

1-1-2013

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KHADIJEH HASHEMI

KAZEM PARIVAR


SAMIRA FAZLIFAR

ALIREZA HAGHPARAST

MEHRDAD MOHRI

See next page for additional authors

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HASHEMI, KHADIJEH; PARIVAR, KAZEM; FAZLIFAR, SAMIRA; HAGHPARAST, ALIREZA; MOHRI, MEHRDAD; AZGHADI, NASER MARGAN; SHIRAZI, MOHAMMADHOSSEIN NAZEM; and GOLI, AMIR AFKHAMI (2013) "Pomegranate (*Punica granatum* L.) reduces endoplasmic reticulum stress induced by renal ischemia/reperfusion injury in rat," *Turkish Journal of Biology*. Vol. 37: No. 4, Article 11. <https://doi.org/10.3906/biy-1211-17>

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KHADIJEH HASHEMI, KAZEM PARIVAR, SAMIRA FAZLIFAR, ALIREZA HAGHPARAST, MEHRDAD MOHRI, NASER MARGAN AZGHADI, MOHAMMADHOSSEIN NAZEM SHIRAZI, and AMIR AFKHAMI GOLI

Pomegranate (*Punica granatum* L.) reduces endoplasmic reticulum stress induced by renal ischemia/reperfusion injury in rat

Khadijeh HASHEMI¹, Kazem PARIVAR¹, Samira FAZLIFAR², Alireza HAGHPARAST³, Mehrdad MOHRI⁴,
Naser MARGAN AZGHADI⁵, Mohammadhossein NAZEM SHIRAZI⁵, Amir AFKHAMI-GOLI^{2,*}

¹Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

²Department of Basic Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

³Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

⁴Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

⁵Khorasan Razavi Veterinary Office, Mashhad, Iran

Received: 09.11.2012 • Accepted: 12.01.2013 • Published Online: 30.07.2013 • Printed: 26.08.2013

Abstract: Ischemia/reperfusion (I/R) is one of the main causes of acute renal failure, leading to generation of reactive oxygen species (ROS) and ensuing oxidative stress, which results in unfolded/misfolded protein accumulation and its dependent response pathways, generally known as unfolded protein response (UPR), in the endoplasmic reticulum (ER). We studied the effects of renal I/R on the expression of ER-stress genes, as well as concomitant effects of oral pretreatment with pomegranate (*Punica granatum* L.) pith and carpellary membrane (PPCM). An in vivo model of rat kidney I/R followed by conventional and real-time RT-PCR was used to evaluate the modulation of GRP78 and XBP1 as central UPR and ER-stress markers. Ischemia followed by reperfusion (60 and 150 min, respectively) resulted in decreased antioxidant properties of plasma (lowered ferric reducing ability of plasma [FRAP] value) and GRP78 transcript levels. Oral administration of PPCM aqueous extract for 10 days with or without ischemia (PPCM and PPCM/Isc groups, respectively) was able to further decrease the GRP78 expression, while it increased the FRAP value. PPCM pretreatment also reduced the XBP1 splicing in the PPCM/Isc group compared to the Isc group. These results suggest that UPR is activated during oxidative insults induced by I/R, while PPCM can reduce I/R-induced ER stress in rat kidneys via antioxidant defense mechanisms.

Key words: Antioxidant, ER stress, GRP78, pomegranate, unfolded protein response, XBP1

1. Introduction

Ischemia/reperfusion (I/R) is a common cause of acute renal failure occurring in various clinical situations such as trauma, renovascular surgery, hypovolemic shock, and renal transplantation (1). Excessive reactive oxygen species (ROS) generated in the reperfusion phase and reduced antioxidant capacity lead to a situation known as oxidative stress (2,3). The endoplasmic reticulum (ER), as an important intracellular organelle responsible for protein synthesis, folding, trafficking, and modification, is susceptible to many stresses such as ischemia, oxidative stress, nutrient depletion, toxins, and hypoxia. These endogenous or exogenous disturbances can result in unfolded/misfolded protein accumulation and the subsequent activation of an unfolded protein response (UPR) (4-7). Glucose-regulated protein (GRP) 78 (also known as BiP: immunoglobulin heavy-chain-binding protein) is a central modulator of the UPR that normally binds to and prevents aggregation of unfolded/misfolded proteins in the ER (6,8). It is a

