

## ORIGINAL ARTICLE

# Silencing HMGB1 expression inhibits adriamycin's heart toxicity via TLR4 dependent manner through MAPK signal transduction

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## Summary

**Purpose:** Adriamycin (ADR) is a commonly used anti-cancer drug. ADR has toxic effects on cardiomyocytes and leads to heart failure. However, the underlying mechanism(s) by which ADR causes heart failure is still not clarified exactly. The aim of present study is to investigate whether ADR-induced heart failure is mediated via HMGB1/TLR4 to initiate the apoptosis through MAPK/AMPK pathways.

**Methods:** H9c2 cell line was used to create four groups as a control, HMGB1 inhibition, ADR, ADR+HMGB1 inhibition. Silencing HMGB1 was performed with specific small interfering RNA. ADR was used at 2  $\mu$ M concentration for 36 and 48 hours. Protein and genes expressions, apoptosis was measured.

**Results:** Although ADR decreased AMPK, pAMPK, ERK1/2, pERK1/2, p38, JNK protein expression, ADR+HMGB1 inhibition led to change those protein expressions. The effect of silencing of HMGB1 prevented apoptosis induced by ADR in

the cells. HMGB1 caused changes a kind of posttranscriptional modification on the TLR4 receptor. This posttranscriptional modification of TLR4 receptor led to decreased AMPK protein level, but phosphorylated-AMPK. This alternation of AMPK protein caused enhancing of JNK protein, resulting from the decline of p38 and ERK protein levels. Eventually, JNK triggered apoptosis by a caspase-dependent pathway. The number of TUNEL positive and active caspase 8 cells at ADR was high, although HMGB1 silencing could decrease the cell numbers.

**Conclusions:** Inhibition of HMGB1 might prevent the lose of the cardiac cell by inhibition of apoptotic pathway, therefore HMGB1 plays an essential role as amplifying on ADR toxicity on the heart by TLR4.

**Key words:** adriamycin, HMGB1, AMPK, TLR4, apoptosis, cardiac muscle cell

## Introduction

Adriamycin (ADR), also called doxorubicin, is an anticancer drug widely used in the treatment of cancer since 1960s. However, it has adverse effects on many tissues such as liver, kidney and especially on the heart. Its toxic effect on the heart limits the use of this drug in the treatment of cancer patients.

ADR-induced cardiotoxicity and -heart failure can be observed shortly or years later after its administration [1]. Heart failure develops in 5-65% of cancer patients treated with ADR [2,3]. Free reactive oxygen species (ROS) overproduction, disruption of cellular and mitochondrial  $Ca^{+2}$  homeostasis,

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induction of mitochondrial DNA breaks, impaired mitochondrial energy production, destruction of contraction proteins and cytoskeletal proteins including titin and dystrophin are suggested to be underlying mechanisms for ADR-induced cardiotoxicity. Each of these pathways can contribute to cardiac cell damage and ultimately apoptosis or necrosis [4,5]. However, the mechanisms that lead to the development of ADR-induced heart failure have not yet been fully elucidated. Therefore, an exact treatment strategy for the prevention or attenuation of the severity of ADR-induced heart failure in cancer patient has still not been developed.

High-mobility group box 1 (HMGB1) is a well-preserved nuclear protein among species, acting as an alarm signal [6]. In addition to its nuclear role, HMGB1 also functions as an extracellular signaling molecule in several processes such as tissue regeneration, infection, cell differentiation, and tumor development [7]. HMGB1 is passively secreted from necrotic cells. Also, HMGB1 has been shown to be secreted under pathological conditions [8]. HMGB1 can bind to at least five different cell receptors such as advanced glycosylation end-product receptor (RAGE), TLR2, TLR4 [7]. It has been reported that HMGB1 is also released in oxidative stress and activates MAPKs (ERK, P38, JNK, SAPKs), NF- $\kappa$ B, calcium/calmodulin-dependent protein kinase (CaMKK $\beta$ ) after interaction with its receptors [8, 9]. Moreover, HMGB1 exerts its cellular actions in infiltration related apoptosis mediated by lipopolysaccharide-induced myocardial insufficiency and ischemia-reperfusion injury via TNF- $\alpha$  [8]. A recent study indicates that HMGB1 also takes part in cardiotoxicity induced by ADR via autophagy [10].

The debate is going on the role of HMGB1 in apoptotic pathways in ADR-induced cardiotoxicity. A previous study indicated that cardiac apoptosis and dysfunction was diminished via blockage of HMGB1 in ADR exposed cardiac cells [8]. However, another study showed that up-regulation of HMGB1 expression in ADR-induced cardiotoxicity resulted in increased expression of heat shock protein beta 1 via attenuating cardiac apoptosis and mitochondrial dysfunctions [11]. In other words, ADR could trigger intracellular pathway(s) to amplify its impact on tissues. According to scientific ranking, HMGB1 can be a good candidate for participation in ADR-induced toxicity. In the light of these knowledges, the underlying mechanisms mediated by HMGB1 in cardiac apoptosis after exposure of cardiac myocytes to ADR is not still clear. In this study, it is aimed to investigate the possible role of HMGB1 in ADR-induced apoptotic pathways in H9c2 cell line.

## Methods

### *Cell culture and treatment*

H9c2 cells was grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% bovine serum albumin (BSA), 2 mM glutamine and antibiotics (100 IU/mL penicillin, 0.1 mg/mL streptomycin) in aseptic cell culture with various litter cell culture flasks at 37°C and 5% carbon dioxide. The cells in the flask started to work when they grew by 80%.

### *Experimental groups*

In the present study, our hypothesis is that HMGB1 participate in ADR-induced cytotoxicity in cardiomyocytes. To test the hypothesis, HMGB1 gene was silenced by using siRNA [12] and four main experimental groups were created; (I) Control: DMEM, (II) siRNA: HMGB1 silenced (10 nM), (III) ADR: adriamycin treatment (2  $\mu$ M), (IV) siRNA + ADR: adriamycin treatment and HMGB1 silenced. The siRNA inhibition of HMGB1 by lipofectamine and ADR treatment were done at the same time. If HMGB1 really involves ADR's toxic effect on tissues, the synergetic effects could be evaluated when the silence of HMGB1 opted the beginning of ADR treatment. That is why, both treatments was preferred simultaneously to start.

