Activation of Nrf2 by Ischemic Preconditioning and Sulforaphane in Renal Ischemia/Reperfusion Injury: A Comparative Experimental Study

By

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Abstract

Objectives: To investigate impact of ischemic preconditioning (Ipre) and sulforaphane (SFN) and combination of them on nuclear factor -2 erythroid related factor 2 (Nrf2) gene and its dependent genes; heme oxygenase-1 (HO1) and NADPH-quinone oxidoreductase1 (NQO-1) and inflammatory cytokines TNF-α, IL1β, and intercellular adhesion molecule-1 (ICAM1) and caspase-3 in renal ischemia/reperfusion (I/R) injury. **Methods:** Ninety male Sprague Dawely rats were classified into 5 groups (each consists of 18 rats): sham, control, Ipre, sulforaphane and Sulfo+Ipre. Each group was subdivided into 3 subgroups each containing 6 rats according to time of harvesting kidney and taking blood samples; 24 hrs, 48 hrs, and 7 days subgroups. Renal functions including serum creatinine, BUN were measured at basal conditions and by the end of experiment. Expression of Nrf2, HO-1, NQO-1, TNF-α, IL-1β, and ICAM-1 was measured by real time PCR in kidney tissues by the end of experiment. Also, immunohistochemical localization of caspase-3 and chemical assay of malondialdehyde (MDA), GSH and SOD activity were measured in kidney tissues. **Results:** both Ipre and SFN improved kidney functions, enhanced the expression of Nrf2, HO-1, and NQO-1, attenuated the expression of inflammatory (TNF-α, IL-1, and ICAM-1) and apoptotic (caspase-3) markers. However, the effect of sulforaphane was more powerful than Ipre. Also, a combination of them caused more improvement in antioxidant genes expression and more attenuation in inflammatory genes but not caspase-3 than each one did separately. **Conclusion:** sulforaphane showed more powerful effect in renoprotection against I/R injury than Ipre as well as there might be a synergism between them at the molecular but not at the function level.

Background

Renal warm ischemia/reperfusion (I/R) injury is a common problem in many clinical situations such as kidney transplantation and renal vascular surgery (Weight et al., 2001; Saito and Miyagawa, 2000). It is complex inflammatory condition, including ATP depletion, accumulation of intracellular Ca^2 and reactive oxygen species (ROS), pro-inflammatory cytokine production and apoptotic pathway activation. During kidney transplantation, renal (I/R) injury is a common cause of renal cell death, renal failure, delayed graft function (Pirsch et al., 1996) and renal graft rejection (Carpenter, 1995). So, it is essential to protect the kidney against I/R injury by exogenous agents or by enhancing the endogenous ability of the kidney cells to withstand this injury.

During evolution, cells have developed inducible defense systems against harmful the toxic and hypoxic insults. Several transcription factors are involved in boosting the cell's defenses. One of them is the transcription nuclear factor-erythroid 2-related factor 2 (Nrf2) which was identified as a regulator of expression of the beta-globin genes (Moi, et al., 1994). Soon it was discovered that Nrf2 is a positive regulator of the human Antioxidant Response Element (ARE) that drives expression of antioxidant enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) (Venugopal and Jaiswal, 1998). Later on, it was found that Nrf2 plays a crucial role in the cellular protection against oxidative stress. Nrf2 is referred to as the "master regulator" of the antioxidant response due to the fact that it modulates the expression of several genes including phase 2 and antioxidant enzymes playing an important role in detoxification of reactive oxygen species (ROS) and electrophilic species, including heme oxygenase-1, NAD(P)H:quinone

oxidoreductase, glutathione-S-transferase, gamma-glutamyl cysteine ligase, glutathione reductase, etc. Recent studies demonstrate that dysfunction of Nrf2-driven pathways impairs cellular redox state thus oxidative stress (reviewed in Silva-Islas et al., 2012). So, up regulation of the ARE-gene battery has a significant impact on the ability of the cell to withstand and survive sustained oxidative insults.

Recent studies by our group demonstrated the protective action of ischemic preconditioning (Ipre) against renal I/R injury (Shokeir et al., 2012) and activation of Nrf2 system and its dependent genes by ischemic preconditioning in renal I/R injury rat model (Shokeir et al., 2014). Also, it was reported that, sulforaphane, a natural dietary isothiocyanate present in cruciferous vegetables as broccoli, brussel sprouts, cauliflower, cabbage, is an excellent inducer for nrf2 gene and its pathway (Hong et al., 2005). Several studies have shown the protective properties of sulforaphane against ischemia/reperfusion damage in brain (Zhao et al., 2006; Ping et al., 2010, Chen et al., 2011) and kidney (Yoon et al., 2008). However, which is more powerful in activation of NRF2, sulforaphane or Ipre is not studied before. Moreover, we hypothesized that a combination of Ipre and sulforaphane could have a synergistic effect on activation NRF2, hence could confer more protection of kidney against I/R injury. So, this study was designed to investigate the combined effects of Ipre and sulforaphane on NRF2 activation as well as to compare their effects.

Materials and Methods

Experimental animals and ethical considerations

The material of this work included 90 male Sprague Dawely rats weighing 200-250 gm aging 4-6 months which were bred in the animal research facility in the Urology & Nephrology Center at Mansoura, Egypt. Experiments were performed according to the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council, Washington, DC: National Academy Press, no. 85-23, revised 1996). All protocols were approved by our ethical committee of Mansoura, Faculty of Medicine.