chaperone member of the heat shock protein 70 (HSP70) family (9), and it normally binds to 3 ER-stress transducers and transmembrane proteins including inositol-requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6). With unfolded protein aggregation, GRP78 releases these proteins and activates the UPR pathways, depending on the severity and duration of ER stress. While the induction of UPR reestablishes ER homeostasis, the ER-associated degradation (ERAD) system tries to clear misfolded proteins from the ER (10). In response to ER stress, IRE1, having endoribonuclease and kinase activity, cleaves X-box binding protein 1 (XBP1) mRNA unconventionally to splice out 26 nucleotides and activate XBP1, which is a member of the leucine zipper protein (bZIP) transcription factor family that can be induced by ATF6 (11). In addition, XBP1 has an important role in cell differentiation (12) and oxidative stress prevention (13), and it is necessary for survival in hypoxic conditions (14).

* Correspondence: a-afkhami@um.ac.ir

Since excessive ROS generation results in various cellular damages, every agent with antioxidant capacity might be of beneficial value in ROS detoxification and ER-stress reduction (2,3,15).

Pomegranate (*Punica granatum* L.), belonging to the family Punicaceae, is a medicinal and ancient fruit with high antioxidant capacity and total polyphenol contents including gallic acid, ellagic acid, gallagic acid, punicalin, and punicalagin (16,17). Punicalagin, mainly found in pomegranate peel and carpellary membrane, is the most abundant polyphenol (18–20). Although it is well known for antioxidant properties (17,21–23), due to its high molecular weight (MW = 108) and the presence of 16 hydroxyl groups in its structure, it can also exhibit antiinflammatory (24), hepatoprotective (22), antigenotoxic (25), anticarcinogenic (26,27), inhibition of tumor growth (28,29), antimicrobial (30), and antiviral (31) activities.

Considering the antioxidant capacity of pomegranate and also the critical role of antioxidants in modulating ER stress, this study was conducted to underscore the molecular changes of ER-stress profile during I/R and the effect of pomegranate pith and carpellary membrane (PPCM) aqueous extract in an in vivo model of renal I/R injury.

2. Material and methods

2.1. Pomegranate extract

Pith and carpellary membranes of pomegranate (*Punica granatum* L.) fruit (Kashmar district, Khorasan province) were separated and dried at room temperature. A 6.25% suspension of the resulting powder was prepared in sterile distilled water and stirred for 36 h. The undissolved particles were discarded through centrifugation and filtering to obtain a 3.75% filtrate, as revealed through measuring the dry mass of the remaining solution.

2.2. Total phenolics measurement

The total phenolics concentration of PPCM was determined by the Folin–Ciocalteu method (32). Briefly, 50 mg of the dried powder was extracted with 100 mL of acidified water (0.3% HCl) before subsequent filtration. Filtrate was then mixed with equal amounts of the Folin–Ciocalteu reagent (Sigma), while 2.0 mL of sodium bicarbonate was added and mixed thoroughly. Absorbance was measured at 725 nm and the values were derived from a standard curve prepared using tannic acid (0–1.0 mg/mL in acidified methanol:water) after 2 h. Values were expressed as mg/g tannic acid equivalents (mg/g of TAE).

2.3. Animals, treatment, and experimental design

The study was performed in adult male Wistar rats weighing 250 to 300 g, prepared from the Faculty of Veterinary Medicine Animal House (Ferdowsi University of Mashhad, Iran) after 3 days of acclimatization upon

arrival. Animals were subjected to standard conditions of temperature and humidity and they had free access to food and water during the treatment period. The rats were randomly assigned to 4 groups (n = 6), named Control (Ctrl), Ischemic (Isc), pomegranate pith and carpellary membrane (PPCM) aqueous extract treatment, and an ischemic group that received PPCM (PPCM/Isc).

In the PPCM and PPCM/Isc groups, rats received PPCM aqueous extract (250 mg/kg BW) by oral intubation twice a day for 10 days, while in the Isc and Ctrl groups, rats received similar volumes of distilled water as a placebo.