### *Transfection of H2c9 cells with HMGB1 siRNA*

Cells were transfected with HMGB1 siRNA (Cat No: L-114889-00-0005; GE Healthcare Dharmacon) to suppress HMGB1 gene expression in cells. Non-targeting siRNA (Cat No: D-001810-10-05; GE Healthcare Dharmacon) was used as a negative control. A hyperfect transfection agent (Cat No. 301702; Qiagen) was used for the transfection. Transfection optimization procedures were performed using different siRNA, transfection agent ratio. As a result of this trial, it was determined that 24 hours were not enough to silence the HMGB1 gene with siRNA. There was no significant difference in gene expression levels between the control and treatment group (data not shown). Therefore, the relationship between HMGB1 and ADR were investigating depending on two periods: 36 and 48-hour interactions. After the time that is necessary for obtaining the efficient effect of siRNA was determined, the effective concentration was determined. For this purpose, siRNA was administrated at 10, 25 and 50 nM concentrations by using 6- $\mu$ L transfection agent. The ratio of HMGB1 gene suppression was 82% when 10 nM siRNA was used, 79% when 25 nM siRNA was used, and 85% when 50 nM siRNA was used. We obtained that there was no significant difference between the concentrations of 10 and 50 nM siRNA. As a result, it was decided to use 10 nM siRNA in concentration.

### *Determination of gene expression*

RNA was isolated from cells and cDNA was synthesized and then gene expression levels were determined by Quantitative RT-PCR.

### *RNA isolation*

The cultured cells were first centrifuged at 1500

rpm and the cells remaining at the bottom were washed 2 times with PBS after the medium was discarded. After the supernatant was poured, RNA was isolated from the remaining cells using the Roche RNA isolation kit according to the protocol. The RNA concentration and purity were calculated by taking 1  $\mu$ L of the samples at the 260 nm wavelength by a Nanodrop device. It was also checked by running on a 1% gel.

#### RT-PCR

cDNA was synthesized using 1  $\mu$ g of total RNA in 20  $\mu$ L volume and random primers using the Roche Transcriptor cDNA synthesis kit according to the manufacturer's protocol.

#### Analysis of gene expression by real-time quantitative PCR

Real-time quantitative PCR (Light Cycler, Roche Diagnostics GmbH, Germany) was used for quantitation. Primers for quantitative PCR were designed. For the PCR reaction, Light Cycler 480 SYBR Green Master Kit (Roche, Mannheim, Germany) and 250 ng cDNA sample were used. For the normalization of RNA quality and quantity in the samples, beta-actin was used as a control. PCR was run at 95°C for 60 second (1 cycle) after preliminary denaturation, at 95°C for 10 seconds, at 62°C for 5 seconds, and at 72°C for 20 seconds (45 cycles). After the amplification, the program applied for melting curve analysis was 0 seconds at 95°C, 10 seconds at 65°C and 0 seconds (1 cycle) at 95°C. For each PCR reaction, water was used as a negative control instead of cDNA. Serial dilutions were prepared for each standard and each dilution and each sample was run in duplicate.

#### Apoptotic cell count

TUNEL kit (Roche, Cat no: 11684795910) was used to determine the number of apoptotic cells. 150,000 cells were plated on glass coverslips for this purpose. Experimental groups were formed by applying ADR and/or siRNA for 36- and 48-h to H9c2 cells, which were expected to adhere to glass for 24 h. DMEM is applied to control and negative control groups. Following the establishment of the experimental groups, the manufacturer's protocol was followed. Briefly, freshly prepared 2% paraformaldehyde was added to 2 mL coverslips and incubated for 40 min and fixation of the cells was achieved. Paraformaldehyde was removed by washing with phosphate buffer (PBS) 3 times for 2 min. Then, permeabilization (expansion of cell pores) with 0.02% Triton X-100 detergent was performed on ice for 10 min to allow easy entry of the dye in TUNEL kit into the cell. After, washing was performed 3 times with PBS for 2 min each. The labeling solution and the enzyme solution were mixed to freshly prepared master mixture. Enzyme-free labeling solution was used for negative control. Incubation was made in the dark and humidified atmosphere for 80 min. Finally, in the dark, the washing process was again carried out 3 times for 2 min each. Field scanning was performed using immunofluorescence microscopy at 20x, 40x and 100x objectives. The number of these red-stained nuclei was counted using the ImageJ program.

#### Determination of active caspase 8 by immunofluorescence method

In this analysis, IETD-FMK (FITC-IETD-FMK), a FITC-linked caspase 8 (Cas-8) inhibitor, was used as a marker. FITC-IETD-FMK is non-toxic and binds irreversibly to activated Cas-8 found in apoptotic cells. Active Cas-8 was determined by immunofluorescence method according to the manufacturer's protocol (Roche). Briefly, for each coverslip, 1  $\mu$ L FITC-IETD-FMK was added to 500  $\mu$ L of DMEM. Cells were incubated for 75 min in a 37°C incubator in FITC medium. Following the completion of the incubation period, washing was carried out twice using a washing solution for 5 min each. Then, the samples were left to dry in the dark at room temperature for 10 min. Then, samples were scanned and imaged at 20x, 40x and 100x by using a fluorescence microscope.