Study design

Animals were randomly divided into 5 groups; ***1) Sham group (18 rats)***: rats were subjected to right nephrectomy, exposure of left renal pedicle with no ischemia, ***2) Control group (18 rats):***rats were subjected to right nephrectomy and left renal ischemia for 45 minutes (definitive ischemia), ***3) Ischemic preconditioning (Ipre) group (18 rats):*** like control group, but three cycles of 2 min ischemia followed by 5-min reperfusion period to I/R were done before the definitive 45-min ischemia (Ambros et al., 2007), ***4) Sulforaphane group (18 rats)***: like control group, but sulforaphane (500 µg/body weight kg, i.v.) was given to the rats 1 hour before clamping of renal pedicle and ***5) Ipre + Sulforaphane group (18 rats)***: like control group, but Ipre was done plus sulforaphane was given to the rats.

The rats in each group were subdivided into 3 subgroups (each was 6 rats), 24 hr, 48 hr and 7 days subgroups, according to the time of harvesting of kidney tissues. Blood samples were taken from each subgroup at the end of the experiment i.e. at 24 hr, 48 hr and 7 days after surgery. Blood (1 ml) was obtained from the ophthalmic venous plexus using a fine-walled Pasteur pipette. The rat was anaesthetized using halothane inhalation and the pipette was positioned at the inner corner of the eye beside the eyeball, and pushed gently but firmly along the side of the orbit to the ophthalmic venous plexus. Blood was centrifuged and serum stored at 20°C for measurement of serum creatinine and blood urea nitrogen (BUN) later on.

By the end of experiment in each subgroup, the left kidney was harvested and divided into 2 halves, one half was placed in formalin 10% for immunohischemical localization of Nrf2 and caspase-3, while the remaining half was stored at -80 C° for real time PCR assay of inflammatory markers, TNF-α, IL-1β, and ICAM-1, Nrf2 gene and antioxidant genes HO-1 and NQO-1.

Assessment of Renal functions

Kidney functions were assessed by measurement of serum creatinine, and serum BUN at basal conditions and end of experiment. The concentrations of creatinine and BUN in serum were measured using an auto-analyser (CX 7; Beckman, Foster City, CA, USA).

Chemical assay of oxidative stress markers (MDA, SOD and GSH)

Kidney tissue was perfused with a PBS (phosphate buffered saline) solution, pH 7.4 containing 0.16 mg / ml heparin to remove any red blood cells and clots. Then, kidney

was weighed, minced, homogenized in 5 – 10 ml cold buffer (i.e. 50 mM potassium phosphate, pH 7.5. 1 mM EDTA). Homogenates were centrifuged at 10000 x *g* for 15 minutes at 4°C and the supernatant was kept at −80 ◦C till used for analysis of lipid peroxides (malondialdehyde, MDA), superoxide dismutase (SOD) , and reduced glutathione (GSH). MDA, SOD, and GSH were measured by using colorimetric kit (Bio-Diagnostics, Dokki, Giza, Egypt) according to manufacturer's instructions.

Real time PCR for TNF-α, IL-1β and intercellular adhesion molecule-1 (ICAM-1), Nrf-2, HO-1 and NQO-1 genes

RNA extraction and cDNA synthesis

According to the manufacturer's instructions, total RNA from kidney tissue specimens was isolated by disruption of 50-100 mg tissues in 1 ml of Trizol (Invitrogen Corporation, Grand Island, NY, USA). RNA was quantified spectrophotometrically, and its quality was determined by agarose gel electrophoresis and ethidium bromide staining. Only samples that were not degraded and showed clear 18 S and 28 S bands under ultraviolet light were used for real-time RT-PCR. Reverse transcription was done using 1 µg total RNA and a cDNA kit (high-capacity cDNA archive kit). The primer sequences for tested genes were , TNFα (295 bp) forward 5'-TACTGAACTTCGGGGTGATTGGTCC-3' reverse 5'-CAGCCTTGTCCCTTGAAGAGAACC-3', ICAM-1 (409 bp) 5'-TGTTTCCTGCCTCTGAAGC-3', Nuclear erythroid-related factor 2 (Nrf2) (109 bp), forward: 5′-GCTATTTTCCATTCCCGAGTTAC-3′, reverse: 5′-ATTGCTGTCCATCTCTGTCAG-3′. NAD (P) H: quinine oxidoreductase-1 (NQO1)

(197 bp), forward: 5′-CATCATTTGGGCAAGTCC-3′, reverse: 5′-ACAGCCGTGGCAGAACTA-3′. Heme oxidase-1 (HO-1) (102 bp), forward: 5′-CTTTCAGAAGGGTCAGGTGTC-3′, reverse: 5′-TGCTTGTTTCGCTCTATCTCC-3′. IL-1β (131 bp), forward: 5′-TGTGATGTTCCCATTAGAC-3′, reverse: 5′-AATACCACTTGTTGGCTTA-3′. GAPDH (140 bp) forward: 5′-TATCGGACGCCTGGTTAC-3′, reverse: 5′-CTGTGCCGTTGAACTTGC-3′

Real time PCR reaction

The reaction was performed in a total volume of 50µl containing 25 µl from 1x TaqMan® Universal PCR with 2.5 µl from 20x TaqMan® Gene Expression Assay Mix and 22.5 µl of cDNA diluted in RNase-free water.The cycling parameters were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation 95°C for 15 seconds, annealing at 60°C for 1 minute, extension at 72°C for 1 minute. Data analysis was carried out using ABI prism 7000 by equation $2^{-\Delta\Delta}$ ct (Livak, and Schmittgen, 2001).

Immunohistochemical examination of caspase-3

Explanted kidneys were bisected along the long axis and were fixed in 10% formalin solution for 24 hours. After automated dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin. For immunohistochemistry to assess the apoptotic index, 3-µm-thick sections were prepared on charged slides and deparaffinized. All sections were incubated for 30 min with 0.3% hydrogen peroxide in methanol and microwave-heated in

10 mM citrate buffer, pH 6.0, for 10–20 min. Subsequently, an indirect immunoperoxidase technique was applied, using monoclonal antibodies for: **anti-caspase-3** (Abcam, catalogue number: ab79123). Indirect immunoperoxidase was performed using ImmunoPure UltraSensitive ABC Peroxidase (Thermo Scientific, catalogue number: 32052) with (DAB) as chromogen. Proper positive and negative controls were performed. Tonsils were used as positive control for caspase-3. In negative control, sections were stained without the addition of a primary antibody. The apoptotic index of caspase-3 was assessed with a standard point-counting method for the percentage of labelled tubular cells (excluding necrotic tubules) in non-overlapping, randomly selected 10 high power fields of each slide (Duan et al., 2003) .