On posttreatment day 11, animals were anesthetized using ketamine (40 mg/kg) and xylazine (10 mg/kg) intraperitoneal injections and underwent a midline abdominal incision followed by isolation of renal vascular pedicles. The right renal artery was permanently occluded in all groups, in order to abolish the interference of right kidney activity in the measurement of serum biochemical factors. In groups Isc and PPCM/Isc, the left renal vessels were occluded to induce ischemia for 60 min using a transient suture, followed by a reperfusion phase for 150 min. In groups Ctrl and PPCM, the same surgical protocol was performed without inducing I/R in the left kidney. During the experiment, rats were reanesthetized if necessary and kept at a steady body temperature. After taking the intracardiac blood samples, the left kidney was harvested, washed in RNase free water and stored at –80 °C. All experiments followed Ferdowsi University of Mashhad Animal Care Committee guidelines and were approved by the Ferdowsi University of Mashhad Animal Care Committee.

2.4. Renal function assays

Serum was obtained from intracardiac blood samples by centrifugation (Eppendorf, 5810R, Germany). Plasma urea nitrogen and creatinine levels were measured using an autoanalyzer (Targa 3000, Biotecnica Instruments, Rome, Italy) in order to assure the efficacy of the I/R procedure.

2.5. Ferric reducing/antioxidant power assay

The ferric reducing/antioxidant power (FRAP) assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe(II)-tripyridyltriazine compound from the colorless oxidized Fe(III) form by the action of electron-donating antioxidants (33).

The FRAP reagent consisted of 300 mM acetate buffer (3.1 g sodium acetate + 16 mL glacial acetic acid, made up to 1 L with distilled water; pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM FeCl₃·6H₂O in the ratio of 10:1:1.

Briefly, 50 µL of blood serum was added to 1.5 mL of freshly prepared and prewarmed (37 °C) FRAP reagent in a test tube and incubated at 37 °C for 10 min. The absorbance of the blue-colored complex was read against reagent blank (1.5 mL FRAP reagent + 50 µL distilled water) at 593 nm. Standard solutions of Fe(II) in the range of 100 to 1000

mM were prepared from ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) in distilled water. The data were expressed as mmol ferric ions reduced to ferrous form per liter (FRAP value) (34).

2.6. Total RNA extraction and RT-PCR

For RNA extraction, 50–100 mg of kidney tissue was homogenized and total RNA was isolated from 200 μL of homogenized suspension using the High Pure RNA Isolation Kit according to manufacturer's directions (Roche Applied Science, Germany). cDNA was synthesized from 1 μg of the RNA using MMLV reverse transcriptase, Random Hexamer Primer, and RNase inhibitor (all reagents from Fermentas, Canada).

For quantity assessment, RNA and cDNA samples were analyzed by a NanoDrop spectroscopy analyzer (Epoch, Biotech, USA). Extracted total RNA was also checked for 18S and 28S rRNA band visualization by 2% agarose gel electrophoresis.

2.7. PCR and real-time RT-PCR

Amplification of XBP1, GRP78, and GAPDH mRNA using Primus 96 gradient (Biotech, Germany) with Taq polymerase (Fermentas) was assessed and normalized against GAPDH (housekeeping gene). The primers were forward (5' -ATAATCAGCCCACCGTAA - 3') and reverse (5' - CCAATTCATTCTCGTGT - 3') for GRP78 (Bip), which were designed (Primer Premier v5, Biosoft International) according to the NCBI mRNA sequences bank (GenBank ID: S63521.1); forward (5' -AGTTCAACGGCACAGTCAAG - 3') and reverse (5' -TACTCAGCACCAGCATCACC - 3') for GAPDH (GenBank ID: NM_017008.3) (35); and forward (5' -TTACGAGAGAAAACATCATGGGC - 3') and reverse (5' -GGGTCCAACCTGTCCAGAATGC - 3') for XBP1 (GenBank ID: NM_001004210.1) (36). All PCR products were separated by 2% (GAPDH and GRP78) or 4% (XBP1) agarose gel electrophoresis and detected by a Syngene gel imaging system (Ingenius LHR, UK).