#### Western blot analysis

The cell lysate proteins were loaded into the wells of the gels in such a way that 100  $\mu$ g of protein was uniformly sampled from the samples to be separated into bands according to their molecular weights by electrophoresis. After loading, the proteins were run in gel electrophoresis (Invitrogen) for approximately one hour under 130 volts. Proteins carried in gel electrophoresis were transferred to the membrane using the iBlot device (Invitrogen). A signal enhancer kit (Super Signal™, Invitrogen) was applied in order to make the protein bands to be obtained clearer and prominent before proceeding with the standard western blot protocol. The membrane was sacrificed with a solution containing BSA (5%) and blocked for 1 h at room temperature with an orbital mixer to minimize the nonspecific binding between membrane and antibody. Each of the primary antibodies (AMPK, TLR4, HMGB1, P38, SAPK / JNK, ERK 1/2, Cytochrome C, AIF, Cas-3, APAF-1,  $\beta$ -actin, pAMPK, pP38, pJNK, pERK 1/2) was diluted according to the manufacturer's protocol and the enhancer was added as blocking buffer. Membranes were then incubated with primary antibodies with orbital mixer at +4°C overnight. PVDF membranes were washed three times with PBS-T for 10 min. PVDF membranes were incubated in secondary antibody (anti-rabbit and anti-mouse, 1/3000) and blocking buffer (5% BSA + PBS-T) for 1 h at room temperature using an orbital mixer. Then, membranes were washed three times for 10 min with PBS-T solution. Alkaline phosphatase (BCIP / NBT) was used for imaging. Targeted proteins were identified using markers with standard molecular weights. Then, protein bands were photographed and analyzed with the Image J program.

#### Statistics

Data were evaluated using SPSS 21. The distribution of the data was determined by Kolmogorov-Smirnov and Levene tests. In the evaluation of the significance of the difference between the averages of more than two groups; normal distribution conditions, one-way ANOVA followed by Tukey test as a *post-hoc* test was performed. The significance level was accepted as  $p < 0.05$ . Values are given as mean  $\pm$  standard error (mean  $\pm$  SE).

## Results

### Gene expression change in 36 hours

We explored the role of ADR in apoptosis via HMGB1 gene. As shown in Figure 1, the finding suggested that ADR treatment resulted in a three-fold reduction of HMGB1 gene expression. Next, to detect the role of HMGB1 in ADR-induced cardiac apoptosis, we silenced HMGB1 gene in control and ADR treated cardiomyocytes. Silencing of HMGB1 gene by siRNA resulted in a 3.5-fold reduction of HMGB1 gene expression compared to the control group. However, HMGB1 gene expression was suppressed seven-fold in cardiomyocytes treated with ADR and siRNA. ADR treatment led to a two-fold increase in TLR4 gene expression, 3.5-fold reduction in AMPK, a two-fold increase in ERK1 and 1.7-fold decrease in ERK2 gene expression and a five-fold increase in cytochrome-C gene expression. Silencing of HMGB1 gene resulted in a 0.5-fold increase in TLR4, 0.5-fold reduction in AMPK, 0.2-fold reduction in ERK1 and 0.1-fold decrease in cytochrome-C gene expression. Treatment of cardiomyocytes with ADR and siRNA led to a three-fold increase in TLR4, a 4.5-fold decrease in AMPK, a 2.2-fold increase in ERK1 and 3.8-fold decrease in ERK2 and a seven-fold increase in cytochrome-C gene expression.

### Gene expression change in 48 hours

As shown in Figure 1, siRNA transfection to cardiomyocytes resulted in a 2.5 fold reduction in HMGB1 gene expression. ADR treatment cause HMGB1 gene expression to decrease 1.1-fold. HMGB1 gene expression was reduced three-fold in cardiomyocytes treated with ADR and siRNA. ADR treatment resulted in a one-fold increase in TLR4, a 1.6-fold increase in ERK1 and 1.2-fold increase in ERK2. In addition, AMPK and cytochrome-C gene expression were almost suppressed in ADR treated cardiomyocytes. The silencing HMGB1 gene by

siRNA led to a 0.3-fold increase in TLR4, a 0.3-fold decrease in AMPK, a 0.5-fold decrease in ERK1 and 1.2-fold increase in ERK 2 and one-fold increase in cytochrome-C gene expression. Co-treatment of cardiomyocytes with ADR and siRNA resulted in a five-fold increase in TLR4, a 1.4-fold increase in ERK1 and 1.3-fold increase in ERK2 and 1.3-fold increase in cytochrome-C gene expression. In addition, AMPK gene expression was completely suppressed in cardiomyocytes treated with ADR and siRNA.

### Protein expression

#### HMGB1 protein

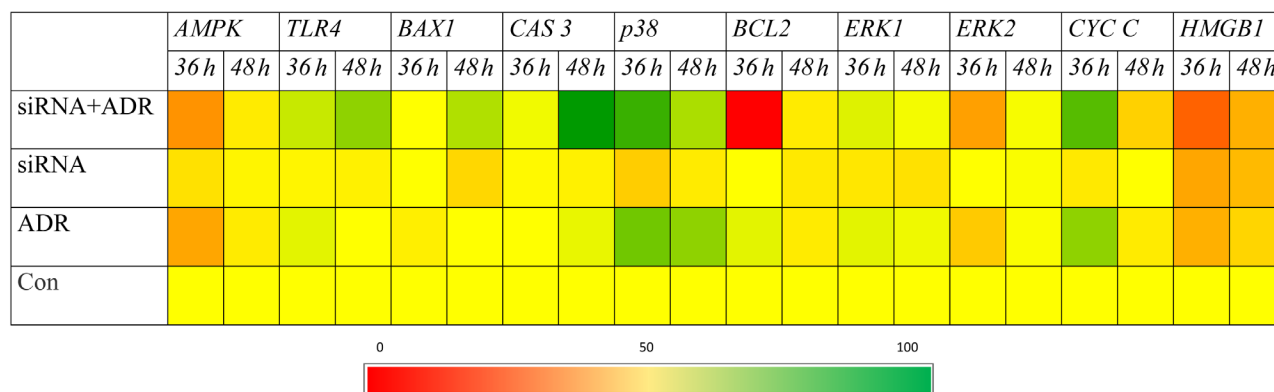
The silencing of HMGB1 gene resulted in a decrease in expression of HMGB1 protein at 36h and 48h (Figure 2a). The protein expression of HMGB1 showed a significant enhance in ADR treated cardiomyocytes compared to control cell at 36h and 48h. Co-treatment of cardiomyocytes with ADR and siRNA caused HMGB1 protein expression to decrease at both exposed time.