Statistical analysis

Statistical analysis was done by using SPSS computer program (version 10). One-way analysis of variance (ANOVA) was done to study test of significance within treated groups, within Scheffe's posthoc test. Significance was considered when P values were less than 0.05.

Results

Effects of Iprecond and sulforaphane and combination of them on kidney functions parameters (serum creatinine and BUN)

Compared to the sham group, I/R and all studied groups had significant increase in serum creatinine and BUN at all-time points of the study (24 hrs, 48 hrs and 7 days) ($p< 0.05$). Compared to the I/R group, the Iprecond, sulforaphane and Ipre + sulfo groups

showed significant improvement in serum creatinine and BUN at all-time points of the study ($p< 0.05$). Nevertheless, the percentage decrease in the Iprecond group was significantly less compared with sulforaphane group ($p< 0.05$). Moreover, addition of Iprecond to sulforaphane did not cause more significant improvement in serum creatinine and BUN except serum creatinine at 48 hrs group (table 1).

Effects of Iprecond and sulforaphane and combination of them on the expression of Nrf2, HO-1 and NQO-1

Real time PCR showed significant increase in the expression of Nrf2, HO-1 and NQO-1 in all studied groups (I/R, Ipre, sulforaphane and Ipre + sulfo) compared to sham group at all time periods of follow up ($p< 0.05$). Compared to I/R group, the other studied groups (Ipre, sulforaphane and Ipre + sulfo) showed significant increase in expression of these genes at all-time points of the study ($p< 0.05$). However, the degree of rise in the expression of these genes was significantly higher in sulforaphane group than Ipre. Moreover, the degree rise in the expression of Nrf2, HO-1 and NQO-1 was significantly high in Ipre + sulfo group compared to other treated groups at 48 hrs and NQO-1 at 2hrs and 24hrs ($p< 0.05$) (fig.1a, b and c).

Effects of Iprecond and sulforaphane and combination of them on the oxidative stress markers (MDA, SOD and GSH)

The results of oxidative stress markers showed significant increase in the levels of MDA and significant decrease in the activity of SOD in kidney tissues in all studied groups (I/R, Ipre, sulforaphane and Ipre + sulfo) compared to sham group at all time

periods of follow up ($p< 0.05$). However, GSH showed significant decrease in I/R group and increase in other groups compared to sham group ($p< 0.05$). Compared to I/R group, the other studied groups (Ipre, sulforaphane and Ipre + sulfo) showed significant increase in the levels of GSH and SOD activities with significant decrease in MDA levels in kidney tissues at all-time points of the study ($p< 0.05$). However, the degree of improvement in these markers was significantly higher in sulforaphane group than Ipre. Moreover, the degree of improvement in the levels of GSH and SOD at all times of follow up and MDA at 24 hrs was significantly higher in Ipre + sulfo group than sulforaphane alone group ($p< 0.05$). Unfortunately, MDA showed significant rise in its level in kidney tissues in Ipre + sulfo group than sulforaphane group alone at 48 hrs and 7 days ($p< 0.05$) (table 2).

Effects of Iprecond and sulforaphane and combination of them on inflammatory cytokines (TNF-α, IL-1β, and ICAM-1)

Compared to the sham group, I/R , Ipre and sulforaphane groups showed significant increase in the expression of TNF-α and ICAM-1 in kidney tissues at all-time points of the study ($P< 0.05$). On the other hand, IL-1β showed significant increase I/R and Ipre groups only at early times (24 hrs and 48 hrs) compared to sham group ($P< 0.05$), without any significant difference between sham group and Ipre group at 7 days. Also, compared to I/R group, Ipre, sulforaphane and Ipre+sulfo groups showed significant decrease in the expression of all of these markers at different times of follow up ($P< 0.05$). Moreover, the degree in reduction of these markers was marked in sulforaphane and Ipre+sulfo groups compared to Ipre and in Ipre+sulfo group compared to sulforaphane ($P< 0.05$)

(table 3).

Effects of Iprecond and sulforaphane and combination of them on expression of caspase-3

Kidneys from all studied groups (I/R, Ipre, sulforaphane and Ipre+sulfo) showed significant increase in the score of caspase-3 expression when compared with that from sham group ($p< 0.05$). Compared to I/R, all other studied (I/R, Ipre, sulforaphane and Ipre + sulfo) groups showed significant reduction in the caspase-3 expression score ($p< 0.05$). However, there were no statistical significant differences among all treated groups i.e. Ipre, sulforaphane and Ipre+ sulfo groups (fig.2a). Figures 2c-e are representative samples of immunostaining for caspase-3 of different groups.

Discussion

The findings of the present study showed that a) using of Ipre or sulforaphane alone improved the kidney functions, enhanced the expression of Nrf2 factor and its dependent genes, improved redox state in kidney tissues, attenuated the inflammatory process in kidney tissues and inhibited apoptosis in kidney tissues in case of I/R injury b) sulforaphane had a powerful effect in induction of Nrf2 and its dependent genes than Ipre c) a combination of both Ipre and sulforaphane conferred more enhancement in the expression of antioxidant genes and more improvement in inflammatory state and antioxidants but not the apoptotic markers.

