

## EFFECTS OF THE DIRECT RENIN INHIBITOR ALISKIREN ON OXIDATIVE STRESS IN ISOLATED RAT HEART

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## EFEKTI DIREKTOG RENINSKOG INHIBITORA ALISKIRENA NA OKSIDATIVNI STRES IZOLOVANOG SRCA PACOVA

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### ABSTRACT

Increased activity of the renin-angiotensin-aldosterone system (RAAS) plays a significant role in the development and progression of various cardio-metabolic diseases, such as hypertension, atherosclerosis and heart failure. Aliskiren is the newest antihypertensive drug and the first orally active direct renin inhibitor to become available for clinical use. This study investigated the acute and direct effects of Aliskiren on different parameters of oxidative stress on isolated rat heart. The hearts of male Wistar albino rats ( $n = 24$ , 8 per experimental group, age 8 weeks, body mass 180–200 g), were excised and retrogradely perfused according to the Langendorff technique at a gradually increasing perfusion pressure (40–120  $\text{cmH}_2\text{O}$ ). Markers of oxidative stress ( $\text{NO}_2^-$ , TBARS,  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$ ) were measured spectrophotometrically after perfusion with three different concentrations of Aliskiren (0.1  $\mu\text{M}$ , 1  $\mu\text{M}$ , and 10  $\mu\text{M}$ ). The results demonstrated possible dose-dependent cardioprotective properties of Aliskiren, particularly with higher CPP. Lipid peroxidation (TBARS) levels decreased with the highest dose of Aliskiren and higher CPP, and the same trend was observed in nitrite ( $\text{NO}_2^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) levels. These findings indicate that the acute effects of Aliskiren do not likely promote the production of reactive oxygen species upon higher pressure with the highest dose. Aliskiren may exert beneficial effects on oxidative stress biomarkers.

**Keywords:** Aliskiren, Isolated rat heart, Langendorff technique, Oxidative stress

### SAŽETAK

Povećana aktivnost renin-angiotenzin-aldosteron sistema (RAAS) može da ima značajnu ulogu u razvoju i progresiji različitih kardio-metaboličkih bolesti kao što su hipertenzija, ateroskleroza i srčana insuficijencija. Aliskiren se ubraja u najnovije antihipertenzivne lekove i prvi je oralni direktni inhibitor renina koji je uveden u kliničku upotrebu. Prema tome, cilj ove studije je bio da ispita akutne i direktne efekte aliskirena na različite parametre oksidativnog stresa izolovanog srca pacova. U studiji su korišćeni Wistar albino pacovi ( $n = 24$ , 8 životinja u svakoj grupi) muškog pola, starosti 8 nedelja, telesne mase 180–200g. Nakon izolovanja, srca ovih pacova su retrogradno perfundovana prema Langendorff-ovoj tehnici uz postepeno povećanje perfuzionog pritiska (40–120  $\text{cmH}_2\text{O}$ ). Marker oksidativnog stresa ( $\text{NO}_2^-$ , TBARS,  $\text{H}_2\text{O}_2$  i  $\text{O}_2^-$ ) su mereni spektrofotometrijski nakon perfuzije sa tri različite koncentracije aliskirena (0.1  $\mu\text{M}$ , 1  $\mu\text{M}$  i 10  $\mu\text{M}$ ). Rezultati su pokazali da aliskiren pri najvišim vrednostima perfuzionog pritiska ostvaruje izvesna dozno-zavisna kardioprotektivna svojstva. Naime, nivoi indeksa lipidne peroksidacije (TBARS), nitrita ( $\text{NO}_2^-$ ) i vodonik peroksida ( $\text{H}_2\text{O}_2$ ) su bili sniženi nakon administracije najveće doze aliskirena pri višim perfuzionim pritiscima. Ova saznanja ukazuju da akutno primenjeni aliskiren (pri višim dozama i perfuzionim pritiscima) ne stimuliše proizvodnju slobodnih radikala. Aliskiren može da ima pozitivne efekte na biomarkere oksidativnog stresa.

