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ORIGINAL ARTICLE

The role of vasopressin V1A and oxytocin OTR receptors in protective effects of arginine vasopressin against H_2O_2-induced oxidative stress in H9C2 cells

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ABSTRACT

Background: Oxidative stress, has been shown to play an important role in the pathophysiology of cardiac remodelling and heart failure. The aim of study is effect of arginine vasopressin (AVP) on apoptosis of cardiomyocyte via its receptors.

Materials and methods: The cell viability effect of AVP in H9C2 cardiomyocytes was assayed using the MTT method. The transcription and translation level of apoptosis genes (Bax, Bcl-2, caspase-3) were discovered with qRT-PCR and western blotting.

Results: The results showed that vasopressin could reduce apoptosis in cardiomyocytes cell line through downregulation of caspase-3, BAX and upregulation of Bcl-2 ($p < .001$). Also, there was a decrease in anti-apoptosis effect of vasopressin when V1A and OTR receptors were blocked with their antagonists.

Discussion: These results suggest that activation of V1A and OTR receptors in H9C2 cells mediate protective effect of vasopressin via regulating apoptosis marker that lead to cell survival under conditions of stress oxidative.

KEY POINT

- AVP may contribute to the improvement of heart ischaemia through its actions on V1A and OTR receptors.

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Vasopressin; H9C2; V1A; OTR; apoptosis

Introduction

Reactive oxygen species (ROS), derived from O_2, are important in the maintenance of normal physiological function. However, at high concentration ROS that produced through an uncontrolled chain reaction cause damage to biomolecules and potentially lethal. Therefore, at low/moderate concentration there is an appropriate balance between production and their quenchers, antioxidants (Singh *et al.* 1995). When ROS cellular overproduction overwhelms intrinsic antioxidant capacity, cause oxidative damage to the DNA, proteins, and lead to cellular dysfunction, irreversible cell damage and death, which is implicated in a wide range of disease (Takimoto and Kass 2007). Also, experimental evidence suggests that ROS can induce apoptosis by a variety of mechanisms, including increased protein expression of pro-apoptotic members (Giordano 2005).

It has been shown that certain oxidative stress activities in the cardiovascular system in response to various stressors causes failing heart (Cesselli *et al.* 2001, Sawyer *et al.* 2002, Sabri *et al.* 2003, Suematsu *et al.* 2003). Studies have shown that high level of ROS activates the proteins of apoptosis signalling pathway in adult cardiomyocytes (Kwon *et al.* 2003). Cardiomyocyte apoptosis occurs in hypertrophied, ischaemic, and failing hearts and may contribute to the development and progression of cardiac dysfunction and heart failure that relevant potential link between heart failure and ROS excessive production (Cesselli *et al.* 2001).

Arginine vasopressin (AVP) is a peptide hormone traditionally known for its role in the hypothalamic-pituitary-adrenal (HPA) axis that released via the posterior pituitary in response to hyperosmolarity, hypotension, or hypovolemia (Land *et al.* 1982, Wasilewski *et al.* 2016). Vasopressin mediate its effects through three distinct cellular receptors that have been identified in an increasing number of tissues (Koshimizu *et al.* 2012, Juul *et al.* 2014). The main endocrine functions of AVP are the maintain body water content by facilitation of water reabsorption of kidney (V2 vasopressin receptors) and the contraction of smooth muscle cells in arteries (V1A vasopressin receptors) and cardiac sympathetic activation (OTR oxytocin receptors) (Holmes *et al.* 2003, Coote 2005). Also, studies have been demonstrated that the antiapoptotic effect of AVP on apoptosis markers is mediated through the V2 and V1A receptor (Higashiyama *et al.* 2001, Miller *et al.* 2013). In this regard, AVP induce phosphorylation

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of pro-apoptotic proteins and resulting causes change function of upstream proteins involve in apoptosis including caspase-3, Bax and Bcl-2 that promotes cell survival.