The first objective of this study was to examine the effect of Ipre and sulforaphane (single dose 500 ug/kg i.v. one hour before ischemia) alone or in combination on renal functions. The results of this study showed that using of Ipre or sulforaphane alone improved the kidney functions, and the effect of sulforaphane was more powerful than Ipre. These findings were in agreement with previous studies that reported the renoprotective for Ipre (Shokeir et al., 2012; 2014; Hernandez et al., 2008; Timsit et al., 2008) and sulforaphane (Yoon et al., 2008) against renal I/R injury. However, in the present study, a combination of Ipre and sulforaphane did not confer more improvement in kidney functions parameters (serum creatinine and BUN) than sulforaphane alone did at different times of follow up. Failure of synergistic effect between sulforaphane and Ipre could be explained on base of single low dose used for sulforaphane in the present study. In consistence with this hypothesis, Cui et al., (2012) found that administration of sulforaphane at a dose of 0.5 mg/kg/day in 5 days/week for 3-months significantly attenuated the progression of renal disease in a mice model of diabetic nephropathy and this effect disappeared after 6 months of sulforaphane treatment, while Zheng et al. (2011) found that administration sulforaphane in diabetic nephropathy at 12.5 mg/kg/day in 3 times/week caused improvement for 4 months.

Nrf2 transcription factor coordinates the regulation of over 200 genes in humans and animals (Hu et al., 2006; Zhu et al., 2008; Hayes and McMahon, 2009), largely related to mechanisms of endogenous cellular defense and survival (Niture et a., 2010). Nrf2 has been variously described as “the master redox switch (Surh et al., 2008) an “activator of cellular defense mechanisms (Lee and Johnson, 2004),” and “a guardian of health span

and gatekeeper of species longevity (Lewis et al., 2010).” As a mediator for amplification of the mammalian defense system against various stressors, Nrf2 sits at the interface between prior understanding of oxidative stress and the endogenous mechanisms used by cells to respond to oxidative stress. This factor can be induced by many factors such as sulforaphane and Ipre. So, the second objective of this study was to examine the effect of Ipre and sulforaphane and combination of them on induction of this factor and its dependent antioxidant genes. This study demonstrated both Ipre and sulforaphane had the power to enhance the induction of Nrf2 and its dependent genes HO-1 and NQO-1 and the effect of sulforaphane alone in induction of them was more powerful than Ipre alone. Similar studies reported this effect for sulforaphane and Ipre in induction of Nrf2 and HO-1 and NQO-1 genes (Shokeir et al., 2014; Yoon et al., 2008) in renal I/R rat model. Moreover, this study demonstrated that a combination of both of Ipre and sulforaphane caused more enhancement of induction of these genes than each one did separately. However, this enhancement was not reflected on the renal functions. These findings suggested that the improvement in kidney functions is partly dependent on induction the antioxidant genes.

Also, this could be explained on base of low dose Also, assessment of oxidative stress markers in kidney tissues showed significant reduction in MDA (marker of lipid peroxidation) with improvement in the antioxidants (SOD activity and GSH concentration) in Ipre and sulforaphane groups. And addition of Ipre to sulforaphane led to more improvement in the redox state than sulforaphane alone. A previous study done

by our group reported similar results for Ipre on oxidative stress markers on kidney tissues in renal I/R injury rat model (Shokeir et al., 2012). Also, recently Banday and and Lokhandwala, (2013) demonstrated that inhibition of oxidative stress and NF-κB activation through stimulation of a redox-sensitive transcription factor (nuclear factor E2-related factor 2- (Nrf2-) phase II antioxidant enzyme pathway) by sulforaphane maintained dopamine (D1) receptor functionality and prevents the development of hypertension.

Renal I/R is complex inflammatory process in which inflammatory cytokines such as ICAM-1, IL1β and TNF-α play crucial role (Beckman et al., 1990). Therefore, reduction of inflammatory reaction may be potential mechanisms of renoprotective effect of Ipre and sulforaphane against renal I/R injury. The present study demonstrated that reduction of the expression of genes of inflammatory cytokines (ICAM-1, IL1β and TNF-α) by sulforaphane and Ipre intervention, however the effect of sulforaphane was more pronounced than that of Ipre. We reported in a recent study by our group similar results for Ipre on inflammatory cytokines in renal I/R injury. Also, Zhao et al., (2010) reported reduction of myeloperoxidase activity (index for inflammatory process) in liver tissues exposed to I/R injury by sulforaphane treatment. Also, the present study demonstrated more attenuation in the expression of tested inflammatory cytokines by a combination of sulforaphane and Ipre than each one separately. These findings suggested synergistic action for sulforaphane and Ipre on reduction of inflammatory cytokines in renal I/R injury.

Finally, we examined the effect of sulforaphane and Ipre on expression of apoptotic marker (caspase-3) in kidney tissues. We found that giving either sulforaphane or Ipre alone caused significant attenuation of caspase-3 expression. Unfortunately, addition sulforaphane to Ipre in a combination did not cause more attenuation in caspase-3 expression than did each one alone. Moreover, sulforaphane did not confer more effect than Ipre did. Shokeir et al (2012) reported attenuation of capase-3 expression by Ipre in renal I/R and Negrette-Guzmán et al.,(2013) reported reduction of caspase-9 (another executor of apoptosis) by sulforaphane in gentamycin induced nephrotoxicity. However, up to the best of our knowledge, no body investigated the effect of combination of them on apoptotic markers. Absence of synergistic effect for sulforaphane and Ipre on caspase-3 expression does not exclude this synergistic effect for the combination on apoptosis as this may be due to specific effect on caspase-3. This is considered as one of the limitations of this study as there was no actual assessment of apoptosis in kidney tissues and we relayed on assessment of the expression of caspase-3.

Conclusion

Both Ipre and sulforaphane protect kidney against I/R injury through improvement of oxidative stress, enhancement of antioxidant genes such as Nnrf2, HO-1 and NQO-1 , reduction of inflammatory cytokines such as TNF-alpha, IL1b and ICAM-1 and apoptotic protein, caspase-3. There was a synergistic effect for both on induction of antioxidant genes and reduction of inflammatory cytokines but not on caspase-3 and this synergistic effect is not reflected on kidney functions parameters.