**Ključne reči:** aliskiren, izolovano srce pacova, oksidativni stres, tehnika po Langendorff-u

### ABBREVIATIONS

|   |   |
|---|---|
| ACEI - Angiotensin converting enzyme inhibitors | DRI - Direct renin inhibitor                |
| ARB - Angiotensin receptor blockers             | MDA - Malondialdehyde                       |
| AT - Angiotensin                                | NO - Nitric oxide                           |
| CF - Coronary flow                              | RAAS - Renin-angiotensin-aldosterone system |
| cGMP - Cyclic guanosine 3',5'-monophosphate     | ROS - Reactive oxygen species               |



## INTRODUCTION

Increased activity of the renin-angiotensin-aldosterone system (RAAS) plays a significant role in the development and progression of various cardiometabolic diseases, such as hypertension, atherosclerosis, diabetes and heart failure (1). RAAS promotes vasoconstriction, sodium reabsorption, cardiac remodelling and other potentially detrimental effects (2, 3). Angiotensin II interaction with the AT1 receptor subtype and promotes oxidative stress, vascular smooth muscle migration, cardiomyocyte proliferation, hypertrophy and ventricular dilatation (4). Direct renin inhibitor blockade of RAAS at the rate-limiting step via reduction in plasma renin activity and levels of circulating angiotensin I and angiotensin II may be beneficial for cardiovascular risk in patients with essential hypertension and associated clinical conditions, such as diabetes and nephropathy (5, 6).

Aliskiren is the newest antihypertensive drug and the first orally active direct renin inhibitor (DRI) to become available for clinical use. The US Food and Drug Administration approved Aliskiren for clinical use in March 2007 (7). Essential hypertension is an important cause of death worldwide. Blockade of RAAS, which plays a significant role in the development of essential hypertension, is suboptimal in combination with angiotensin II (AT II)-converting enzyme inhibitors (ACEIs) or ATII type 1 receptor blockers (ARB) (8). However, whether Aliskiren is superior to conventional RAAS blockers in the prevention of heart and renal diseases is not known (9). Aliskiren is generally well tolerated, and it exhibits a placebo-like profile at doses from 75 mg to 300 mg (5). The plasma concentration of Aliskiren increases in a dose-dependent fashion, with peak concentrations after 3–6 hours. The average plasma half-life of Aliskiren is 23.7 hours, oral bioavailability is approximately 5% (95% is excreted unchanged in faeces), and plasma steady-state levels are achieved after 5–8 days of treatment (1). Aliskiren is primarily eliminated unmetabolised via biliary excretion, and less than 1% is excreted in the urine (5). Several clinical trials confirmed the efficacy of Aliskiren on blood pressure reduction as a monotherapy (10, 11) and in combination therapy (12, 13).

Oxidative stress is a well-known phenomenon that plays an important role in the pathogenesis of various diseases and syndromes. Any imbalance between pro- and anti-oxidants in which pro-oxidants prevail is known as oxidative stress (14). Existing evidence supports the view that oxidative stress may play a crucial role in cardiac and vascular abnormalities in different types of cardiovascular diseases, and antioxidant therapy may be beneficial (15). Few studies investigated the influence of Aliskiren on oxidative stress, but recent studies in different models suggested that Aliskiren monotherapy or in combination reduces blood pressure by increasing NO (nitric oxide)-cGMP (cyclic guanosine 3',5'-monophosphate) production (16), superoxide anion production and malondialdehyde (17).

Previous data on reactive oxygen species (ROS), such as superoxide anion radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), index of lipid peroxidation and nitrites ( $NO_2^-$ ), are not sufficient. Therefore, this study investigated the acute and direct effects of Aliskiren on different parameters of oxidative stress on isolated rat hearts from adult male rats.

## MATERIALS AND METHODS

### Isolated rat heart preparation

The hearts of male Wistar albino rats ( $n = 24$ , 8 per experimental group, age 8 weeks, body mass 180–200 g) were euthanized via cervical dislocation (Schedule 1 of the Animals/Scientific procedures, Act 1986, United Kingdom) after short ketamine/xylazine narcosis. Emergency thoracotomy and sudden cardiac arrest were performed via superfusion with ice-cold isotonic saline. Normal heart rhythm was restored, and an entrance to the left atrium of the heart was created through the damaged mitral valve. Hearts were retrogradely perfused according to the Langendorff technique at a gradually increasing perfusion pressure (40  $cmH_2O$  – 120  $cmH_2O$ ). Hearts were perfused with a Krebs-Henseleit solution composed of: NaCl 118 mM, KCl 4.7 mM,  $CaCl_2 \cdot 2H_2O$  2.5 mM,  $MgSO_4 \cdot 7H_2O$  1.7 mM,  $NaHCO_3$  25 mM,  $KH_2PO_4$  1.2 mM, and glucose 5.5 mM, equilibrated with 95%  $O_2$ /5%  $CO_2$  and warmed to 37°C (pH 7.4).