Transcript levels of GRP78 in real time were assessed using SYBR Green Maxima (SYBR Green/ROX qPCR

Master Mix, Fermentas) and the Real-Time Rotor-Gene 6000 Rotary Analyzer (Corbett, Australia) according to the manufacturer's directions (Fermentas) and were normalized against mRNA levels of GAPDH as housekeeping gene.

2.8. Statistical analysis

Relative fold changes in GRP78 transcript levels were assessed via the $2^{-\Delta\Delta\text{-Ct}}$ method and analyzed with one-way ANOVA followed by the Tukey–Kramer test (GraphPad InStat Version 3.0, GraphPad Software Inc., USA). $P < 0.05$ was considered as significant.

3. Results

3.1. Total phenolics of PPCM

Polyphenols, including hydrolysable tannins and ellagitannins, account for the main known antioxidant properties of pomegranate (37). The PPCM aqueous extract was found to contain 224 ± 5 mg/g total polyphenolics, expressed as tannic acid equivalents (TAE, mg/g of TAE).

3.2. Ischemia causes renal injury

Plasma urea and creatinine concentrations were measured in all experimental animals after inducing I/R, revealing that 60 min of ischemia followed by 150 min of reperfusion phase can acutely damage the kidney tissue, as suggested by an increase in plasma urea and creatinine in ischemic groups compared to sham operated littermates (Figures 1A and 1B, $P < 0.001$).

3.3. PPCM increased the antioxidant capacity of plasma

Renal I/R caused a significant reduction in the FRAP value of plasma as compared with sham-operated animals (2.53 ± 0.04 versus 1.48 ± 0.05 $\mu\text{mol}/\text{mL}$, $P < 0.001$). The aqueous extract of pomegranate PPCM increased the antioxidant power (FRAP value) of plasma (Figure 2, $P < 0.001$).

3.4. I/R lowers GRP78 (BiP)

After RNA extraction and reverse transcription into cDNA, the mRNA levels of GRP78, as a central regulator of UPR, were evaluated in real time. Results suggested that

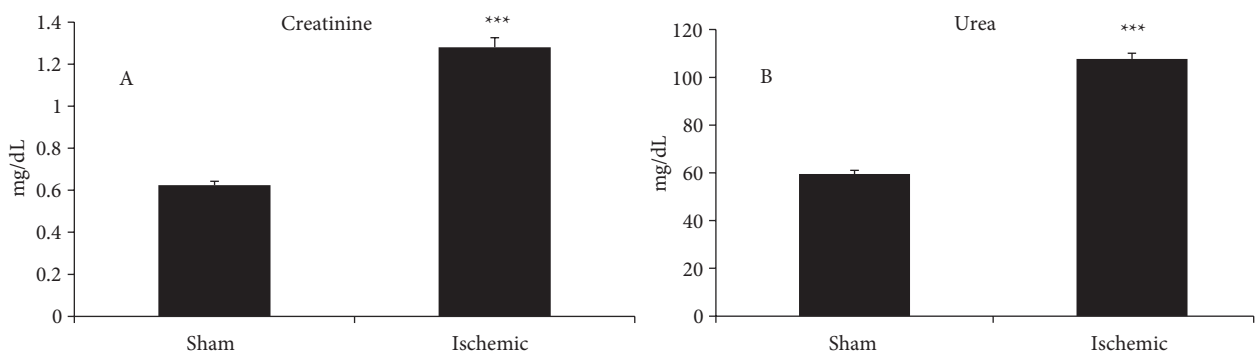


Figure 1. Renal injury induced by I/R. Rats were subjected to 60 min of ischemia and 150 min of reperfusion in the ischemic group. After surgery, serum clinical parameters (urea and creatinine) were measured in the ischemic and sham operated groups. Results are expressed as means \pm SEM. ***: $P < 0.001$ as compared to sham group, $n = 6$.