Effects of AVP are important for cardiovascular homeostasis such as regulation of blood volume, and vascular tone (Chandrashekhar *et al.* 2003, Thibonnier 2003). Other studies reported that AVP provides cardioprotective effect against heart ischaemia/reperfusion injury by antioxidant effect and reduction of stress oxidative (Nazari *et al.* 2011, 2015). Cardiac effects of vasopressin be mediated through the V1R (vascular smooth muscle/calcium-dependent effect) or OTR (endothelial/NO effect) (Holmes *et al.* 2004). Few studies have examined the effect of vasopressin on H_2O_2-induced oxidative stress injury and its molecular mechanism in H9C2 cells. Therefore, we investigated whether cardioprotective effect of vasopressin on apoptosis factors such as Bcl2, Bax and caspase-3 is exerted by V1A and OTR receptors in H9C2 cardiomyocyte.

Materials and methods

Reagents

3–(4,5-Dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT), arginine vasopressin (AVP), SR49059 (V1A receptor antagonist) and H_2O_2 solution (35 wt.% in water) were purchased from (Sigma–Aldrich, St Louis, MO, USA). OTA (OTR receptor antagonist) was purchased from bachem (Bubendorf, Switzerland). Western blot reagents and complete protease inhibitor tablet (S8820) were obtained from Sigma. Antibodies against Caspase-3 (9665), Bax (2772), β-actin (4970) and HRP-linked secondary antibody (7074) were purchased from Cell Signalling. Anti-Bcl-2 antibody (ab59348), Prism protein ladder (115832) was purchased from Abcam. Polyvinylidine difluoride (PVDF) membrane and Enhanced chemiluminescent (ECL) detection kit (RPN 2235) were purchased from Immobilon Millipore and Amersham Biosciences respectively.

Cell culture and treatment

The H9C2 embryonal rat heart-derived cell line were obtained from Pasteur Institute (Tehran, Iran). The cells were cultured and maintained in Dulbecco's Modified Eagles Medium (DMEM) with 10% FBS and 1% penicillin/streptomycin at 37˚C in humidified atmosphere of 5% CO2.

The culture medium was changed every 2 or 3 days. After 4 to 5 days, cells were passaged at a 1:5 ratios and seeded at the density of 1.2×10^6 cells per 100-mm dish, 0.15×10^6 cells per well of 6-well plates, 3×10^4 cells per well of 24-well plates. After incubation of cells for 24 h, the oxidative stress model was established by exposure to a 100 μM H_2O_2 solution in DMEM without FBS for 3 h. Cells were grouped in the following order:

1. Control group (C): The cells were placed under normoxic conditions for 4 h.
2. H_2O_2 group (H): The cells were exposed to H_2O_2 (100 μM) for 4 h.
3. AVP + H_2O_2 (AH): The cells were pre-treated with AVP (1 μM) for 10 min before being exposed to H_2O_2 (100 μM).
4. SR49059 + AVP + H_2O_2 (SAH): The cells were exposed to SR49059 (100 nM) for 10 min before were pre-treatment with AVP.
5. OTA + AVP + H_2O_2 (OAH): The cells were exposed to OTA (1 nM) for 10 min before were pre-treatment with AVP.
6. OTA + SR49059 + AVP + H_2O_2 (OSAH): The cells were exposed to OTA (1 nM) and SR49059 (100 nM) for 10 min before pre-treatment with AVP.

Cell viability assay

Cell viability was examined by the trypan blue (Gibco BRL, Grand Island, NY, USA) exclusion assay and 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) uptake assay (20). Cultured H9C2 cells were dispersed by trypsinization and seeded at 10^4 cells/well in a 96-well plate (Nunc, Denmark) and incubated overnight before being treated. After treatments period, 20 μL MTT solution (5 mg/mL) was added to each well and incubated at 37 °C for 4 h. The supernatant was removed, and the insoluble produced formazan crystals was dissolved in 150 μL DMSO. Absorbance of each culture well was measured with a microplate reader (Statfax–2100, Awareness Technology, USA) at a wavelength of 570 nm (Qin *et al.* 2006).