### Physiological assay and experimental protocol

A 30-min perfusion stabilisation period was performed. CPP was lowered to 60  $cmH_2O$  after an equilibration period (70  $cmH_2O$ ) and gradually increased to 80  $cmH_2O$ , 100  $cmH_2O$ , and 120  $cmH_2O$ , then finally lowered to 40  $cmH_2O$ . Measurements were performed at each perfusion pressure using pure a Krebs-Henseleit solution and immediately followed by perfusion with active components (different concentrations of Aliskiren) to avoid time-dependent adverse effects. Groups were assigned by the concentration of Aliskiren given in the Krebs-Henseleit perfusate: the hearts of the first group were perfused with 0.1  $\mu M$  Aliskiren; the second group with 1  $\mu M$ ; and the third group with 10  $\mu M$ . Each heart was its own control. Coronary flow (CF) was considered stable when three repeated values of CF were identical. The following markers of oxidative stress were measured spectrophotometrically in the collected samples of coronary venous effluent:

1. Nitrites ( $NO_2^-$ )
2. Index of lipid peroxidation (measured as TBARS - thiobarbituric acid-reactive substances)
3. Hydrogen peroxide ( $H_2O_2$ ) and
4. Superoxide anion radical ( $O_2^-$ )



The Faculty of Medical Sciences Ethics Committee for the welfare of experimental animals, University of Kragujevac approved the experimental protocol.

### Biochemical assays

#### Determination of nitrites ( $\text{NO}_2^-$ )

Nitric oxide decomposes rapidly to form stable metabolite nitrite/nitrate products. The nitrite level ( $\text{NO}_2^-$ ) was measured and used as an index of nitric oxide (NO) production using Griess's reagent. A total of 0.5 ml of perfusate was precipitated with 200  $\mu\text{l}$  of 30 % sulphosalicylic acid, vortexed for 30 min, and centrifuged at 3000 x g. Equal volumes of the supernatant and Griess's reagent, containing 1 % sulphanilamide in 5 % phosphoric acid/0.1 % naphthalene ethylenediamine dihydrochloride, was added and incubated for 10 min in the dark and measured at 543 nm. Nitrite levels were calculated using sodium nitrite as the standard (18).

#### TBARS determination (index of lipid peroxidation)

The degree of lipid peroxidation in the coronary venous effluent was estimated by TBARS using 1 % thiobarbituric acid in 0.05 NaOH incubated with the coronary effluent at 100°C for 15 min and measured at 530 nm. The Krebs–Henseleit solution was used as a blank probe (19).

#### Determination of hydrogen peroxide ( $\text{H}_2\text{O}_2$ )

Measurements of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) were based on the oxidation of phenol red by hydrogen peroxide, in a reaction catalysed by horseradish peroxidase (HRPO). A volume of 200  $\mu\text{l}$  of perfusate was precipitated with 800  $\mu\text{l}$  of a freshly prepared phenol red solution, and 10  $\mu\text{l}$  of (1:20) HRPO (made ex tempore) was added. An adequate volume of Krebs–Henseleit solution was used in blank probes (instead of coronary venous effluent). The level of  $\text{H}_2\text{O}_2$  was measured at 610 nm (20).

#### Determination of superoxide anion radical ( $\text{O}_2^-$ )

Superoxide anion radical ( $\text{O}_2^-$ ) levels were measured using a nitro blue tetrazolium reaction in TRIS buffer with coronary venous effluent at 550 nm. The Krebs–Henseleit solution was used as a blank probe (21).

### Drug

Aliskiren was used as pure drug (Hangzhou Holypharm Biotech CO., LTD).