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Legends of figures

Fig. (1): Real time PCR expression of Nrf2 gene and its dependent genes HO-1 and NQO-1 at 24 hrs, 48 hrs and 7 days after ischemia. *Significant vs sham group of the same time interval, # Significant vs I/R of the same time interval, $ significant vs Iprecond group of the same time interval, § significant vs sulforaphane group of the same time interval.

Fig. (2): Expression of caspase-3. a) Scoring index for caspase-3 expression at 24 hrs, 48 hrs, and 7 days after ischemia, samples of kidney sections stained immunohistochemically using anti caspase-3 antibody from (b) control group with (c) Ipre group, (d) sulforaphane group and (e) Ipre+sulfo group (immunoperoxidase DAB X400). *Significant vs sham group of the same time interval, # Significant vs I/R of the same time interval, $ significant vs Iprecond group of the same time interval, § significant vs sulforaphane group of the same time interval.

Table (1): Effects of Iprecond and sulforaphane and combination of them on kidney functions (serum creatinine and BUN) at 24 hrs, 48 hrs and 7 days after ischemia

Parameter	Group	24 hr group (n=10)	48 hr group (n=10)	7 days (n=10)
Serum creatinine (mg/dl)	**Sham**	0.60 ± 0.05	0.55 ± 0.11	0.51 ± 0.10
	I/R	2.18 ± 0.22*	2.75 ± 0.17*	1.61 ± 0.16*
	Iprecond	1.12 ± 0.17*# (- 48.62%)	1.05 ± 0.09*# (- 61.82%)	1.00 ± 0.12*# (- 37.89%)
	Sulforaphane	1.06 ± 0.21*# (- 51.38%)	0.88 ± 0.13*#$ (- 68.00%)	0.77 ± 0.17*#$ (- 52.17%)
	Ipre + Sulfo	1.01 ± 0.03*# (- 53.67%)	0.75 ± 0.02*#$§ (- 72.72%)	0.80 ± 0.17*#$ (- 50.31%)
Serum BUN (mg/dl)	**Sham**	25.74 ± 3.48	25.02 ± 3.31	25.46 ± 2.70
	I/R	76.71 ± 3.40*	61.03 ± 2.90*	45.73 ± 4.11*
	Iprecond	40.60± 2.49*# (- 47.07%)	36.09 ± 1.53*# (- 40.86%)	31.20 ± 1.29*# (- 31.77%)
	Sulforaphane	36.60 ± 3.16*#$ (- 52.29%)	29.10 ± 4.77*#$ (- 52.32 %)	28.90 ± 3.24*#$ (- 36.80%)
	Ipre + Sulfo	34.16 ± 2.97*#$ (- 55.47 %)	28.10 ± 5.02*#$ (- 53.95%)	29.52 ± 2.31*#$ (- 35.44%)

All data were expressed as mean ± SD. % = percent change from I/R group value of the same time interval, One way ANOVA test with post-hoc Scheffe's test (significant if $p \leq 0.05$). *Significant vs sham group of the same time interval, # Significant vs I/R of the same time interval, $ significant vs Iprecond group of the same time interval, § significant vs sulforaphane group of the same time interval.

Table (2): Effects of Iprecond and sulforaphane on oxidative stress markers (MDA, SOD and GSH) at 24 hrs, 48 hrs and 7 days after ischemia

	Group	MDA (nmol/g tissue)	GSH (mg/g tissue)	SOD (U/g tissue)
24 hrs	**Sham**	22.28 ± 1.24	214.28±8.56	91.27± 3.30
	I/R	367.09±16.01*	114.30 ± 14.06*	67.27± 10.40*
	Iprecond	152.06 ±12.97*#	295.03 ± 13.74*#	79.15 ± 4.84*#
	Sulforaphane	100.45 ±4.92*#$	370.59 ± 15.50*#$	82.60 ± 3.07*#
	Ipre + Sulfo	80.56 ± 5.05*#$§	359.06 ± 10.01*#$§	82.05 ± 2.45*#
48 hrs	**Sham**	26.19 ± 2.25	204.29± 14.13	94.50±6.63
	I/R	265.07±22.11*	32.26±8.47*	52.28± 12.36*
	Iprecond	79.04 ± 5.26 *#	341.06 ± 21.08*#	77.39 ± 2.06 *#
	Sulforaphane	50.30± 4.86*#$	380.67 ± 7.10*#$	84.06± 2.06*#$
	Ipre + Sulfo	60.73 ± 3.87*#$§	539.07 ± 20.01 *#$§	85.07 ± 1.97*#$
7 days	**Sham**	16.72 ± 3.59	225.50 ± 11.90	96.93 ± 4.61
	I/R	154.25± 12.07*	132.04±12.15*	83.29±12.40*
	Iprecond	42.01 ± 7.11*#	370.14 ± 11.60*#	81.05 ± 2.44*
	Sulforaphane	30.46 ± 3.67*#$	421.60 ± 5.06*#$	91.49 ± 1.25*#$
	Ipre + Sulfo	39.05 ± 4.09*#§	452.07 ± 5.39*#$§	92.06 ± 3.06*#$

All data were expressed as mean ± SD. % = percent change from I/R group value of the same time interval, One way ANOVA test with post-hoc Scheffe's test (significant if $p \leq 0.05$).*Significant vs sham group of the same time interval, # Significant vs I/R of the same time interval, $ significant vs Iprecond group of the same time interval, § significant vs sulforaphane group of the same time interval.

