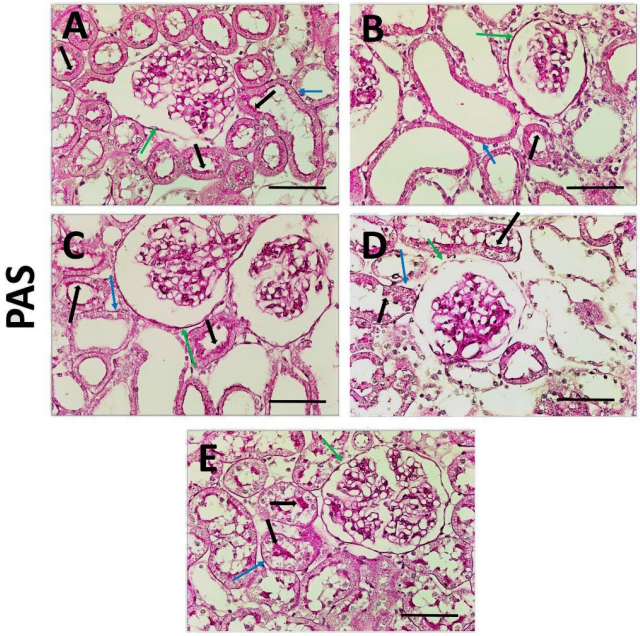

Figure (2): Representative photographs of kidney histopathology with PAS staining for glycogen and mucopolysaccharides (×400) magnification (A-E) in kidney tissues of negative control, IR, IR+Met, IR+ABS and IR+Met+ABS group (A-E; respectively). Magnification, ×400. Scale bar = 50um. Negative control group shows normal tubular epithelial brush border (Arrows). IR group shows loss of tubular epithelial brush border (black arrows), weak PAS-positive reaction in the capsular basement membrane (green arrows) and tubular basement membrane (blue arrows). IR+Met only and IR+ABS only groups showed moderate affection of the tubular epithelial brush border (black arrows) with partial restoration of capsular basement membrane (green arrows) and tubular basement membrane (Blue arrows). However, IR+Met+ABS group showed mild affection of tubular epithelial brush border with strong restoration of capsular basement membrane (green arrows) and tubular basement membrane (Blue arrows).

3.4 Results of EGTI histopathological score:

The median for the EGTI histopathological score was significantly higher in IR group (10) when compared to the other groups, with insignificant decrease among IR+Met group (7), IR+ABS group (7.5) and a significant decrease in IR+Met+ABS group (4) when compared to IR group. Moreover, IR+Met and IR+ABS groups were still significantly higher than the negative group (0). Meanwhile, IR+Met+ABS group showed insignificant difference from the negative group **(Table 3).**

3.5. Metformin and ABS attenuated the level of expression of apoptotic marker BAX and increased the level of expression of anti-apoptotic BCl2 in kidney tissues of I/R rats

Weak BAX +ve expression was seen in the negative control groups. Kidneys of IR group showed an intense expression for BAX in the renal tubular epithelium as a pro-apoptotic marker. In contrast, immunoreactivity was reversed in IR+Met and IR+ABS groups showing weak BAX expression, with weaker expression in IR+Met+ABS group **(Figure 3).** However, Bcl2 immunoreactivity showed an inverted manner for expression as compared to BAX; it showed strong staining in the renal tubules in the negative control group, mild staining in IR group and moderate staining in IR+Met and IR+ABS groups with more intense expression in IR+Met+ABS group **(Figure 4).** Morphometric analysis of BAX, and Bcl2 immunopositive area showed statistically significant difference among the studied groups ($P<0.0005$) **(Figs. 3 and 4).** Post-hoc analysis for the mentioned proteins showed that the percentage of BAX immunopositive area was significantly increased in IR group in proportional to the other groups, with a significant decrease among IR+Met, IR+ABS and IR+Met+ABS groups as compared to IR group. Moreover, there has been insignificant difference between IR+Met and IR+ABS groups, and there was significant reduction in IR+Met+ABS group when compared to IR+Met and IR+ABS groups. However, IR+Met+ABS group was still significantly increased ascopared to the negative group **(Figure 3).** On the other hand, Bcl-2 showed the opposite; it showed the most significant reduction in IR group as compared to the other groups, with a significant increase in IR+Met, IR+ABS and IR+Met+ABS groups as compared to IR group. In addition, there was insignificant difference between IR+Met and IR+ABS groups, and there was a significant increase in IR+Met+ABS group when compared to both IR+Met and IR+ABS groups. However, IR+Met+ABS group was still significantly reduced as compared to the negative group **(Figure 4).**