### Statistical analysis

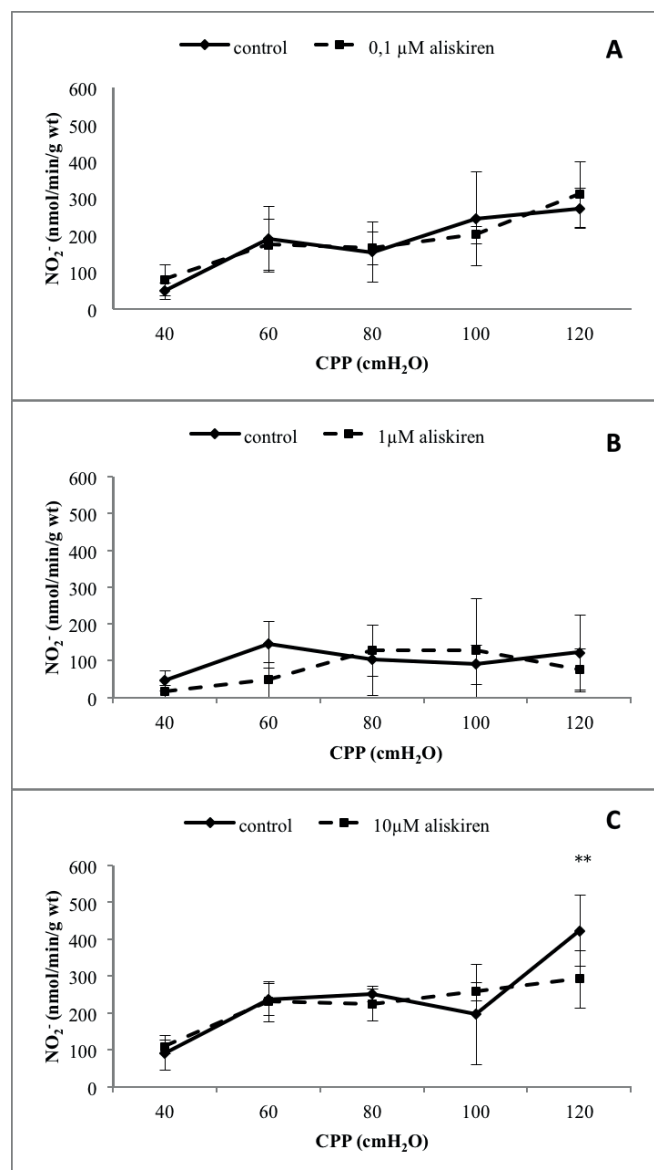
Experimental data are expressed as the mean value ( $\bar{X}$ )  $\pm$  standard deviation (SD). Paired samples t tests were used to test the statistical significance of the results and confirm the hypotheses. A database analysis of the results was performed using the software package SPSS 18th version (SPSS Inc., Chicago, IL, USA). P values lower than 0.05

( $p < 0.05$ ) were considered significant, and p values lower than 0.01 ( $p < 0.01$ ) were considered highly significant.