Table (3): Effects of Iprecond and sulforaphane and combination of them on the expression of inflammatory cytokines (TNF-α, ICAM-1 and IL-1β) at 24 hrs, 48 hrs and 7 days after ischemia

	Group	TNF-α	ICAM-1	IL-1β
24 hrs	**Sham**	1.22 ± 0.15	0.87 ± 0.14	0.92 ± 0.16
	I/R	10.11*± 0.41	3.29* ± 0.46	2.66* ± 0.15
	Iprecond	5.15*#± 0.23	2.50*# ± 0.10	1.66*# ± 0.13
	Sulforaphane	2.39*#$± 0.18	1.99*#$ ± 0.11	0.92#$ ± 0.04
	Ipre + Sulfo	0.50*#$§ ± 0.07	0.43*#$§ ± 0.07	0.37*#$§ ± 0.04
48 hrs	**Sham**	1.41 ± 0.23	0.86 ± 0.10	0.83 ± 0.11
	I/R	11.96* ± 0.36	4.25* ± 0.37	3.19* ± 0.12
	Iprecond	4.12*# ± 0.44	2.06*# ± 0.10	1.92*# ± 0.09
	Sulforaphane	1.71#$ ± 0.30	1.46* ± 0.04	0.53#$ ± 0.05
	Ipre + Sulfo	0.13*#$§ ± 0.03	0.09*#$§ ± 0.01	0.12*#$§ ± 0.03
7 days	**Sham**	1.30 ± 0.26	0.78 ± 0.13	0.97 ± 0.14
	I/R	7.21* ±0.53	2.38* ± 0.37	1.52* ± 0.39
	Iprecond	3.29*# ±0.24	1.91*# ± 0.05	1.22# ±0.10
	Sulforaphane	1.03#$ ± 0.37	1.64*# ± 0.02	0.98# ±0.12
	Ipre + Sulfo	0.13*#$§ ± 0.03	0.08*#$§ ± 0.01	0.07*#$§ ±0.01

All data were expressed as mean ± SD. % = percent change from I/R group value of the same time interval, One way ANOVA test with post-hoc Scheffe's test (significant if $p \leq 0.05$). *Significant vs sham group of the same time interval # Significant vs I/R of the same time interval, $ significant vs Iprecond group of the same time interval, § significant vs sulforaphane group of the same time interval.

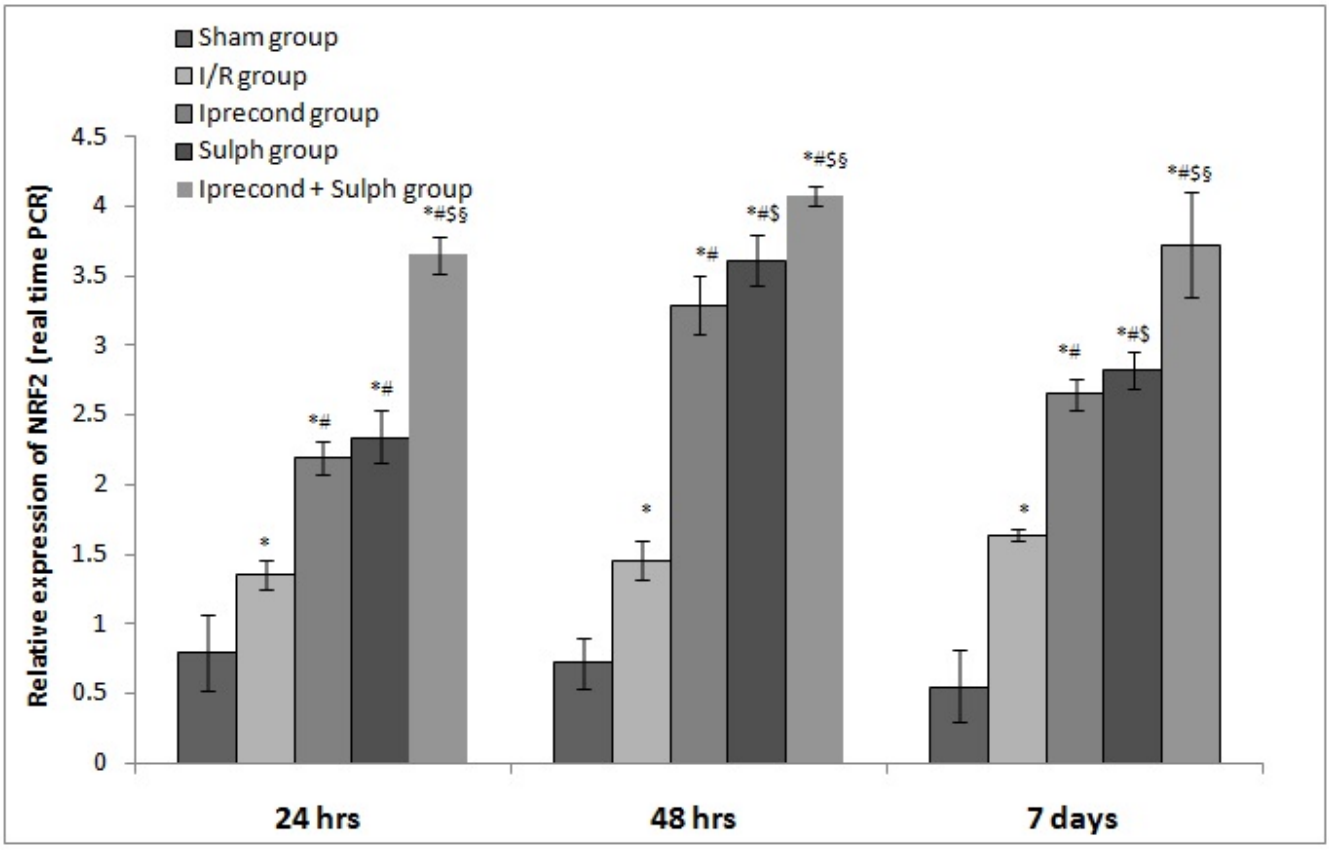
Sham group
I/R group
Iprecond group
Sulph group
Iprecond + Sulph group
Relative expression of NRF2 (real time PCR)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*#
*#
*#$
*#$§
*
*#
*#$
*#$§
*
*#
*#$
*#$§
24 hrs
48 hrs
7 days