Table (3). Results of the histopathological EGTI score of the renal injury:

Parameter	Group					H	P value
	negative (n=6)	IR (n=6)	IR+Met (n=6)	IR+ABS (n=6)	IR+Met+ABS (n=6)		
Endothelial	1(0-0.75) A	3(2-3) B	2(1-2) B	2(1-2) B	0(0-1.5) A	55.309	**<0.0005**
Glomerular	0(0-1) A	3(2-3) B	2(2-2.75) BC	2(2-2) BC	0(0-2) AC	53.455	**<0.0005**
Tubular	0(0-1) A	3(2-3) B	2(1.25-2) B	2(1-2) BC	0(0-2) AC	54.712	**<0.0005**
Interstitial	0(0-0) A	3(2-3) B	2(1-2) BC	2(1-2) BC	0(0-2) AC	53.687	**<0.0005**
Total score	1(1-1) A	10(9-11.75) B	7(6.25-8) B	7.5(7-8) B	4(2-6) A	82.664	**<0.0005**

Results are tabulated as median and interquartile range. P value was determined by Kruskal-Wallis H test, different letters = statistically significant difference). Significant p values (≤0.05).

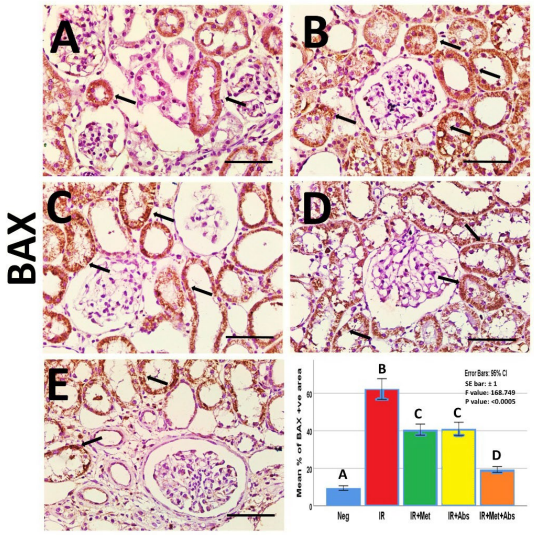

Figure (3): (A-E): Light-microscopic image of renal tissue with BAX immunopositive staining (×400) in kidney tissues of negative control, IR, IR+Met, IR+ABS and IR+Met+ABS group (A-E; respectively). Scale bar = 50 µm. **(F) Morphometric analysis of the mean percentage of BAX immunoreactive area.** Results are mentioned as mean ± standard error. Different letters mean statistically significant difference = P value is ≤0.05. Arrows = BAX +ve cells.

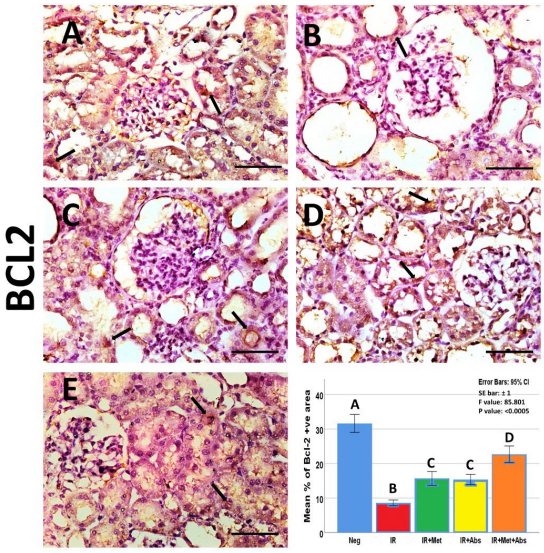

Figure (4): (A-E): Light-microscopic image of renal tissue with Bcl2 immunopositive staining (×400) in kidney tissues of negative control, IR, IR+Met, IR+ABS and IR+Met+ABS group (A-E; respectively). **Scale bar = 50 µm. (F) Morphometric analysis of the mean percentage of LC3 immunoreactive area.** Results are mentioned as mean ± standard error. Different letters mean statistically significant difference = P value is ≤0.05. Arrows = Bcl-2 +ve cells.