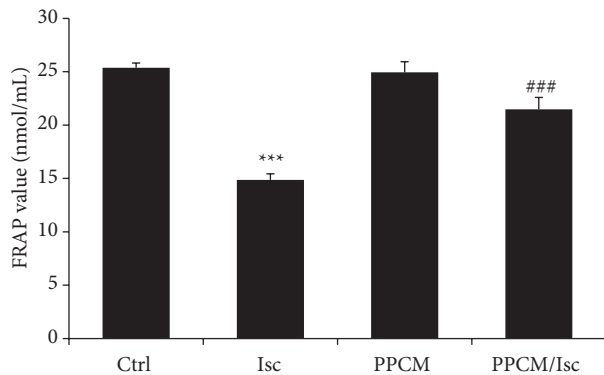


Figure 2. FRAP value comparison in 4 experimental groups following 60 min of ischemia and 150 min of reperfusion (I/R). FRAP value of plasma (nmol/mL) as an indicator of the total antioxidant power was determined. Results are expressed as means \pm SEM. ***: $P < 0.001$ compared to sham group, ###: $P < 0.001$ compared to ischemic group, $n = 6$. Ctrl: control, Isc: ischemic, PPCM: pomegranate pith and carpellary membrane (PPCM) aqueous extract treatment, PPCM/Isc: PPCM aqueous extract treatment/ischemic.

I/R can significantly down-regulate GRP78 in rat kidneys compared to control littermates ($P < 0.05$), as evidenced by lowered mRNA relative fold change, as well as band comparisons on a 2% agarose gel electrophoresis (Figure 3A and 3B).

3.5. GRP78 expression is down-regulated by PPCM aqueous extract

Real time RT-PCR underlined that PPCM aqueous extract administration (250 mg/kg) for 10 days significantly down-regulated GRP78 expression compared to the control group ($P < 0.001$). Electrophoresis on a 2% agarose gel also revealed the same results emphatically (Figure 3A and 3B).

3.6. XBP1 is activated via mRNA splicing during I/R in the rat kidney

Ischemia evokes ER stress, which can lead to the induction of XBP1 mRNA transcription (11,38). This activation is characterized by unconventional XBP1 splicing and removal of 26 nucleotides. In order to examine the role of XBP1 mRNA as a UPR target for IRE1 activation in ER-stress conditions, we used conventional PCR followed by a 4% agarose gel electrophoresis, showing a single unspliced band for XBP1 (U) (289 bp) in the control and PPCM treatment groups, emphasizing the absence of ER stress. In the ischemic group, on the other hand, the spliced XBP1 (S) (263 bp) revealed the activation of IRE1 during ER stress in the kidneys (Figure 4).

3.7. PPCM extract decreased the XBP1 splicing

While splicing of XBP1 mRNA was visible in groups that underwent I/R, PPCM treatment decreased the XBP1 spliced (S) form, underscoring its protective effects in

contributing to the subsequent lessened ER stress in the treatment group (Figure 4).

4. Discussion

I/R is one of the main causes of acute renal failure (39), which occurs during different cardiovascular disorders such as hypovolemic shocks, renal transplantation, trauma, and emboli. Besides the direct effects of ischemia, reactive oxygen species produced during the reperfusion phase play the main critical role in pathogenesis of tissue disorders accompanied by I/R (3,40). Considering the key role of the ER in protein synthesis and proper folding, cellular stresses such as hypoxia, glucose depletion, and oxidative damage may lead to ER dysfunction and the accumulation of unfolded/misfolded proteins, which are categorized as ER stress (41). In response to these linked events, UPR as an adaptive or proapoptotic response will be activated (5,6).

Herein, we have focused on transcription levels of the UPR markers GRP78 and XBP1 after induction of I/R in rat kidneys. One hour of ischemia followed by 150 min of reperfusion phase could significantly reduce the expression of GRP78 mRNA, while XBP1 splicing was obviously activated during this time period. Bilecová-Rabajdová et al. reported that the levels of GRP78 as an antiapoptotic gene decreased after 1 h of ischemia and 1 h of reperfusion phase, while these levels increased up to 24 h after I/R. The mRNA levels of Gadd153 as a proapoptotic gene, on the other hand, were increased at the first time point and decreased to the lowest levels 24 h after the induction of I/R (42). In another study, pretreatment with UPR inducers such as tunicamycin and thapsigargin induced XBP1 splicing at 1 or 2 h after ischemia in the rat kidney, while the peak levels of GRP78 mRNA were seen at 6 h after I/R. This up-regulation could protect proximal tubular cells from I/R-induced injuries (43). Moreover, treatment with $CdCl_2$ was able to induce GRP78 and ATF4 mRNA levels in a time- and dose-dependent manner. Although ATF4 transcript levels are increased after 2 h of I/R, GRP78 mRNA began to increase after 4 h and was at maximum levels at 6 h after I/R (44). While down-regulation of GRP78 transcript levels in our study was consistent with these results, there was no possibility for subjecting in vivo models to longer periods of reperfusion and different time courses. In contrast to these findings, studying the activation of UPR under hypobaric hypoxia conditions at different time points revealed decreased XBP1 splicing along with down-regulation of GRP78 (45).