#### TLR4 receptor protein

TLR4 protein expression in the ADR group at 250 kDa decreased compared to the others, except siRNA+ADR group (Figure 2a). Although TLR4 protein expression in 100 kDa in siRNA+ADR attenuated, there was seen a new band at around 150 kDa. Moreover, the new band at 150 kDa in ADR group was seen, but the expression of new 150 kDa in ADR was higher than the siRNA+ADR group in both exposure time.

#### AMPK- $\alpha$ 1 protein

ADR treatment attenuated the protein expression of AMPK- $\alpha$ 1 when compared to control and siRNA at 36h and 48h treatment (Figure 2a). AMPK protein expression increased in cardiomyocytes treated with ADR and siRNA compared to ADR treatment at 36h and 48h treatment.



**Figure 1.** The mRNA expressions in the groups. Con: control group; siRNA: HMGB1 silence group; ADR: Adriamycin group; siRNA+ADR: HMGB1 silence plus adriamycin group. Data are expressed relative to that of  $\beta$ -actin.



#### *Phosphorylated AMPK- $\alpha$ 1 protein*

It was interesting that the silencing of HMGB1 gene by siRNA did not change the protein expression of pAMPK compared to control (Figure 2a). However, ADR treatment increased pAMPK protein expression. The co-treatment of cardiomyocytes with ADR and siRNA decreased pAMPK protein expression when compared to ADR.

#### *Phosphorylated AMPK- $\alpha$ 1*

Interestingly, western blotting showed pAMPK- $\alpha$ 1 bands at 65 and 50 kDa. siRNA increased the protein expression of pAMPK- $\alpha$ 1 at both 65 and 50 kDa compared to control (Figure 2a). However, ADR decreased the protein expression of pAMPK- $\alpha$ 1 at 65 kDa though increased it at 50 kDa in contrast to control. pAMPK- $\alpha$ 1 protein expression was found to increase at 65 kDa and decrease at 50 kDa in cardiomyocytes treated with ADR and siRNA compared to ADR.

#### *SAPK / JNK protein*

ADR decreased SAPK/JNK protein expression at both exposed duration although there was no change in SAPK/JNK protein expression in control and siRNA groups (Figure 2b). SAPK/JNK protein expression increased in cardiomyocytes treated with ADR and siRNA compared to ADR at both exposed duration.

#### *Phosphorylated JNK protein*

The silencing of HMGB1 gene by siRNA alleviated phosphorylated JNK (pJNK) protein levels compared to control at 36h and 48h treatment (Figure 2b). Although ADR treatment attenuated pJNK protein levels compared to the control group, the protein expression levels of pJNK were higher in cardiomyocytes treated with ADR and siRNA than that in the ADR group at 36h and 48h treatment.

#### *P38 protein*

The silencing of HMGB1 by siRNA elevated P38 protein expression compared to control at 36h and 48h (Figure 2b). Although ADR decreased P38 protein expression, the co-treatment with ADR and siRNA increased P38 protein expression compared to ADR alone at 36h and 48 h.

#### *Phosphorylated P38*

Phosphorylated P38 (pP38) protein expression was lower in siRNA group compared to the control group at 36h treatment (Figure 2b). ADR treatment decreased pP38 protein expression although ADR administration with siRNA increased pP38 protein expression compared to ADR at 36h and 48h.

#### *ERK 1/2 protein*

siRNA administration increased ERK 1/2 protein expression compared to control. ADR decreased ERK1/2 protein expression compared to control. Co-treatment with ADR and siRNA increased ERK1/2 protein expression compared to ADR (Figure 2b).

#### *Phosphorylated ERK 1/2*

Phosphorylated ERK 1/2 protein (pERK 1/2) in siRNA groups was more apparent than that in the control group at 36 and 48h treatments. ADR treatment decreased pERK 1/2 protein expression in contrast to control groups at 36 h but increased at 48h treatment. The treatment of cardiomyocytes with ADR and siRNA resulted in a decrease in the expression of pERK1/2 protein when compared to ADR at 48h treatment (Figure 2b).

#### *Cytochrome-C*

Cytochrome-C is one of the most important regulatory proteins in the initiation of the apoptotic pathway. Therefore, we explored the change in protein expression of cytochrome-C. The result indicated that there were no significant changes in cytochrome-C protein expression among groups at 36h treatment. But, the protein expression of cytochrome-C at 48 h in the ADR group decreased compared to the others (Figure 2c).

