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Original Article

Valproic acid restores the down-regulation of SDF-1 following kidney ischemia; experimental validation of a mathematical prediction

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Abstract

Background and purpose: Stromal-derived factor (SDF)-1, a chemokine recruiting leucocytes and stem cells, plays an essential role in tissue regeneration. In a previous study, we have unexpectedly found that the expression of this chemokine declines following kidney ischemia reperfusion (IR). To explain this observation, a mathematical model was constructed which proposed histone deacetylase (HDAC) as the main driver of SDF-1 down-regulation. To experimentally verify this prediction, the effect of valproic acid (VPA), a potent HDAC inhibitor, on the kinetics of kidney SDF-1 expression was here assessed.
Experimental approach*:* Adult mice were subjected to IR or sham operation and received VPA or vehicle. Next, SDF-1 expression as well as tissue repair indices were measured in a time course manner.
Findings / Results: The transcriptional expressions of *Sdf-1 alpha*, *beta*, and *gamma* isoforms were noisy in the sham groups but the fluctuations disappeared following IR where a continuous declining trend was observed. VPA induced the over-expression of gamma, but not alpha and beta mRNA in IR mice which was accompanied with protein upregulation. Remarkably, VPA deteriorated kidney injury.
Conclusion and implications: HDAC inhibition restores SDF-1 down-regulation following kidney IR. The present study is a classic example of the potential of computational modeling for the prediction of biomedical phenomena.

Keywords: Acute kidney injury; Histone deacetylase, Ischemia reperfusion injury; SDF-1; Valproic acid.

INTRODUCTION

Acute kidney injury is a common complication in hospital-admitted patients. It affects up to two thirds of intensive care unit patients and increases the length of stay, costs, and mortality rate (1). In spite of huge efforts, the current therapies are still supportive rather than curative (2-4). Stem cell therapies have been proposed as a promising alternative approach (5,6). However, several concerns such as the scarcity of homing to kidneys after systemic transplantation or even direct injection to target tissues has hindered their clinical application (7-10).

It is commonly believed that stromal-derived factor (SDF)-1 chemokine over-expresses following tissue injury and recruits CXCR4- and CXCR7-expressing stem cells and leucocytes (11-13). In previous studies, we were interested to identify the time in which SDF-1 reaches its maximum expression following kidney injury. This time could potentially be an appropriate time for the transplantation of stem cells genetically engineered to overexpress SDF-1 receptors (14). In order to determine the kinetics of SDF-1 in kidney injury, a time-course study was performed using a mouse model of ischemia reperfusion (IR).

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Unexpectedly, this gene demonstrated a declining trend in both mRNA and protein levels. So as to elucidate such condition, we constructed a Petri nets mathematical model of molecular interactions regulating SDF-1 expression, including hypoxia pathway elements (15). This model proposed that tissue hypoxia directly activates and indirectly inhibits SDF-1 expression. Sensitivity analysis of the model predicted that histone deacetylase (HDAC) is an influential mediator for SDF-1 down-regulation, whose inhibition may result in a rising peak in SDF-1 a few hours following the induction of ischemia. Accordingly, some recent studies have indicated the protective role of HDAC inhibitors in kidney injury (16-18). To the best of our knowledge, however, the effect of HDAC on SDF-1 expression has not yet been experimentally assessed.

The current study was performed to validate the mathematical model predictions. Valproic acid (VPA), a widely used HDAC inhibitor in animal studies (19-23) and FDA-approved drug for neuropsychiatric disorders, was administered to mice subjected to kidney IR or sham operation and the kinetics of SDF-1 was assessed in a time-course manner. In addition, the therapeutic effects of VPA on IR injury were investigated using biochemical and histopathology measures.

MATERIALS AND METHODS

Animal model of IR injury

Male BALB/c mice aged 6-8 weeks were obtained from Pasteur institute of Iran, Tehran, Iran. They were kept in normal light cycle with free access to food and water. All animal experiments were carried out according to the institutional guide for the use of laboratory animals. The study was approved by research ethics committee (approval code: IR.MUI.REC.1395.3.115).