Total RNA extraction, cDNA synthesis and real-time PCR

Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) was performed for determining the expression levels of Bcl2, Bax and caspase-3 genes. Total cellular RNA was isolated using the Trizol reagent according to the manufacture's instruction (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Triplicate assays were performed for each RNA sample. RNA content and purity were measured at a wavelength of 260–280 nm using UV-vis spectrophotometer (WPA Biowave II, UK). According to manufacturer instructions, cDNA synthesis was done by using cDNA synthesis kit (CinnaGen, Tehran, Iran). Briefly, after DNase treatment, total RNA (1 μg) was reversed to cDNA by oligodT primers (1 μL of 100 μM), dNTPS (2 μL of 10 mM), and M-MuLVRevertAid Reverse Transcriptase (1 μL of 100 U/μL), incubated for 10 min at 25 °C, followed by 60 min at 45 °C in a total volume of 20 μL. The reaction was terminated using heating the reactions at 85 °C for 5 min. Primers for real-time PCR, were designed with the perimer3 online software and primer sequences are shown in Table 1. Real-time PCR amplification was performed in a Corbet Rotor Gene 6000 (Corbet life science, Australia) and the following thermal cycling conditions: Initial denaturation in 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 60 °C for 60 s, and 72 °C for 25 s. Expression of each amplicon was analysed by using the $2^{-\Delta\Delta Ct}$ method (Keyhanmanesh *et al.* 2018).

Table 1. Primers used for real-time PCR analysis.

Gene name	Primer sequence	Amplicon length	Gene accession number
Caspase-3	Forward: GCTGGACTGCGGTATTGAGA Reverse: AGGAATAGTAACCGGGTGCG	199	NM_001276711.1
Bax	Forward: CATCCACCAAGAAGCTGAGCG Reverse: CCACATCAGCAATCATCCTCTG	249	NM_012611.3
Bcl-2	Forward: TGGCCTTCTTTGAGTTCGGT Reverse: GTTCCACAAAGGCATCCCAGC	134	NM_006256731.2
GAPDH	Forward: GTCATCCCAGAGCTGAACGG Reverse: ACTTGGCAGGTTTCTCCAGG	202	NM_012675.3

Western blot analysis of Bax, caspase-3, and Bcl-2 expression

Cells from each group were lysed using RIPA buffer containing protease inhibitors (sc-24948, Santa Cruz Biotechnology, Heidelberg, Germany). The lysate was centrifuged at 10,000 g for 30 min at 4 °C to remove the insoluble material and supernatants were collected. The protein concentration was measured by Bradford protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein (50 μg) from each sample mixed with 2× loading buffer and samples were loaded on 12% SDS–PAGE gel and separated protein bands transferred to nitrocellulose membrane (Sigma Chemical Co., MO, USA). The membranes were blocked for 1 h in 3% bovine serum albumin (BSA) (Sigma Chemical Co. MO, USA) in PBS plus 0.1% tween 20 for 1 h with gentle shaking. Subsequently, the blots were incubated overnight at 4 °C with rabbit polyclonal primary antibodies against Bcl-2, or Bax or caspase-3. All of the antibodies were diluted at 1/500 in antibody dilution buffer (1% w/v non-fat dry milk in 0.05% v/v Tris-Buffered Saline (TBS) with 0.05% v/v tween 20). Following a 30-min wash with PBST, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. The membranes were then washed for 30 min and the immune complex visualised by enhanced chemiluminescence kit (ECL kit; Bio-Rad). The protein expression levels were normalised to the beta-actin expression. The density of the sample and reference bands were quantified using Image J software (version 1.48; National Institutes of Health, Bethesda, MD, USA) (Shamekhi *et al.* 2019).

Statistical analysis

Data were expressed as means ± SEM. All parameters were tested for normality using the one-sample Kolmogorov-Smirnov test. Difference was analysed for significance by one-way ANOVA followed by Tukey's test (GraphPad Prism 6.0 statistical software). A value of $p < .05$ was considered statistically significant.

Results

Effect of AVP on cell viability

The viability rate in the H group was significantly lower than the control group ($p < .01$). After treating the cells with AVP in the AH group, the viability of the cells in this group increased significantly compared to the H_2O_2 group ($p < .01$). Treatment with SR49059 and OTA antagonists in SAH and OAH groups significantly reduced cell viability compared to the AH group ($p < .05$). However, in both groups, the survival rate is slightly higher than that of the H group, but not statistically significant. In the OSAH group, which exposed the cells to both the SR49059 and OTA antagonists, viability was significantly reduced compared to the AH group ($p < .05$). Also viability reduction in this group was significant compared to SAH and OAH groups ($p < .05$) (Figure 1).