#### *Apoptosis-inducing factor*

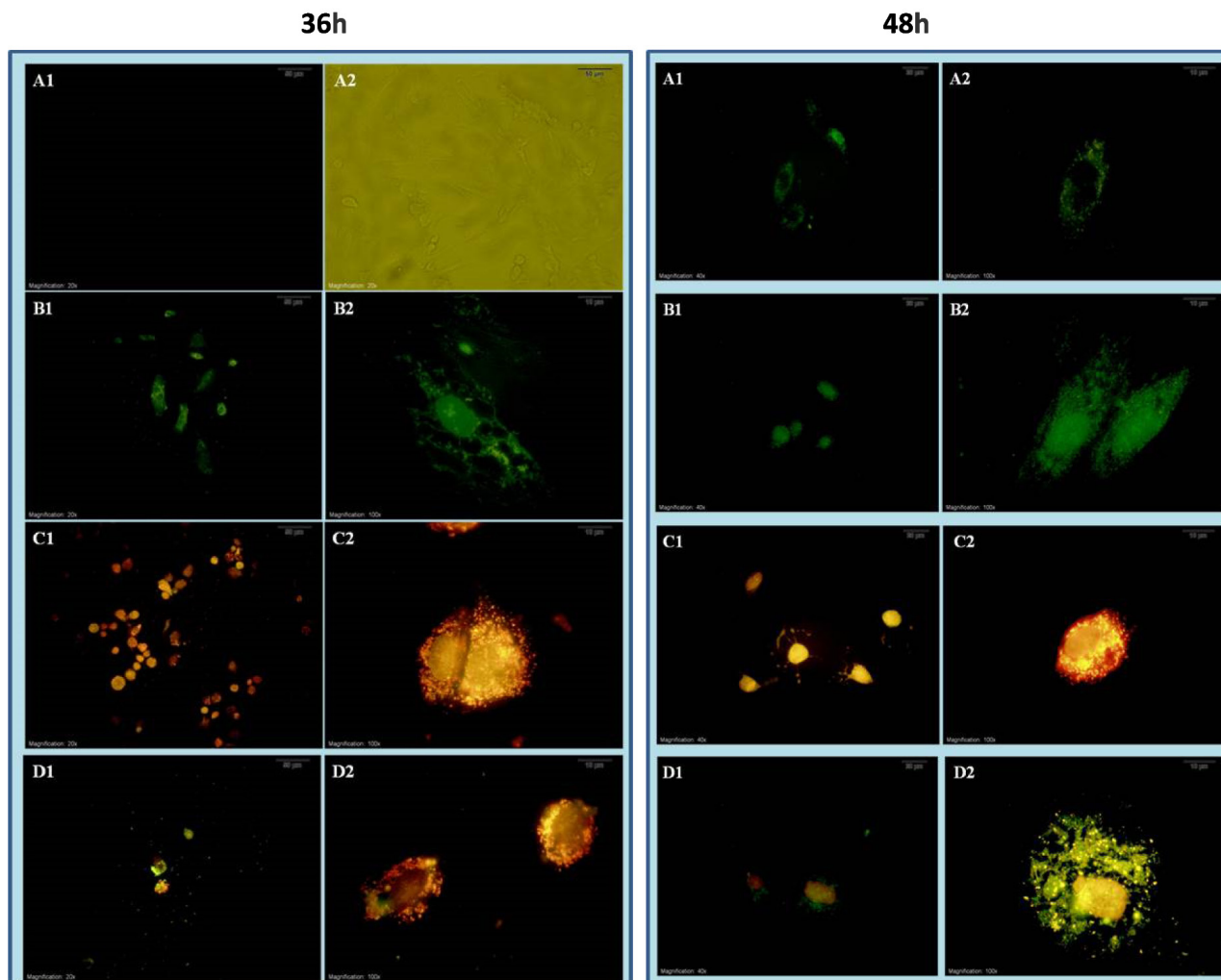
Apoptosis-inducing factor (AIF) is an important protein activating the apoptotic pathways, independently from caspases. AIF protein expression was found to be lower in ADR and co-treatment with ADR and siRNA compared to control at 36h treatment (Figure 2c). But there is no difference between groups at 48 h treatment.

#### *Apoptotic peptidase activating factor 1*

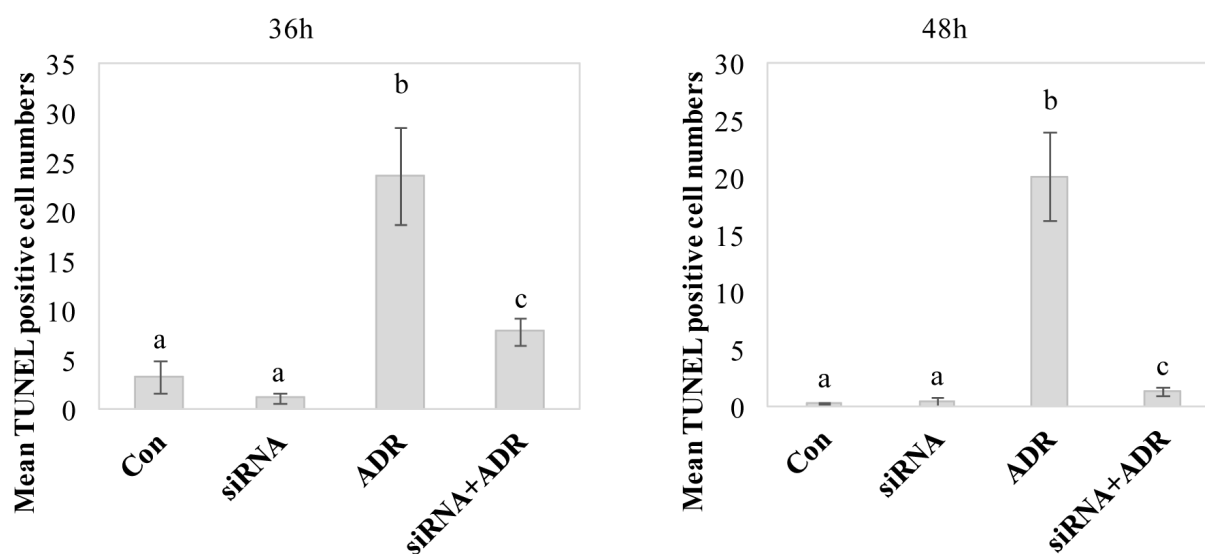
siRNA did not change the protein expression of apoptotic peptidase activating factor 1 (APAF-1) compared to control (Figure 2c). ADR increased the expression of APAF-1 protein at 36 and 48h treatment although the protein levels in co-treatment with ADR and siRNA was found to be lower in the ADR group at both duration.