The mice were anesthetized by intra-peritoneal injection of 115 mg/kg ketamine and 11.5 mg/kg xylazine (Alfasan, Woerden, Netherland) and were kept on a 37.5 °C warm stage with their eyes being covered with tetracycline ointment. The kidneys were assessed through a mid-abdominal incision under sterile conditions. Left kidney vasculatures were ligated for 35 min with an atraumatic vascular clamp (Medicon, Tuttlingen, Baden-Württemberg, Germany), after which the clamp was released and tissue reperfusion was visually inspected. During left kidney ischemia, right total nephrectomy was performed after triple ligation of the vessels with a 4/0 silk.

The abdominal wall incision was sutured and 1 mL dextrose saline was injected subcutaneously to prevent dehydration. For sham operation, the same procedure was performed except that the left kidney vessels were touched but not ligated with the vascular clamp (15).

VPA administration

According to previous studies (21-24), different doses of VPA (50, 150, 300, and 600 mg/kg) were examined in our preliminary experiments. A single injection of 150 mg/kg VPA was selected for further experiments as it was the maximum tolerable dose (data not shown).

VPA was dissolved in normal saline (NS) and administered through intra-peritoneal injection 1 h prior to ischemia. IR and sham mice receiving either VPA or NS were sacrificed at different intervals after operation. Blood was collected and after 24 h incubation in refrigerator, serum was obtained with two rounds of centrifugation at 2000 g for 6 min. In addition, left kidneys were harvested and following a coronal section, the anterior part was stored in 3.7% formaldehyde/phosphate buffered saline for histopathology analysis and the posterior part was divided into upper and lower sections used for RNA and protein extraction, respectively.

Biochemical assay

Blood urea nitrogen (BUN) was quantified with a diagnostic kit based on Berthelot method (Pars-Azmun, Tehran, Iran), according to the manufacturer's instructions. Colorimetric assay of final solutions was conducted using spectrophotometer (UNICO, Dayton, Ohio, USA) at 578 nm. Serum creatinine was measured through an enzymatic method by Cobas Integra analyzer (Roche, Indianapolis, Indiana, USA).

Table 1. The sequences of the primers.

Gene name	Forward sequences (5' to 3')	Reverse sequence (5' to 3')
Sdf1α	CTGTGCCCTTCAGATTGTTGC	CACCACTGCCCTTGCATC
Sdf1β	GCCAGAGCCAACGTCAAGC	GTTCCTCGGGCGTCTGACT
Sdf1γ	CTTCAGATTGTTGCACGGCTG	GTTACAAAGCGCCAGAGCAGA
Hprt	CGTCGTGATTAGCGATGATG	AGTCTTTCAGTCCTGTCCATAA
Tfrc	TGCATTGCGGACTGTAGAG	CCCACCAAACAAGTTAGAGAAT

Histopathological assessment

Formalin-fixed paraffin-embedded tissues were processed to provide 5 µm hematoxylin and eosin (H&E)-stained sections. Kidney slides were examined in a blinded manner and tissue injury was scored as previously described (25) with minor modifications.

Briefly, 100 tubules were inspected using a 40× objective in random cortical fields and a score ranging from 0 to 3 was assigned to each tubule (0: normal histology; 1: tubular cell swelling, brush border loss, and nuclear condensation, with loss of up to one third of the tubule nuclei; 2: same as for score 1, but more than one-third and less than two-thirds of the tubular profile showing nuclear loss; and 3: more than two-thirds of the tubular profile showing nuclear loss). The final injury score was the sum of all 100 tubule scores with a minimum and maximum of 0 and 300, respectively. In addition, 40 random cortical fields were assessed to determine the mean number of hyaline casts per high power field.