Effect of AVP on Bax expression

Bax gene expression in all groups except the AH group was significantly increased compared to the control group. while, pre-treatment of cells with vasopressin in the AH group reduced Bax levels when compared to H group ($p < .001$). Administration of SR49059 and OTA antagonists respectively in SAH and OAH groups, significantly increased the expression of BAX gene compared to the AH group ($p < .001$ and $p < .01$, respectively). In the OSAH group, which received both antagonists, the expression of this gene increased significantly when compared to the AH, SAH and OAH groups (Figure 2).

Effect of AVP on caspase-3 expression

Caspase-3 gene expression in group H was significantly increased as compared to control ($p < .001$). Pre-treatment of cells with AVP in the AH group, significantly reduced the level of this gene compared to the H group ($p < .001$). The separate administration of SR49059 and OTA in SAH and OAH groups significantly increased the expression of caspase-3 expression as compared to the AH group ($p < .01$). In the OSAH group, the combination of these two antagonists significantly increased the expression of caspase-3 gene when compared to the AH group that the protective effect of vasopressin was greatly reduced ($p < .001$). Also, there is a significant difference between OSAH group and SAH, OSAH groups ($p < .001$) (Figure 3).

Effect of AVP on Bcl-2 expression

The expression of Bcl-2 gene in all treatment groups did not differ significantly with the control group. However, the expression of this gene in the AH group significantly increased compared to the H group($p < .001$). Also, the expression of Bcl-2 in SAH, OAH and OSAH groups significantly decreased compared to AH group ($p < .001$). However, there was no significant difference in expression of Bcl-2 in

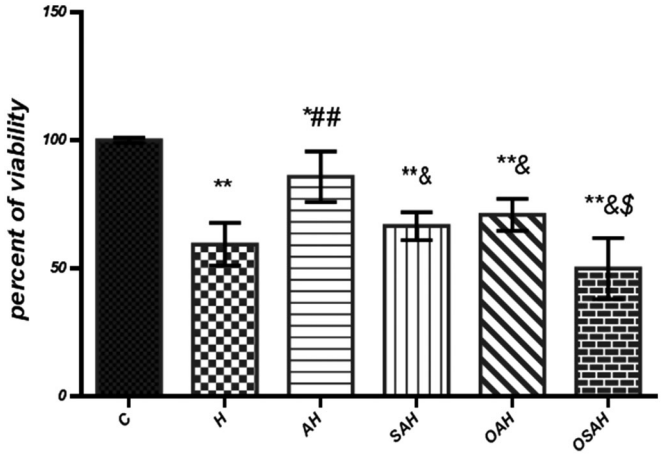

Figure 1. Effect of vasopressin on the cell viability in cardiomyocytes cell line evaluated by MTT assay. MTT values are expressed as mean ± SEM ($n = 3$). $^{**}p < .01$ and $^{*}p < .05$ vs. C; $^{\#\#}p < .01$ vs. H; $^{\&}p < .05$ vs. AH; $^{\$}p < .05$ vs. OAH.

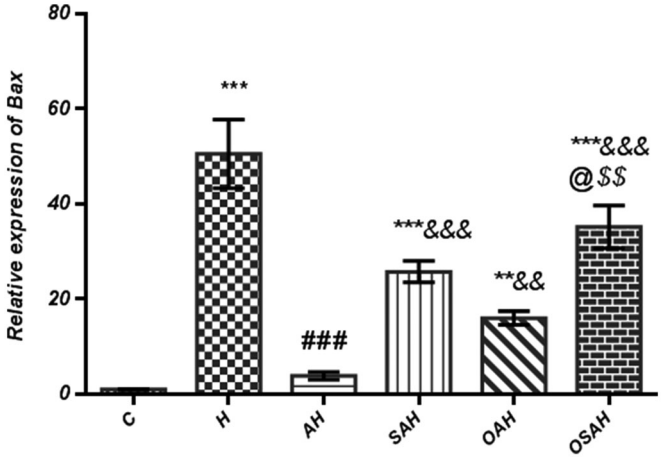

Figure 2. Effect of vasopressin on Bax expression level in cardiomyocytes cell line. Data are shown as mean ± SEM ($n = 7$). $^{***}p < .001$ and $^{**}p < .01$ vs. C; $^{\#\#\#}p < .001$ vs. H; $^{\&\&}p < .01$ and $^{\&\&\&}p < .001$ vs. AH; $^{\$\$}p < .01$ vs. OAH; $^{@}p < .05$ vs. SAH.