#### *Caspase-3*

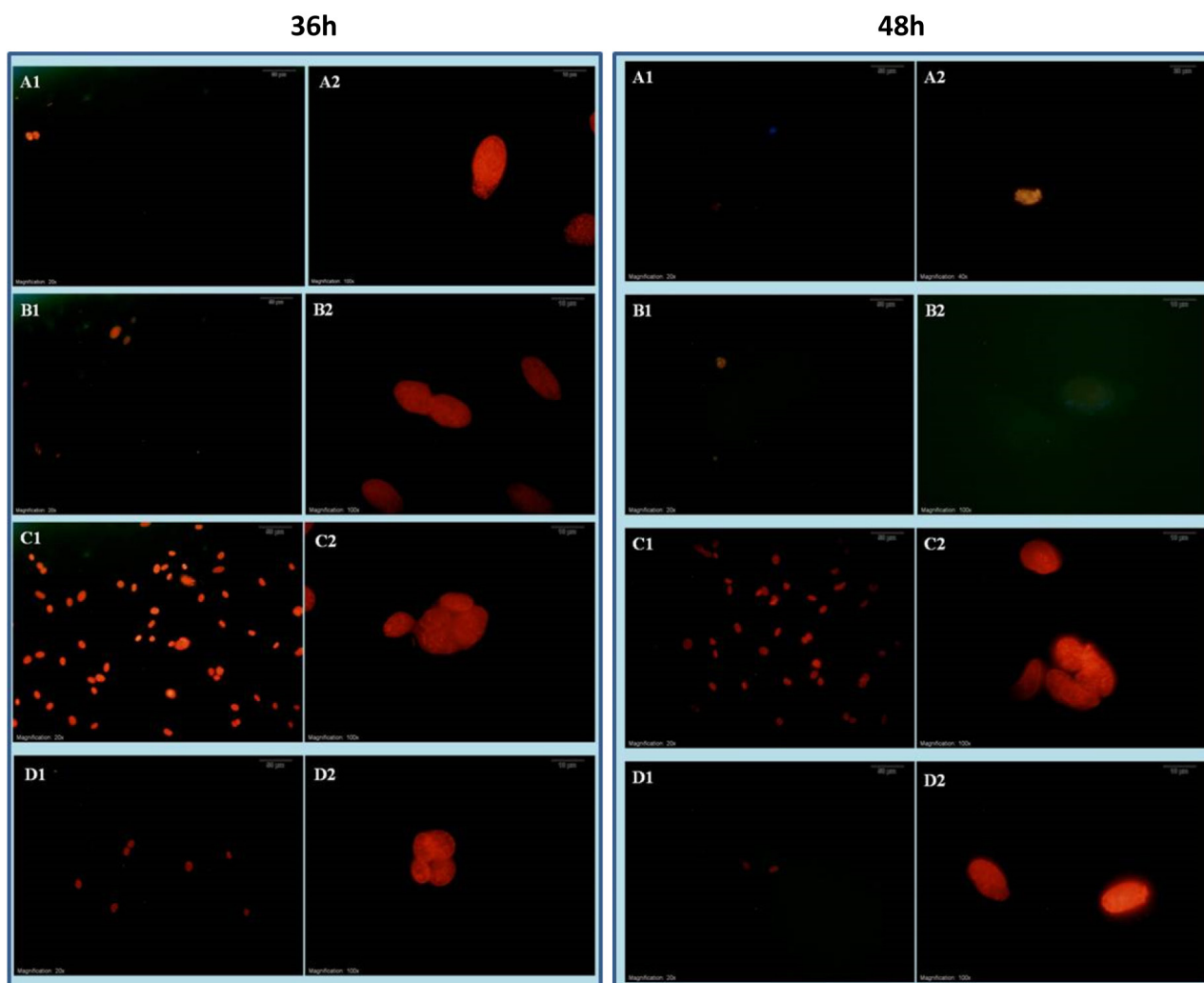
siRNA downregulated caspase-3 (Cas-3) protein in contrast to control at 36 and 48h treatments. ADR upregulated protein expression of Cas-3, although the protein expression level of Cas3 was lower in cardiomyocytes co-treated with ADR and siRNA than that in ADR at both duration (Figure 2c).



**Figure 3.** Active Caspase-8 in H9c2 cells treated with adriamycin after 36 h and 48 h treatment. First number of each alphabet represents 20x and its second number represents 100x magnification. A1-A2: Control group, B1-B2: siRNA group, C1-C2: Adriamycin group, D1-D2: HMGB1 silence plus adriamycin group. Although adriamycin enhanced active Caspase-8 number, HMGB1 silencing caused decrease of active caspase-8 number.



**Figure 4.** Apoptotic cell counts in H9c2 cells treated with adriamycin after 36 h and 48 h treatment. Con: control group; siRNA: HMGB1 silence group; ADR: Adriamycin group; siRNA+ADR: HMGB1 silence plus adriamycin group. Different letters indicate significant differences between groups ( $p < 0.05$ ).



**Figure 5.** Apoptosis in H9c2 cells treated with adriamycin after 36h and 48h treatment. First number of each alphabet represents 20x and its second number represents 100x magnification. A1-A2: Control group, B1-B2: siRNA group, C1-C2: Adriamycin group, D1-D2: HMGB1 silence plus adriamycin group. Although adriamycin enhanced TUNEL-Positive cell numbers, HMGB1 silencing caused decrease TUNEL-positive cell numbers.

#### *Active Caspase 8 analysis after 36- and 48-hour treatments*

After 36h, ADR led to an increase in the number of active Cas-8 (apoptotic cell) compared to control (Figure 3). The administration of siRNA to cell resulted in a decreased number of apoptotic cell in ADR-induced cell in contrast to the ADR group. Similarly, after 48h the cells exposed to ADR had intense active Cas-8. The silencing of HMGB1 gene by siRNA resulted in decreased activity of active caspase in ADR administered cells, supporting the role of HMGB1 gene in ADR-induced apoptotic pathways in cardiomyocytes.

#### *TUNEL positive cell number after 36-hour and 48-hour treatments*

After 36h and 48h, the number of TUNNEL positive cells significantly increased in ADR injected cells compared to control and siRNA groups. The

silencing of HMGB1 gene by siRNA resulted in decreasing number of TUNNEL positive cells in contrast to the ADR group (Figures 4 and 5).