3.6. Metformin and ABS attenuated the increased expression of oxidative stress marker NADPH oxidase homolog (NOX-4) induced by I/R

I/R led to a significant elevation in NOX4 mRNA and protein expression in proportional to control. While they decreased, significantly, in IR+Met, IR+ABS and IR+Met+ABS groups when compared to I/R group. However, IR+Met, IR+ABS and IR+Met+ABS groups showed significant difference from the negative control. In addition, there was no significant difference among IR+Met, IR+ABS and IR+Met+ABS groups in NOX4 protein expression, while mRNA of NOX showed significant difference between IR+Met+ABS groups and both IR+Met and IR+ABS **(Figs. 5 and 6)**

3.7. Metformin and ABS attenuated the increased expression of apoptotic marker p-53 induced by I/R

I/R led to a significant elevation in mRNA P53 gene expression, significantly, in IR+Met, IR+ABS and IR+Met+ABS groups when compared to I/R group. However, IR+Met, IR+ABS and IR+Met+ABS groups showed significant difference from the negative control. In addition, there was no significant difference among IR+Met, IR+ABS and IR+Met+ABS **(Figure 5)**

3.8. Metformin and ABS increased connexin-43 expression

I/R led to a significant elevation in mRNA of Cx43, proportional to control. Moreover, mRNA insignificantly decreased in IR+Met, significantly decreased in IR+ABS and IR+Met+ABS groups ascompared to IR group. Furthermore, there was no significant difference between IR+Met and IR+ABS. Lastly, there was significant difference between the negative group and both IR+Met and IR+ABS. Meanwhile, there was insignificant difference between the negative group and both IR+Met+ABS group**.** As for phosphorylated Connexin 43 (P-Cx34) protein expression, I/R led to a significant elevation, proportional to control. Moreover, P-Cx43 protein expression significantly decreased in IR+Met, IR+ABS and IR+Met+ABS groups. Furthermore, there was no significant difference between IR+Met and IR+ABS. Lastly, there was significant difference between the negative group and both IR+Met and IR+ABS. Meanwhile, there was insignificant difference between the negative group and both IR+Met+ABS group **(Figs. 5 and 6).**

3.9. Correlations between serum K^+, serum creatinine, renal tissue MDA and NOX-4 expression, connexin-43 (CX43) expression, BAX expression and BCl2 expression in renal tubules in I/R group

Table (4) shows significant positive correlations between serum K^+, serum creatinine, renal tissue MDA and NOX- 4 expression and BAX expression and negative correlations with CX -43 and BCL2 in I/R group.

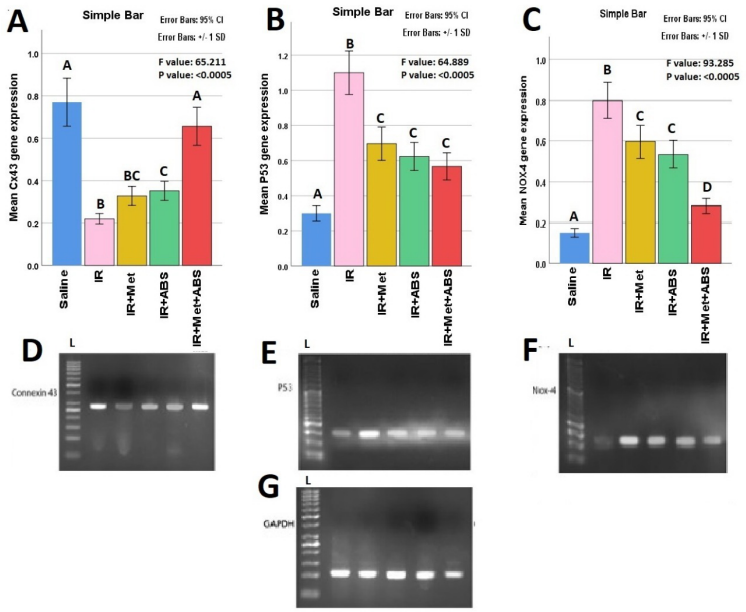

Figure (5): (A-C) Histograms showing the means of the relative gene expression of real-time PCR products of the studied genes. Results are mentioned as mean ± standard deviation. Different letters mean statistically significant difference = P value is ≤0.05. IR: ischemia/Reperfusion, Met: metformin, Abs: Abscisic acid, Cx43: Connexin43 and NOX4: NADPH oxidase4. **(D-G) Gel electrophoresis of the real-time PCR products of the studied genes;** (D) Connexin 43 qPCR product (277 bp). (E) P53 qPCR product (146 bp). (F) NOX-4 qPCR product (119 bp). (G) GAPDH qPCR product (123 bp). **L:** 50 bp ladder**.**