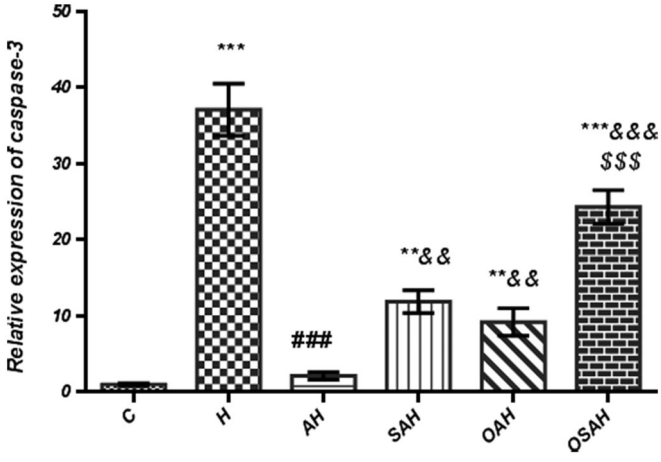

Figure 3. Effect of vasopressin on caspase-3 expression level in cardiomyocytes cell line. Data are shown as mean ± SEM ($n = 7$). $^{***}p < .001$ and $^{**}p < .01$ vs. C; $^{\#\#\#}p < .001$ vs. H; $^{\&\&}p < .01$ and $^{\&\&\&}p < .001$ vs. AH; $^{\$\$\$}p < .001$ vs. OAH.

the OSAH group compared to SAH and OAH groups (Figure 4).

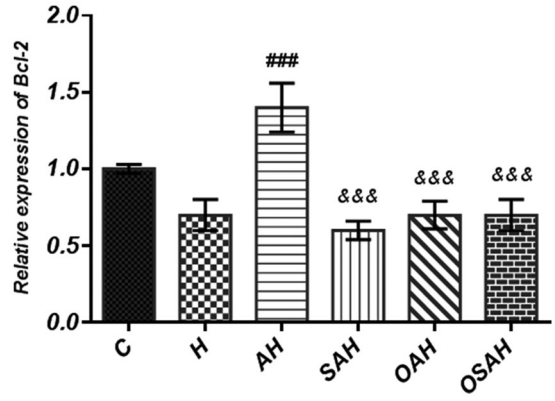

Figure 4. Effect of vasopressin on Bcl-2 expression level in cardiomyocytes cell line. Data are shown as mean ± SEM ($n = 7$). $^{\#\#\#}p < .001$ vs. H; $^{\&\&\&}p < .001$ vs. AH.

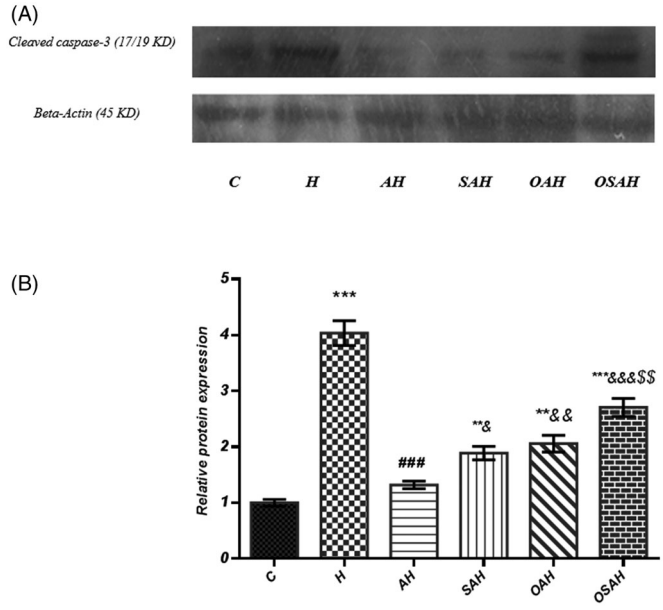

Figure 5. Effect of vasopressin on the protein levels of caspase3 in cardiomyocytes. (A) Representative Western blots showing specific bands for caspase3 and β-actin (as an internal control). All analyses are representative of five independent experiments. (B) Densitometric data of protein analysis. All analyses are representative of five independent experiments. Data are expressed as the mean ± SEM. $^{***}p < .001$ and $^{**}p < .01$ vs. C; $^{\#\#\#}p < .001$ vs. H; $^{\&}p < .05$, $^{\&\&}p < .01$ and $^{\&\&\&}p < .001$ vs. AH; $^{\$\$}p < .01$ vs. SAH and OAH.