## **Discussion**

The most important findings of the present study are (I) ADR exerts its undesired effect on cardiac cells via HMGB1; (II) HMGB1 causes a posttranslational change in TLR4 receptor; (III) TLR4 activates MAPK, resulting in apoptosis by the caspase-dependent pathway.

ADR causes a cytotoxic effect on noncancerous tissue, especially on heart. Therefore, it is important to clarify the underlying mechanism of its toxic effect. The idea of the present study was that ADR may possibly participate in its toxic effect on noncancerous tissue through intracellular pathways. One of candidate agents might be HMGB1



because it is one of the damage-associated molecular pattern (DAMP) [13]. In the other words, HMGB1 acts as a molecule associated with extracellular damage [14]. It acts as an extracellular signaling molecule, especially in acute and chronic inflammation. While the excess of HMGB1 plays a pathological role in cardiovascular diseases, it may be beneficial to decrease the level of HMGB1. So, ADR has been reported to increase the protein levels of HMGB1 [10,15]. The mRNA data of HMGB1 in the present study represented to decrease, whereas the significant elevation on HMGB1 protein levels in ADR. The silence of HMGB1 caused to decrease the protein levels. Thus, HMGB1 gene silencing or its effects may not only be effective in non-cancerous tissues such as heart muscle but may also be effective in the treatment of cancer in the prevention or reduction of the resistance to chemotherapeutic drugs such as ADR. Nevertheless, it should not be forgotten that HMGB1 is indispensable for life because it is indicated that the life span of HMGB1<sup>-/-</sup> silenced mice is shortened [16].

HMGB1 can bind cell receptors such as RAGE, TLR2, TLR4. In our study, we suggested that HMGB1 could participate in ADR's toxic effect on cardiomyocytes via TLR4. In this context, the results obtained from the studies made especially for our hypothesis are exciting. According to the result, ADR cause to increase HMGB1 levels, moreover TLR4 probably involve in HMGB1 effects on cardiomyocytes because TLR4 protein expression has some alternation. The post-translation modification of TLR, such as glycosylation, is so crucial to participate in the receptors localization, signaling capacities, and assembly [17]. Glycosylation is reported to one of most seen and complexes post-translation modification complying to covalent attachment of oligosaccharide chain to -NH<sub>2</sub> group of asparagines (N-glycosylation). It is believed to this glycoprotein generally located in cell membrane or organel lumens, thus participated in many physiologic processes, including immunologic reaction [18]. Moreover, TLR4 is one of  $\alpha$ -2-6- and  $\alpha$ -2,3-sialylated glycoproteins like MD2. So, when sialyl residues in TLR4 is removed, cells could increase NF- $\kappa$ B activation, cytokine releasing and TLR4 dimerization [17]. Also, sialidase Neu 1 is related to TLR2, TLR3 besides TLR4, enhancing following agonist trigger [17]. TLR4 is reported to possess N-glycosylated proteins, meaning that core fucose,  $\alpha$ 1,6-linked fucosylation of the innermost N-acetylglucosamine residue of N-linked glycan, can affect TLR4-dependent signaling [19]. Also, fatty acid modification of TLR4 was reported to be important for its function [20]. The higher bands of the protein in western blot are usually reported to

post-translational modification [21-23]. According to the antibody manufacturer info used in the present study, the higher band of TLR4 than 170 kDa means to the heavily glycosylated or complexed form or TLR4 despite the heat of cell lysate for 10 minutes at 100°C in the present study. Unfortunately, we could not able to confirm the posttranslational modification of TLR4 which is the limitation of the current study, excepting our Western blot result.

The main mechanism underlying heart failure is cardiac cell loss due to apoptosis, resulting in cardiomyocytes death and severe dysfunction in cardiac contractility [8,11]. However, the mechanism of this apoptotic process is still a mystery. So, we investigated intracellular pathways of ADR/HMGB1/TLR4 pathways through MAPK/AMPK. AMPK is a regulator and chief actor in cell energy and nutrition signaling. This kinase is generally activated in cell energy stress induced by either physiological condition such as exercise that increases the intracellular calcium, reactive oxygen, and nitrogen levels or pathological conditions such as ischemia that elevate the ratio of AMP/ATP, ADP/ATP [24]. AMPK is also associated with MAPK [24]. MAPK is a tightly conserved serine/threonine protein kinase family that transitions between species and plays a role in many processes such as infiltration, immune, differentiation, cell death, and survival. This family has three members, ERK, JNK and P38 [16]. ERK1/2 activated by all kind of stress in a cell, plays a role in the survival of the cell (anti-apoptotic). JNK has a pro-apoptotic effect. In our study, we propose as a hypothesis that ADR alleviated the protein expression of pro-apoptotic JNK protein via decreasing ERK1/2 protein level. In addition, it was indicated that ADR suppresses AMPK in the heart, but increases MAPK signaling pathway. Because ADR suppresses AMPK, ATP production capacity is decreasing. While ADR activates AMPK directly by impairing its energy balance, it also suppresses AMPK indirectly by its oxidative stress and its genotoxic effect [24]. A recent study suggests that ADR activates AMPK and, conversely, reduces P38 and mTOR [25]. However, AMPK is reported to activate via P38 and ASK1. Both P38 and ASK1 are activated via extensive cellular responses to stress. Inhibition of ASK1 also phosphorylates P38 and reduces oxidative damage. AMPK inhibits P38 and ASK via inactivating them. This information demonstrates that ADR can affect AMPK through many intracellular signaling pathways. Therefore, this may be a reason for the difference in the result of AMPK expression. It is a sign of the strength to resolve the mechanism of changes in AMPK. In our study, ADR reduced the protein expression