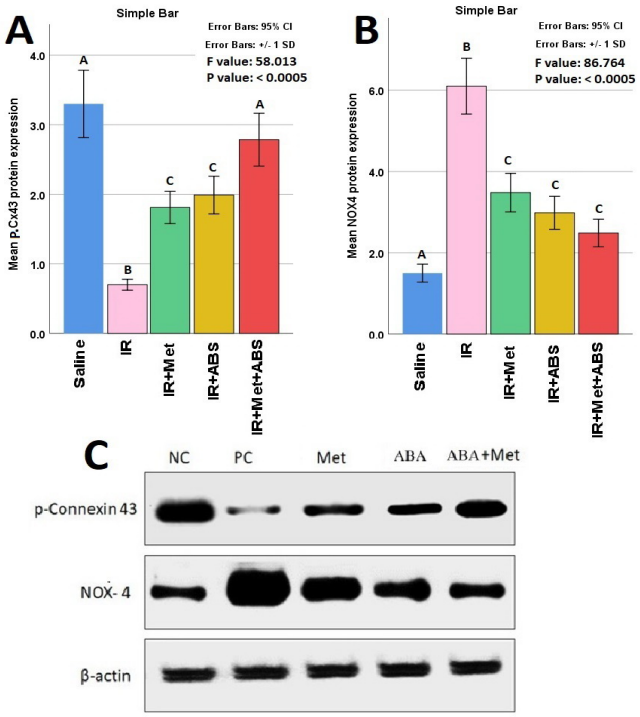

Figure (6): (A, B) Histograms showing the means of the relative protein expression by western blotting of the studied proteins. Results are mentioned as mean ± standard deviation. Different letters mean statistically significant difference = P value is ≤0.05. **(C) Gel electrophoresis of the studied proteins; Phospho-connexin43 and NOX4 by western blotting.** IR: ischemia/Reperfusion, Met: metformin, Abs: Abscisic acid, Cx43: Connexin43 and NOX4: NADPH oxidase4

Table (4): Correlations between serum K^+, serum creatinine, renal tissue MDA and NADPH oxidase homolog (NOX-4) expression, connexin-43 (CX43) expression, BAX expression and BCl2 expression in I/R group

Parameters		Serum K	Serum Creatinine	Renal tissue MDA	NOX-4 expression	CX-43	BAX	BCl2
Serum K^+	r		0.9	0.8	0.8	-0.5	0.8	-0.99
	p		0.000	0.000	0.000	0.010	0.000	0.000
Serum Creatinine	r			0.5	0.4	-0.8	0.9	-0.37
	p			0.013	0.023	0.000	0.000	0.07
Renal tissue MDA	r				0.8	-0.8	0.6	-0.85
	p				0.000	0.000	0.001	0.000
NOX-4 expression	r					-0.46	0.7	-0.62
	p					0.02	0.000	0.001
CX-43	r						-0.44	0.82
	p						0.022	0.000
BAX	r							-0.78
	p							0.000

NOX-4: NADPH oxidase homolog.
r: Pearson's correlation coefficient. P: probability.
P < 0.05: is considered significant. P < 0.001: is considered highly significant.

4.Discussion

In the present study, and similar to previous studies, IRI in rats caused histopathological changes including; thickened basement membrane of Bowman`s capsule, expanded Bowman's space, atrophy of glomeruli, vacuolated tubular epithelium, inflammatory infiltration and increased cast formation **[27].**

In addition, the increased serum creatinine and K+ indicating deteriorated kidney functions in IRI

group could be explained by the initiation of inflammatory process. The injured cells increased ROS production with altered oxidative phosphorylation in mitochondria, lipid peroxidation, depletion of ATP, increased calcium entry and tissue damage **[28],** in accordance with our findings of increase renal MDA and decreased GSH. Moreover, the oxidant impairment of DNA causes apoptosis **[29].** Furthermore, NOX4 plays a key role in Toll-like receptor 4-linked apoptosis in renal IRI **[30]** and hence we`ve found increased expression of renal NOX4, renal apoptotic markers; P53 and BAX with a significant decrease in the anti-apoptotic marker; BCL2 in IRI group.