Western blot analysis of apoptosis associated proteins

Effects of vasopressin on apoptosis were evaluated by western blot analysis. As Figures 5 and 6 indicate, H_2O_2 increased expression level of BAX/Bcl-2 ratio and cleaved caspase-3 in H2C9 cell line. In next treatment group, results showed that vasopressin decrease significantly BAX/Bcl-2 ratio and cleaved caspase-3 protein expression when compared with H group. Also, there is a significant difference between groups received antagonist separately and group administrated with both antagonists.

Discussion

A novel therapy for oxidative stress induced cardiomyocytes apoptosis in the cardiovascular disease urgently needed.

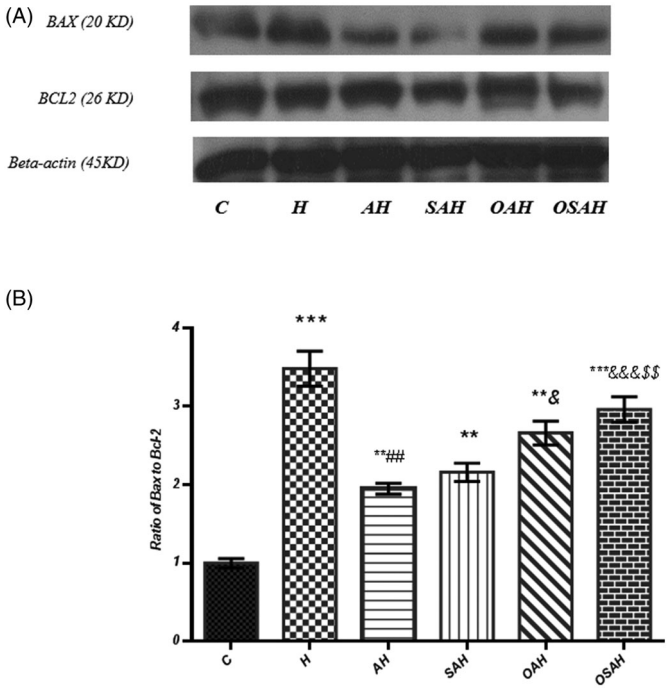

Figure 6. Effect of vasopressin on the expression levels of the Bcl-2 family in cardiomyocytes. (A) Representative Western blots showing specific bands for Bax, Bcl-2 and β-actin (as an internal control). All analyses are representative of five independent experiments. (B) Densitometric data of protein analysis. All analyses are representative of five independent experiments. Data are expressed as the mean ± SEM. $^{***}p < .001$ and $^{**}p < .01$ vs. C; $^{\#\#}p < .01$ vs. H; $^{\&}p < .05$ and $^{\&\&\&}p < .001$ vs. AH; $^{\$\$}p < .01$ vs. SAH.

In the present study we showed that AVP decreased the caspase-3 and Bax expression levels, and increased Bcl2 expression levels that lead to augmentation of cell viability in the oxidative stress generated by H202 addition to the culture medium of cardiomycotes.

Oxidative stress results from an imbalance between ROS generation and antioxidant Defence mechanisms. ROS include free radicals such as hydrogen peroxide (H_2O_2) which is nonradicals capable of generating free radicals (Tsutsui *et al.* 2011). Experimental evidence suggests that H_2O_2 can mediate apoptosis by a variety of mechanisms including activate the Bax and caspase3 (Tamura *et al.* 2003). The apoptosis pathway is largely mediated through Bcl-2 family proteins, which include both proapoptotic members such as Bax and antiapoptotic members such as Bcl-2 (Antonsson *et al.* 1997). Another important component in apoptosis pathway is the caspase-3 that causes DNA degradation or fragmentation (Enari *et al.* 1998). A number of experimental and clinical studies have demonstrated that levels of ROS generation were elevated in the plasma and pericardial fluid of patients with cardiovascular disease and also positively correlated with its severity (Lonn *et al.* 2005, Sugamura and Keaney Jr. 2011).