Exposure to hydrogen peroxide in XBP1-deficient cells resulted in down-regulation of the antioxidant catalase, enhanced ROS generation, and prolonged p38 phosphorylation, which underscores the protective effects of XBP1 via catalase expression (13). Although it seems

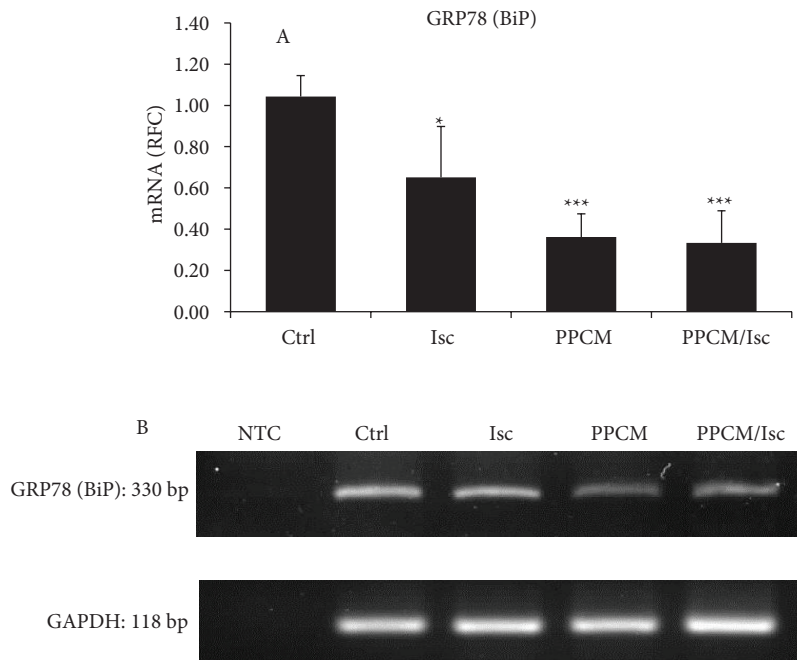


Figure 3. Comparison between mRNA levels of GRP78 (BiP) in 4 experimental groups following 60 min of ischemia and 150 min of reperfusion (I/R). A) GRP78 mRNA levels were assessed by real-time PCR. Results are normalized against GAPDH as a housekeeping gene and are shown as means \pm SEM. *: $P < 0.05$ compared to control group, ***: $P < 0.001$ compared to control group, $n = 6$. RFC: Relative fold change. B) Detected bands by 2% gel electrophoresis for GRP78 and GAPDH in all groups. NTC indicates negative control for PCR reactions. Ctrl: control, Isc: ischemic, PPCM: pomegranate pith and carpellary membrane (PPCM) aqueous extract treatment, PPCM/Isc: PPCM aqueous extract treatment/ischemic.

that XBP1 splicing is dependent on a special region of the GRP78 promoter, an increase in the spliced form of XBP1 occurs before changes in GRP78 transcript levels (11). Herein, we have seen the XBP1 splicing (as UPR activation marker) after 60 min of ischemia and 150 min of reperfusion phase, which emphatically resembles the results of previous investigations. Furthermore, Skhoda et al. suggested that GRP78 redistribution in the cells at early time points occurs independently from GRP78 protein resynthesis (46). Finding a way to study *in vivo* models for longer time periods can offer more detailed information about UPR activation.