Gap junctions (GJ) are essential for transmitting signals between cells. Previous studies reported the presence of GJs in renal vascular endothelium and smooth muscle cells. GJs are altered in renal IRI, in particular, Connexin 43 (Cx43) which is essential for the integrity of endothelium **[8].** Moreover, similar to previous studies, IR group revealed a significant downregulation in Cx43 **[31].** After I/R, the kidney usually restores its configuration and functional activity through adaptive regeneration and repair. Previous studies revealed that Cx43 could play an important role in reducing the renal injury and helping this adaptive regeneration and repair following acute renal injury. This repair includes many physiological events e.g. repair of tubular injury/apoptosis, induction of proliferation of tubular cell, resolution of inflammation and renal fibroblasts, and recovery of microvasculature. It seems that CX43 signaling shares in all these repair events, leading to enhanced recovery **[32].**

Metformin could successfully ameliorate the renal damage after IRI similar to previous studies **[12],** they reported a reduction in the tubular cell death, expansion, epithelial cellular shedding, and cast formation, decreased apoptotic markers, in accordance with our finding of antiapoptotic role of Metformin against IRI with decreased BAX and P53 and increased BCL-2 expression. Moreover, a previous study **[13]** found that tubulointerstitial fibrosis after IRI was ameliorated, with decreased expression of inflammatory cytokines, fibrosis markers in the group treated with continuous, not transient, administration of metformin. Furthermore, another study found that Metformin combined with vildagliptin could reduce myocardial infarct size and Bax, increase Bcl-2, and improve mitochondrial function in High fat diet-fed rats after cardiac IRI through activation and phosphorylation of CX43 **[33]**, similar to our finding of restoration of CX43 expression levels in IRI group treated with metformin.

Abscisic acid (ABS), a phytohormone controlling physiological functions in plants after various stresses **[34],** has been identified in mammalian plasma and several cell types such as human granulocytes and pancreatic β cells **[35, 36].**

We report for the first time, the role of synthetic ABS in ameliorating the kidney damage after IRI, with reduction of the tubular necrosis, epithelial sloughing, and cast formation, too. In addition, ABS could decrease the oxidative stress marker NOX-4, MDA and increase GSH with a decrease in the apoptotic markers BAX and P53, and an increase in the antiapoptotic marker Bcl2 similar to a previous study **[14]** which reported that ABS could suppress thioacetamide-induced liver fibrosis through antioxidant, anti-inflammatory and anti-apoptotic activities in mice.

Moreover, we found that ABS-induced attenuation of IRI renal damage was accompanied by upregulation of the gap junction protein; Cx43, and its phosphorylated form, this is in accordance to the results of a previous study **[15]** reporting that ABS exerted significant improvement in cardiac markers and pro-inflammatory cytokines in a model of diabetic cardiomyopathy with improvement of histopathological changes through antiapoptotic activity as well as through upregulation of myocardial Cx43 mRNA.

Conclusions

This study presents novel protective mechanisms for ABS and/or Metformin in I/R. ABS and Metformin decreased the expression of kidney apoptotic marker BAX, oxidative stress marker NOX-4 and increased renal tissue CX43 expression in I/R model. The data from the current study revealed the protective effect of ABS and Metformin against I/R injury. This effect might be interpreted by their antioxidant activity, anti-apoptotic effects and their role in restoration of renal tissue expression of CX43. However, further experiments will be required to study the detailed molecular mechanism. Finally, our data suggest a renoprotective effect of ABS and Metformin in I/R.

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Author Contributions:

Mohamed Adel: shared in formulation of the idea, induction of IR model, biochemical analysis, statistical analysis and paper writing. **Mohammed R. Rabei** shared in the induction of I/R model**,** biochemical analysis, data analysis and paper writing. **Noha Hazem:** western blot, PCR and biochemical analysis. **Hassan Reda:** shared in experimental design, histopathology and paper writing. **Mohammad El-Nablaway:** western blot, PCR and biochemical analysis and paper writing.

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