AVP, is synthesised by the magnocellular neurons of the hypothalamus, has clearly divergent physiologic activity such as cardiovascular homeostasis (Holmes *et al.* 2003). Recently, this peptide is used in heart surgery so that administration of low-dose AVP is a safe and effective way to manage postoperative vasodilatory shock of patients undergoing cardiopulmonary bypass (Masetti *et al.* 2002). Therefore, recent research has tended to use vasopressin for cardiovascular protection of heart failure patients. In this regard, we used H9C2 cells line with a cardiac phenotype that possess all basic features of V1-vasopressin receptor signalling. Brostrom *et al.* (2000) proposed that treatment of H9C2 cells with AVP, causes cardiac hypertrophy by activation of protein kinase C following with augmentation of $[Ca^{2+}]_i$ that is a primary signal for cardiac hypertrophy. Nazari *et al.* (2011) reported that AVP can induce preconditioning effect probably via V1 vasopressin receptor on cardiac tissue against myocardial ischaemia in rat. Also, these researchers in other study demonstrated that AVP can protect heart in a dose-dependent manner via V1 receptor and seems that the cardioprotective effect of AVP are mediated via inhibiting of mitochondrial transmembrane protein that play a major role in both necrotic and apoptotic cell death (Nazari *et al.* 2015). It is well known that secretion of vasopressin produces activation of suppressors apoptosis proteins such as Bcl-2 and caspase-9 (activator of caspase-3) in animal with acute dehydration as a model for the activation of vasopressin system (Chernigovskaya *et al.* 2005, 2011). Higashiyama *et al.* (2001) reported that AVP inhibits apoptosis of glomerular mesangial cells through V1A receptors and protein kinase C signalling pathway in apoptosis status not in normal condition. Chen *et al.* (2009) showed that AVP mediated its anti-apoptotic effects on Bax or caspase-3 through MAPK signalling and PKC pathway of V1 receptors. Jafari *et al.* (2015) demonstrated that administration of AVP caused a significant decline in the activity of caspase-3, prevented the decrease of viable cells and reduce the oxidative damage in cardiac tissue of rat. These effects of AVP lead to protection of heart from entering the apoptotic phase. This study provides initial evidence that the antiapoptotic effects of AVP in the cardiomyocyte cells mediated through the V1A together OTR receptors in heart failure condition.

In this study, we demonstrated that AVP through the function of the V1 and OTR receptors activate Bcl-2 protein, inactivate Bax and caspase-3 proteins in heart failure. In response to prove the role of both receptors in cardiac protective of AVP, when the V1A and OTR receptor blocked together with their antagonists (SR49059 and OTA, respectively), the antiapoptotic action of AVP for cardioprotective from oxidative stress severely reduced as determined by western blot and real time PCR analysis. Therefore, we can prove that AVP bind to both V1A and OTR receptors in cardiomyocyte cells and increase Bcl-2 function, decrease Bax and caspase-3 activity following trigger antiapoptotic proteins such as Bad (Chen *et al.* 2008). Bad, member of Bcl-2 family as pro-apoptotic protein, is able to dimerise with anti-apoptotic proteins and ultimately regulate mitochondrial cytochrome c, that is a critical event in apoptosis with increasing activity of caspase cleavage (Hsu and Hsueh 2000). AVP with phosphorylation of Bad promotes cell survival by blocking Bad dimerisation with Bcl-2. Regarding the limitations of this study, we did not evaluate the role of AVP receptors on cardioprotective effect of AVP *in vivo*.

In conclusion, our study reported that H_2O_2 causes mitochondrial dysfunction leading to oxidative stress and

apoptosis in H9C2 cells. As illustrated above, administration of AVP can improve cell viability and regulate apoptosis markers including Bax/Bcl-2 ratio and caspase-3 levels by activation of V1A and OTR receptors in cardiomycytes under oxidative stress conditions. Therefore, more experimental evidence is needed to discover the role of V1A and OTR receptors to protect heart failure *in vivo* study.

Disclosure statement

The authors declare there is no conflict of interest.

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