Fig 1a

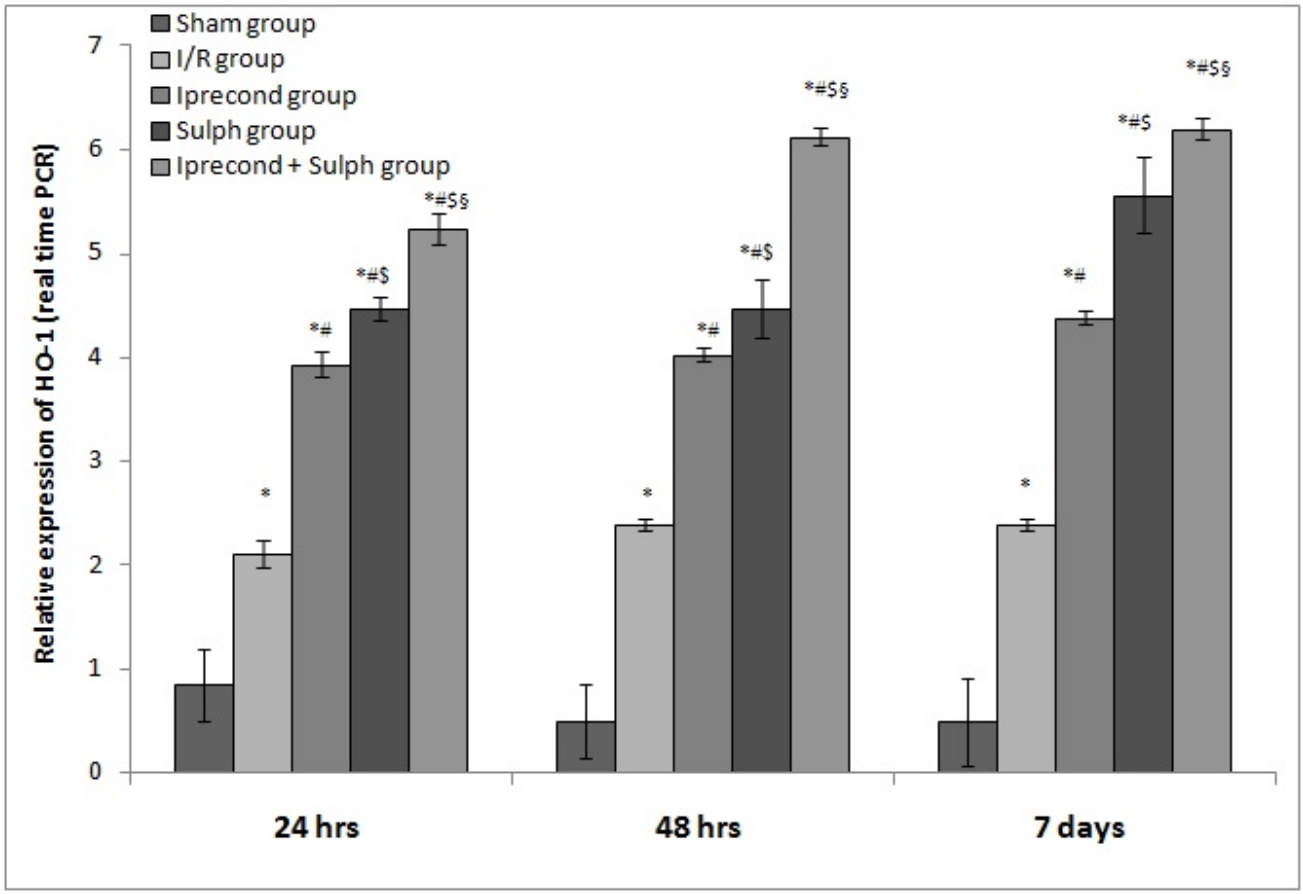
Sham group
I/R group
Iprecond group
Sulph group
Iprecond + Sulph group
Relative expression of HO-1 (real time PCR)
0
1
2
3
4
5
6
7
*
*#
*#$
*#$§
24 hrs
48 hrs
7 days