Real-time polymerase chain reaction

Kidney samples were lysed by Micro Smash™ tissue homogenizer (TOMY Digital Biology, Tokyo, Japan) at a rate of 4500 rpm for 40 sec. RNA extraction was performed with RNX-Plus (CinnaGen, Tehran, I.R. Iran). An equal combination of random hexamer and oligo dT primers was used for cDNA synthesis (Yekta Tajhiz Azma, Tehran, I.R. Iran).

Forward and reverse primers were designed for *Hprt*, *Tfrc*, *Sdf-1α*, *Sdf-1β,* and *Sdf1-γ* (Table 1) using AlleleID® and Gene Runner software. Gene expressions were quantified using SYBR green real time polymerase chain reaction (RT-PCR) kit (Ampliqon, Odense, Denmark) and Rotor Gene 6000 Machine (Corbett Life Science, Uithoorn, Netherlands).

Temperature profile consisted of an initial step of 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 30 sec, followed by melting curve analysis. The data was analyzed with ΔΔCt method using REST 2009 software.

Enzyme-linked immunosorbent assay

Kidney tissues were powdered in liquid nitrogen and lysed with radioimmuno-precipitation assay (RIPA) buffer (Cyto Matin Gene, Isfahan, Iran) according to the manufacturer's instructions. Protein concentrations were measured using Quick Start Bradford kit (BioRad, Hercules, California, USA) with bovine serum albumin, employed as standard. A concentration of 5 µg/mL of all samples was prepared for enzyme-linked immunosorbent assay (ELISA) assay. SDF-1 protein was quantified with RayBio® SDF-1 α ELISA kit (RayBiotech, Norcross, Georgia, USA) and Epoch™ Microplate Spectrophotometer (BioTek, Winooski, Vermont, USA).

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Statistical analysis was conducted with GraphPad Prism 5.01 (GraphPad Software, San Diego, USA). The differences between groups were assessed with Mann-Whitney U test. P values ≤ 0.05 were considered statistically significant.

RESULTS

VPA exhibited no healing effect based on biochemical and histopathological assessments

In this study, we developed a mouse model of kidney IR. The severity of kidney injury in IR particularly depends on ischemia time and body temperature. This model is labile as minimal changes in time and temperature may have a profound effect on outcome (26). Based on our previous studies and preliminary optimizations, we found that 35 min of left

kidney ischemia with the surgery stage temperature controlled at 37.5 °C are appropriate conditions for a reproducible model of kidney injury. In addition, right nephrectomy was performed in order to minimize variations as proposed by previous investigators (26). In all experiments at least 3 mice were incorporated in each group.

BUN and creatinine were significantly higher in IR mice compared to sham and normal controls indicating deteriorating kidney function following the insult. This was associated with kidney structure injury measured by histopathology indices. Therefore, the validity of IR induction protocol was corroborated using biochemical and histopathological parameters (Fig. 1, $P \leq 0.05$).

Both IR and sham-operated mice received a single dose of either VPA or NS one hour prior to the operation. In this regard, the experimental groups were IR + VPA, IR + NS, sham + VPA, and sham + NS. Three mice in each group were sacrificed 4, 12, 24, 36, and 48 h after the vascular clamp was released. In addition, five untreated normal mice were used to determine the baselines (Fig. 2A)..

VPA administration did not protect against kidney injury and even based on serum creatinine, pathology score, and number of hyaline casts, the severity of kidney injury in IR + VPA was significantly higher compared to IR + NS ($P \leq 0.05$, Fig. 2B-J).

The Sdf-1γ mRNA along with the protein were elevated in VPA-treated IR mice

To investigate the kinetics of SDF-1 after kidney ischemia and evaluate the prediction of our previously constructed mathematical model regarding the effect of HDAC inhibitor on SDF-1, the expressions of *Sdf-1α*, *Sdf-1β,* and *Sdf-1γ* were quantified in a time-course manner. In light of our foregoing study (27), we selected *Hprt* and *Tfrc* as stable reference genes.