The excessive generation of ROS and subsequent oxidative stress during I/R can cause a decline in antioxidant enzymatic defense mechanisms (3,40,47-49). In this regard, each antioxidant (endogenous or exogenous) that lessens ROS production or scavenges the free radicals contributing to reduced ER stress can be used as a possible therapy for ameliorating renal I/R injuries (2,3,39,50,51). In recent decades, the effects of various antioxidants (natural or chemical) on I/R accompanied injuries were assessed using the measurement of total antioxidant capacity, catalase, superoxide dismutase, glutathione peroxidase, total oxidant status, oxidative stress index, myeloperoxidase, or malondialdehyde as

a lipid peroxidation marker. Pretreatment with garlic oil (39), *Nigella sativa* (52), NZ-419 (5-hydroxy-1-methylimidazole-2,4-dione) (53), vitamins C and E (54,55), and cepharanthine (56) could ameliorate these cell injuries. *Punica granatum* L. is a rich source of polyphenol contents with antioxidant capacity and various therapeutic effects (57,58). Tzulker et al. demonstrated that the antioxidant activity of the whole pomegranate fruit was higher than the levels found in aril juice and this activity in the peel's homogenate was much greater than that of the aril juice (16). Compared to other common fruit juices, pomegranate showed greater antioxidant capacity (59) with no toxicity in human volunteers and animal models (60,61). Our results underscored the high amount of total phenolic compounds in PPCM (224 ± 5 mg/g). As an indicator of antioxidant capacity, we have also shown the reduction of FRAP values in plasma following I/R, while a significant increase in antioxidant capacity was seen in littermates pretreated with PPCM aqueous extract. These results underline the robust antioxidant properties of this extract in ameliorating the effects of ROS on ER stress pathways.

To date, there is no evidence regarding the ER chaperone's expression and the effect of antioxidants during acute renal failure injuries.

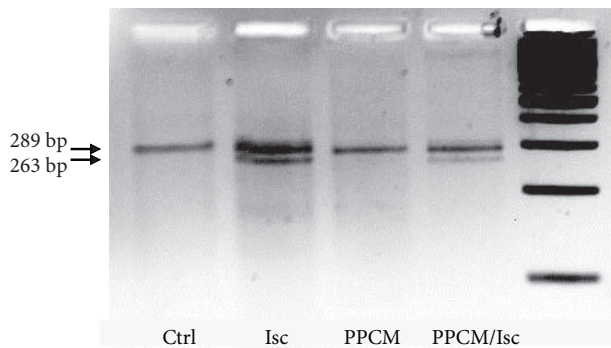


Figure 4. XBP1 splicing during I/R and effects of pomegranate. XBP1 splicing was detected by conventional PCR on a 4% agarose gel electrophoresis system. Sixty minutes of ischemia followed by reperfusion for 150 min induced splicing of XBP1, as revealed by 2 bands of 289 bp and 263 bp, while oral administration of PPCM aqueous extract lowered the spliced XBP1, underscoring its potential beneficial effects during I/R in rat kidney. Ctrl: control, Isc: ischemic, PPCM: pomegranate pith and carpellary membrane (PPCM) aqueous extract treatment, PPCM/Isc: PPCM aqueous extract treatment/ischemic.

In the present study, pretreatment with PPCM aqueous extract (250 mg/kg) for 10 days (twice a day) significantly reduced GRP78 mRNA levels. A possible explanation might be the reduction of GRP78 to the basic levels with

no possibility for further decrease. It is currently unclear if pomegranate extract can reduce GRP78 expression via other pathways, although it is possible that inflammation responses can play a role in this reduction. While I/R could induce the XBP1 splicing, pomegranate extract was able to reduce the XBP1 spliced form compared to unspliced XBP1, underscoring lessened ER stress. Although detailed molecular mechanisms by which PPCM aqueous extract down-regulate GRP78 mRNA levels await future studies, it seems plausible to conclude that pretreatment with PPCM may have potential protective effects against I/R-induced ER stress, although this protection at the molecular level needs further studies using less severe models of renal injury, more brutal pretreatment protocols with higher doses of PPCM, and/or longer periods of pretreatment time in order to achieve complete protection of kidney tissue according to a clinical viewpoint.

Acknowledgment

We thank Dr Hessam Dehghani and Dr Laleh Shayesteh for helpful discussions and technical support, and Mahnaz Kohanghadr and Maryam Torabi for technical assistance. These studies were supported by the Ferdowsi University of Mashhad, as AAG holds an Assistant Professor grant at the Ferdowsi University of Mashhad and a Research Chair at the Institute of Biotechnology.

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