Fig 1b

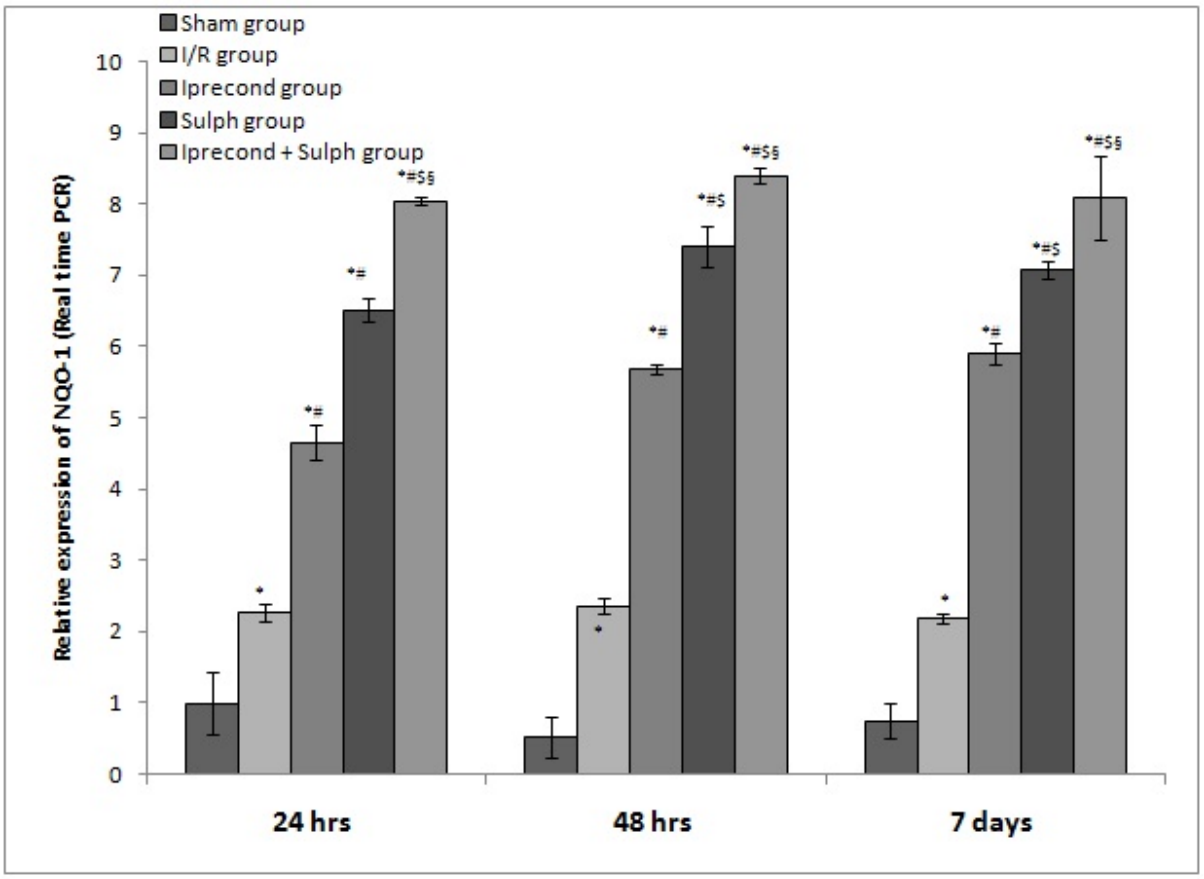
Sham group
I/R group
Ipreccond group
Sulph group
Ipreccond + Sulph group
Relative expression of NQO-1 (Real time PCR)
0
1
2
3
4
5
6
7
8
9
10
24 hrs
48 hrs
7 days

Fig 1 c

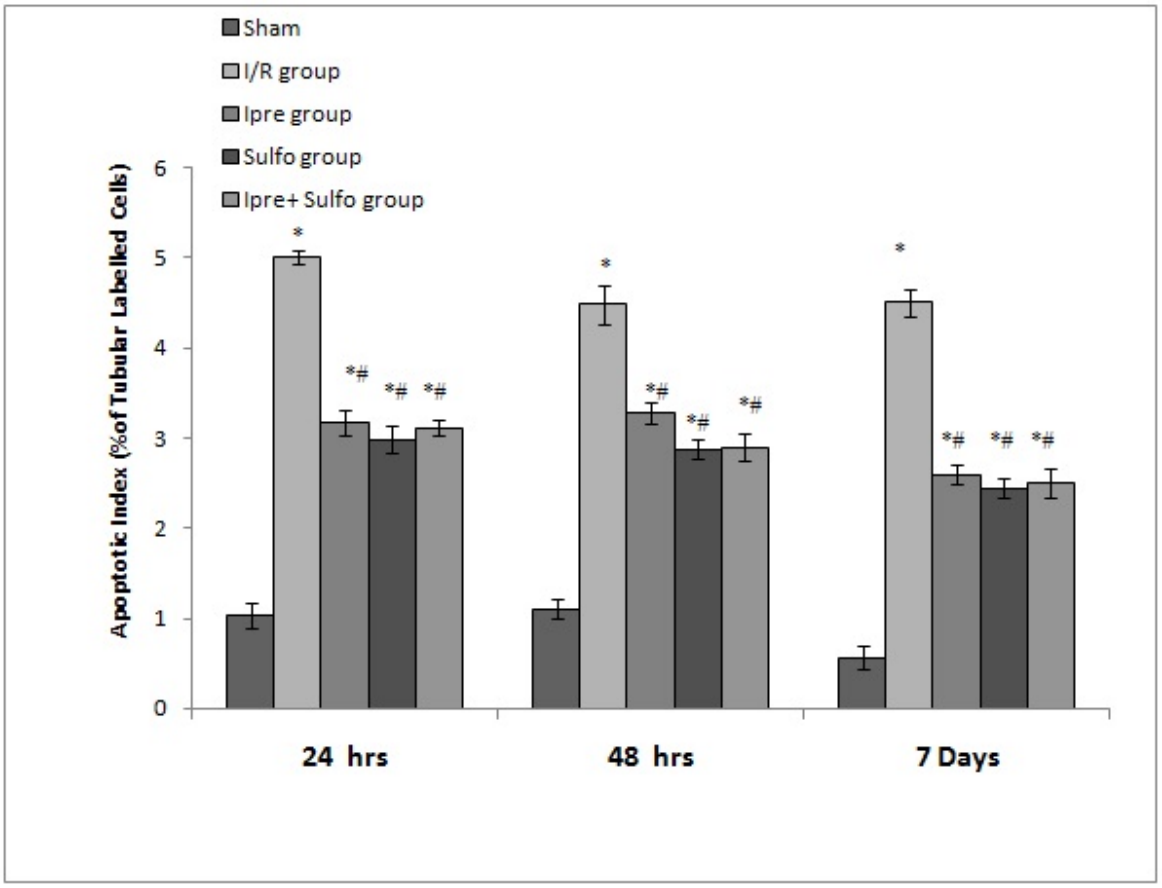
Sham
I/R group
Ipre group
Sulfo group
Ipre+ Sulfo group
Apoptotic Index (%of Tubular Labelled Cells)
0
1
2
3
4
5
6
24 hrs
48 hrs
7 Days

Fig 2 a