Independent of VPA or NS administration, the expression of all three *Sdf-1* isoforms had notable fluctuations in sham-operated mice. Interestingly, these alterations disappeared following kidney injury as gene expression noise was minimal in both IR groups. In agreement with our previous data (15), the expression of all isoforms had a continuous declining trend in IR + NS group. Remarkably, VPA administration resulted in a significant *Sdf-1γ* over-expression with a sharp peak at 12 h post-ischemia ($P \leq 0.05$, Fig. 3A-F).

To investigate if the VPA-induced SDF-1 up-regulation can also be observed in the protein level, ELISA technique was utilized to measure SDF-1 protein concentrations. Similar to mRNA data, considerable fluctuations in the protein level were evident in both sham groups (Fig. 3G). These variations were replaced by a smooth decline after ischemia. In IR + VPA group, the protein expression increased in the first few hours which was followed by a decline at later time points ($P \leq 0.05$, Fig. 3H).

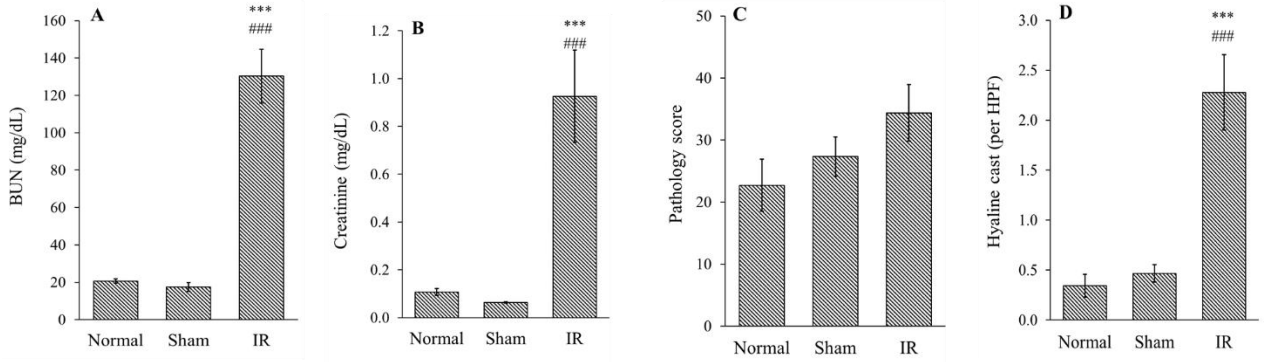

Fig. 1. The animal model of kidney IR was validated using biochemical (A, BUN and B, creatinine) and histopathological parameters (C, pathology score and D, hyaline cast). *, $^{\#}P < 0.05$ and ***, $^{\#\#\#}P < 0.001$ indicates significant differences compared with normal and sham groups, respectively. Data are mean ± SEM. IR, Ischemia reperfusion; BUN, blood urea nitrogen.