levels of P38 and PP38 protein. In addition, the silencing of the HMGB1 gene caused an increase in the level of AMPK protein. A study suggests that AMPK may be protective against cell damage from ADR-induced renal podocytes. It has also been noted that oxidative stress activates AMPK [26]. There are many sources in the literature that oxidative stress is an important mechanism in heart failure caused by ADR [27-33]. Since AMPK is an important protein for cell function, it interacts with many intracellular pathways and mediators. One of these pathways was recently defined. In a previous study, ADR has been reported to inhibit AMPK with time. Interestingly, ADR has also been shown to increase ERK1/2 phosphorylation in this cancer line. In addition, ADR has induced disruption of Caspase-3 (Cas-3). It is emphasized that this is an important mechanism in the development of resistance to ADR [34]. The heart often consumes ATP, and most are used in muscle contraction. Adverse effects of ADR on heart energy metabolism are mediated by impairing oxidative phosphorylation, mitochondrial electron transport system and AMPK functions [35]. AMPK is one of the most important proteins in the maintenance of cellular energy. Thus, AMPK plays a major role in survival [36] and/or apoptotic pathways [37]. Because AMPK is an active cascade that requires energy in apoptosis, we explored the change in protein expression level of AMPK- $\alpha$ 1. Interesting knowledge on AMPK is indicated that TAK1 is one of the upstream activators of AMPK. JNK and S6 kinase leads to an increase in NF- $\kappa$ B expression via IKK. TAK1-P38 in MH60 cell line was reported to trigger apoptosis by BMP-2. Moreover, blocking of TAK1 is reported to enhance susceptibility to ADR in HeLa (adenocarcinoma) and mouse embryonic fibroblasts (MEFs) cell lines [38]. It was also reported that HMGB1's inhibition led to inhibit TLR4/MyD88/ NF- $\kappa$ B pathways in brain ischemia/reperfusion [39].

Caspases are intracellular cysteine-aspartic protease groups, which mediate apoptosis. Caspases are found as inactive proenzymes and are activated by proteolytic enzymes. Cas-8 is thought to be an apoptotic and cytokine-mediated cell death receptor [16]. In our study, the amount of active Cas-8 was determined in live cardiomyocytes because of the ability of HMGB1 to act as a cytokine. Suppression of the HMGB1 gene resulted in a decrease in the amount of ADR-induced active Cas-8. Cas-9 is effective in both the mitochondrial death pathways and the passage of cytochrome-c from mitochondria to cytoplasm. Cas-9 is also effective in the mitochondrial death pathway and is effective in the passage of cytochrome-c from mitochondria to cytoplasm. Cas-3 and Cas-7 serve as an enhanc-

er in this apoptotic process initiated by Cas-8 and Cas-2 [16]. In the present study, we also examined the protein expression level of Cas-3. The results showed that blocking HMGB1 inhibits apoptosis via attenuating Cas-3 and Cas-8 levels, which is an indication that apoptosis occurs in the caspase-dependent pathway. However, although caspase activity plays a critical role in apoptotic cell death pathway, it has been described that apoptosis-inducing factor (AIF) and endonuclease G plays an important role in the translocation from mitochondria to the nucleus, independently from the caspase, which results in DNA breaks [16]. In the present study, it was proposed that Cas-3 is important in ADR-induced heart failure and apoptotic cell death occurs via caspase-mediated pathways depending on the observation that there was no change in the expression of AIF protein. HMGB1 not only regulates endonuclease activity, but it also becomes a part of apoptotic bodies [16]. With the light of this information, we built the main hypothesis of our work on HMGB1.

HMGB1 take part in cell migration via RAGE and mediates inflammation and gene transcription through TLR4 and P53. The pro-inflammatory effects of HMGB1 are mediated by P38, ERK, JNK. The accumulation of HMGB1 in the extracellular fluid causes toxic effects and tissue damage such as apoptosis, necrosis, and cell death. The extracellular HMGB1 causes the formation of giant mitochondrial vacuoles and rapidly mitochondrial DNA break by entering mitochondria in an endocytosis-independent manner. HMGB1's entry into the mitochondria is not via TLR-2, TLR4 or not RAGE signaling pathways. However, it is thought that the activation of JNK through ROS is effective in this process [16], which support ADR-induced apoptotic pathways in the present study. This information enhances the importance of the result of the present study when one of the intracellular targets of ADR is combined with knowledge of mitochondria [27-32]. It is stated that the amount of HMGB1 increases in cancer and decreases in aging [16].

JNK is a kinase involved in cell apoptosis and inflammation. Cardiac cells increase HMGB1 production and release in stress conditions such as sepsis and ischemia/reperfusion. After being released from the cell, HMGB1 binds to its receptor, leading to pro-inflammatory effects and resulting in apoptotic cell death and heart failure. One of the previous study suggested that ADR increased the production of HMGB1 in the heart and triggered apoptosis [8]. JNK, a proapoptotic kinase, is thought to play an important role in apoptosis in the heart. The main mechanism of the JNK activation is oxidative stress. It also indicates that ADR increases

the expression of HMGB1 via JNK in the mentioned study. Therefore, it was found that the amount of HMGB1 decreased when the effect of JNK was suppressed, which demonstrate JNK's importance in the apoptotic pathway caused by ADR. It is emphasized that either genetic (in JNK or TLR4 gene silencing) or pharmacologically suppression of the action of HMGB1 can not completely prevent the toxic effect of ADR seen in the heart [8]. Our result supports the mentioned study. The silencing of the HMGB1 gene significantly reduced apoptotic cell death. We presumably believe that some of these toxic effects of ADR may be mediated through the interaction of HMGB1 specifically with the TLR4 receptor.

## Conclusion

The undesired effect of ADR on heart tissue, noncancerous tissue, participate in HMGB1/TLR4 axis through MAPK/AMPK pathways, resulting in triggering in apoptotic cell death. The most inter-

esting results in the current study was seen on some posttranslational modification of TLR4. It is limitation in the study not to deeply analyze the modification. It is suggested to evaluate the modification of TLR4 by future studies.

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## Conflict of interests

The authors declare no conflict of interests.

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