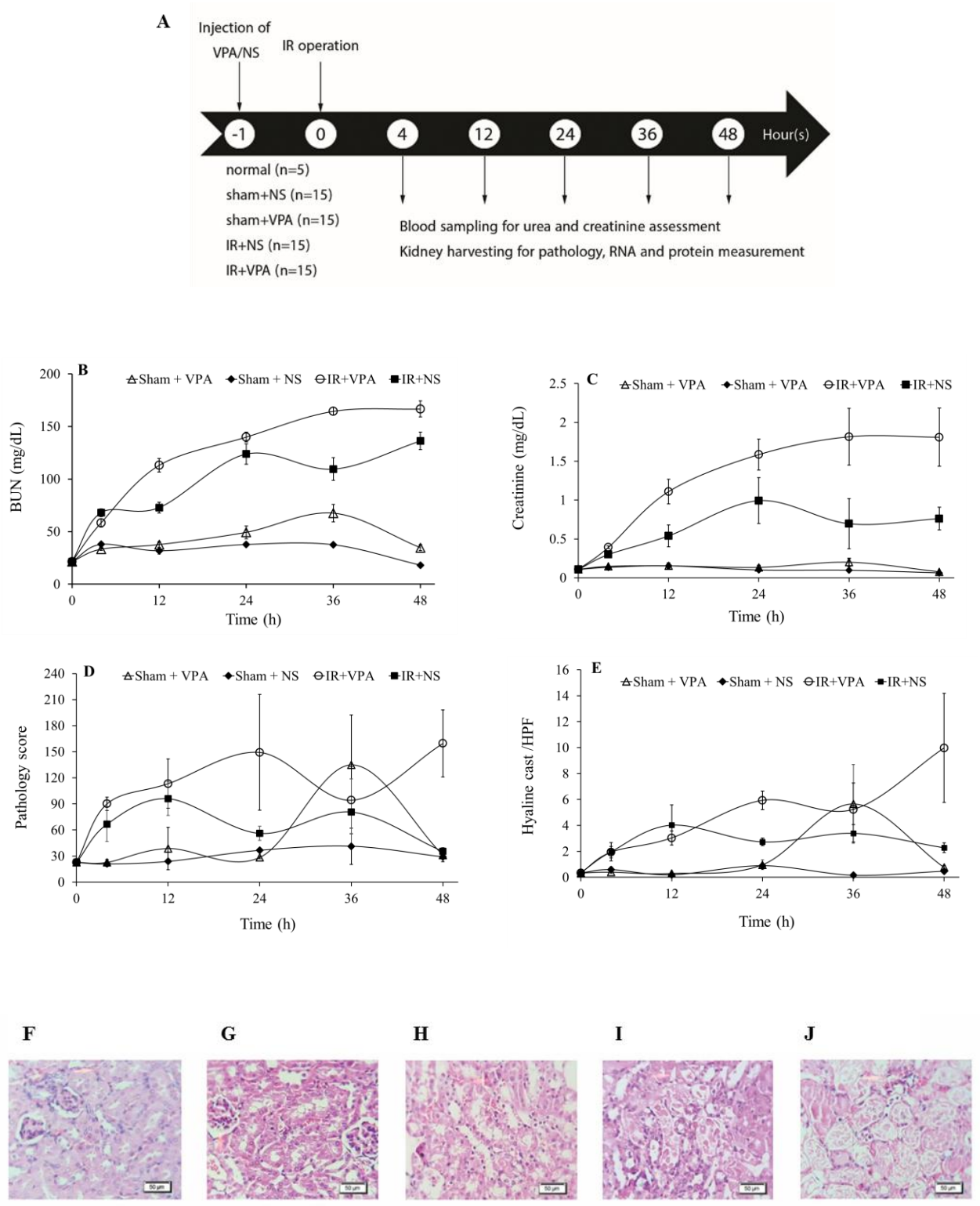

Fig. 2. VPA exacerbated IR-induced kidney injury. (A) IR or sham mice received either VPA or NS 1 h before the operation. Three mice in each group were harvested 4, 12, 24, 36, and 48 h after ischemia. Also, five untreated mice were harvested as normal controls. (B) BUN, (C) serum creatinine, (D) tissue injury score, and (E) number of hyaline casts were measured in IR + VPA, IR + NS, sham + VPA, and sham + NS groups in a time-course manner. Representative images of hematoxylin and eosin (H&E) staining in five groups including (F) normal group, (G) sham + NS, (H) sham + VPA, (I) IR + NS, and (J) IR + VPA are shown. *$P < 0.05$ indicates statistically significant differences between IR + VPA and IR + NS groups. Data are mean ± SEM. IR, Ischemia reperfusion; VPA, valproic acid; NS, normal saline.

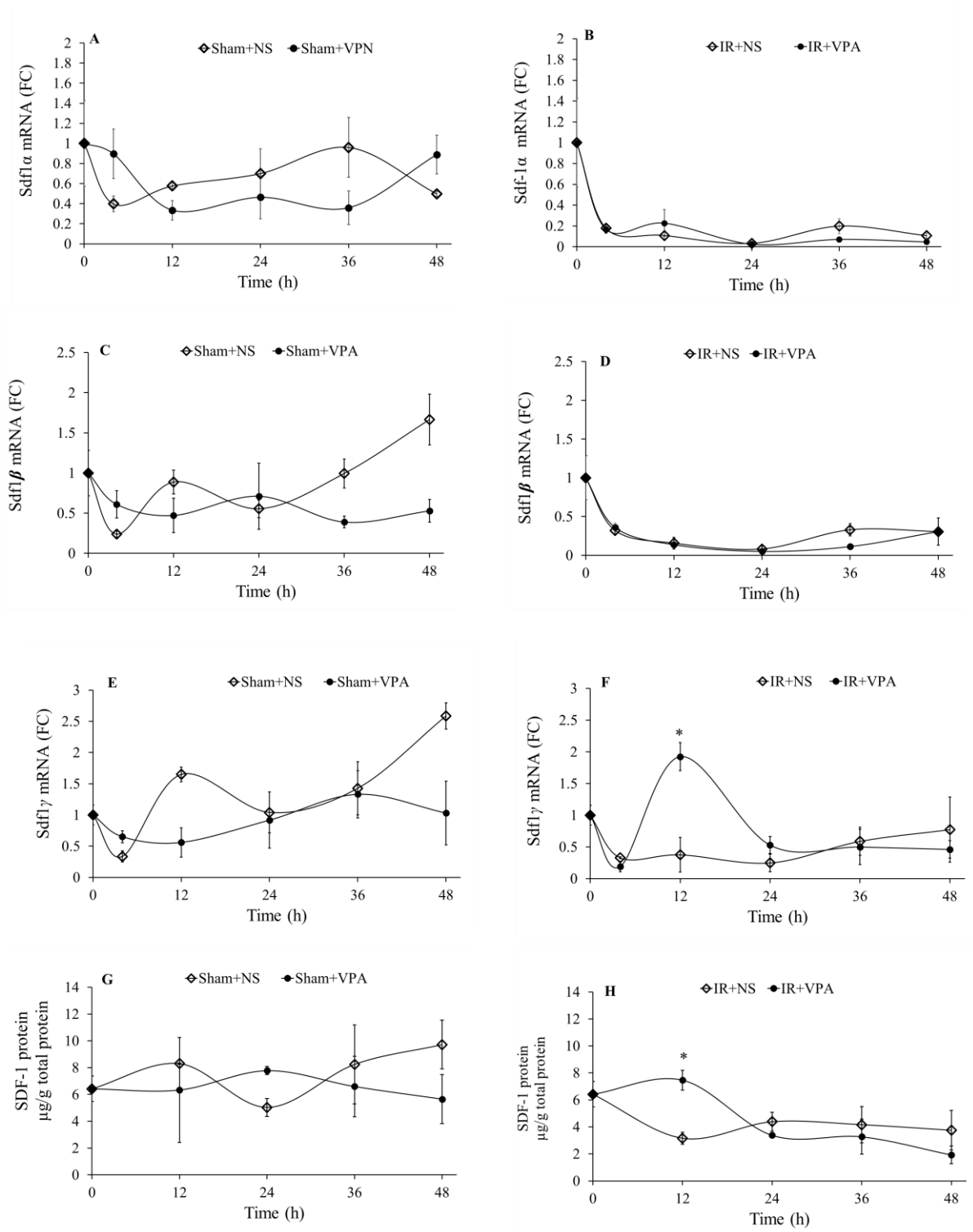

Fig. 3. VPA restores the down-regulation of SDF-1 following kidney ischemia. The expression of Sdf-1 alpha mRNA is measured over time in (A) sham + NS and sham + VPA groups as well as (B) IR + NS and IR+VPA groups. Also, (C and D) Sdf-1 beta mRNA is measured in sham + NS and sham + VPA groups and IR + NS and IR + VPA groups, respectively. In addition, (E and F) the expression of Sdf-1 gamma mRNA is quantified in sham + NS and sham + VPA groups as well as IR + NS and IR + VPA groups, respectively. Similarly, (G and H) SDF-1 is measured at the protein level in in sham + NS and sham + VPA groups as well as IR+NS and IR+VPA groups, respectively. *P < 0.05 indicates statistically significant differences between the groups at the same time points. IR, Ischemia reperfusion; NS, normal saline; SDF-1, stromal derived factor-1; VPA, valproic acid.

DISCUSSION

Acute kidney injury is a critical health problem with insufficient therapies. Although kidney has an intrinsic ability for self-repair, it is not capable of surmounting severe insults. The augmentation of this innate regeneration potential is a wise approach to develop novel therapeutics. In a recent study, we were interested in assessing the kinetics of SDF-1, a key regulator of tissue regeneration, and unexpectedly observed its down-regulation following kidney ischemia. To explain this observation, we developed a stochastic Petri nets-based model of hypoxia pathway that proposed HDAC as a potent negative regulator of SDF-1. The present study was designed to assess the validity of this model.

Our previous findings on SDF-1 decline after kidney IR was reproduced in the current project. In addition, a transient SDF-1 overexpression in both mRNA and protein levels was observed following a single dose of VPA administration. These experimental findings clearly validate the prediction of the Petri nets model. Interestingly, VPA-induced SDF-1 up-regulation was observed only in mice subjected to IR, implying that such mechanism probably depends on hypoxia pathway activation. Furthermore, the transcriptional overexpression of SDF-1 was isoform-specific, which probably specific gene regulatory circuits of each isoform have not yet been discovered. The current experience is a classic example showing the potential of systems biology bottom-up approach for the prediction and quantitative analysis of biological processes.

An interesting observation in this study is the significant variations in the basal transcriptional and translational gene expression in sham groups that disappeared after kidney injury. These alterations could be attributed to gene expression noise or endogenous periodic variations such as circadian rhythms. Interestingly, Wu *et al*. in a recent study, have shown that the hypoxia pathway and the circadian clock regulate each other and hypoxia signals dampen the amplitude of circadian oscillations (28). Although a considerable fraction of genes is estimated to have endogenous variations (29), it is commonly ignored in gene expression analysis experiments. We believe that it is crucial to design these studies in a time-course manner and follow not only test group(s) but also all controls over time to preclude the misinterpretation of data and discriminate between actual responses and those related to innate variations.

A notable observation in this study is that VPA administration was not associated with tissue repair. Instead, we observed that animals receiving VPA were more susceptible to the destructive effects of IR. Given the fact that we included different control groups, animal model validation, and blindness in histopathological assessments, it is unlikely that this result would be affected by technical errors.

Nevertheless, it is in contrast to a few previous studies showing that VPA attenuates kidney injury in rats subjected to IR (21,24,30). However, in line with our findings, it is shown in animal studies that VPA increases reactive oxygen species, lipid peroxidation, and mitochondrial dysfunction in kidneys and results in tubulointerstitial nephritis (31). Similarly, it is reported that chronic VPA therapy in children (32) and adults (33) with epilepsy has adverse effects on kidney function and is associated with subclinical kidney injury. Therefore, it has been suggested to monitor kidney function in high risk patients taking this medication (33).

CONCLUSIONS

In agreement with a previously constructed mathematical model, we have here experimentally shown that VPA up-regulates SDF-1 in mice subjected to kidney ischemia. This study is a scenario exhibiting the value of computational modeling for the analysis and prediction of living organisms. Moreover, this report is in favor of the studies suggesting that VPA has adverse effects on kidney function.

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CONFLICT OF INTEREST STATMENTS

The authors declare no conflict of interest for this study.

AUTHORS' CONTRIBUTION

K. Moradzadeh performed the experiments, collected and analyzed the data, and prepared the first draft of the manuscript. S.M. Nassiri performed histopathological assessments and contributed in the preparation of the manuscript. Y. Gheisari designed the experiments, supervised data collection and analysis and critically edited the text. All authors actively contributed in this work, revised final manuscript and approve it.

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