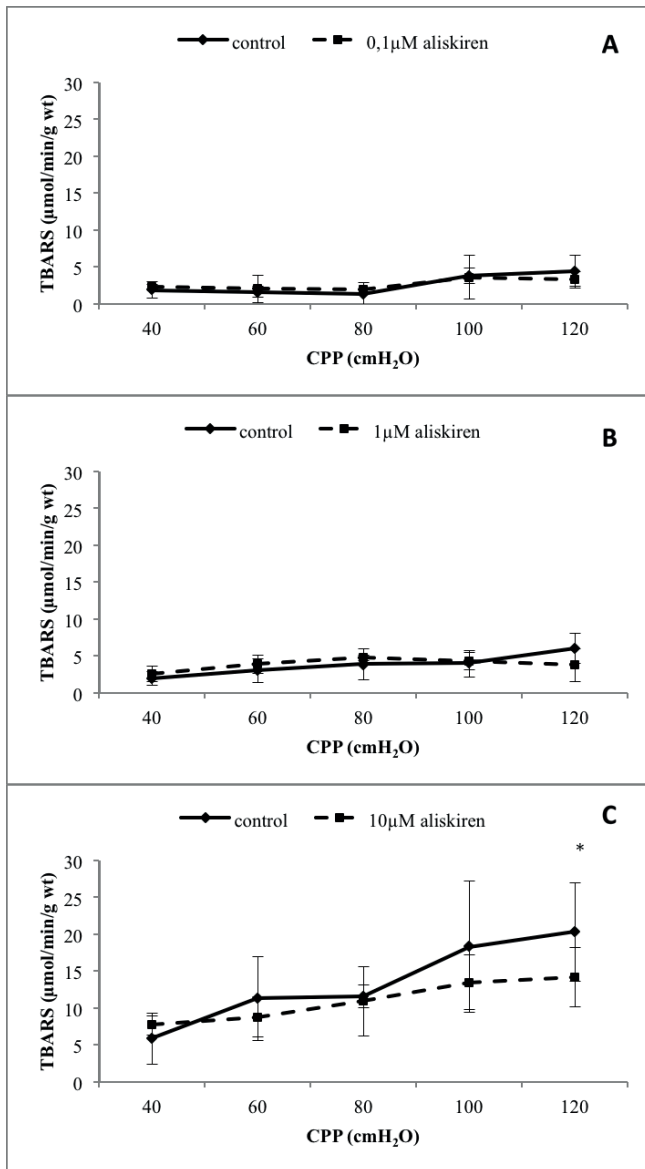
### RESULTS

#### Nitrites ( $\text{NO}_2^-$ )

The administration of 10  $\mu\text{M}$  Aliskiren induced a statistically significant decrease in  $\text{NO}_2^-$  release at CPP = 120  $\text{cmH}_2\text{O}$ .  $\text{NO}_2^-$  release did not change significantly during the administration of 0.1  $\mu\text{M}$  and 1  $\mu\text{M}$  Aliskiren for any CPP value compared with the control conditions (Figure 1A, 1B, 1C).



**Figure 1A-C.** The effects of 0.1  $\mu\text{M}$  Aliskiren (1A), 1  $\mu\text{M}$  Aliskiren (1B) and 10  $\mu\text{M}$  Aliskiren (1C) on the oxidative stress parameter  $\text{NO}_2^-$ . Values are represented mean as the means  $\pm$  SE; \* $p < 0.05$ , \*\* $p < 0.01$ .



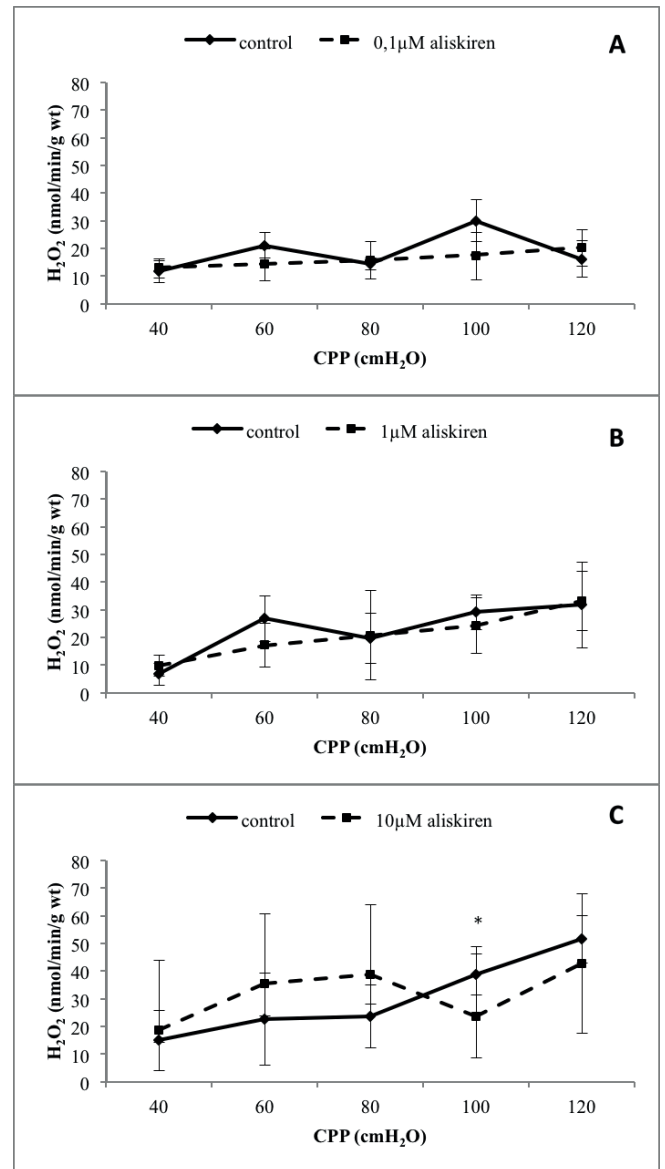
**Figure 2A-C.** The effects of 0.1 µM Aliskiren (2A), 1 µM Aliskiren (2B) and 10 µM Aliskiren (2C) on the oxidative stress parameter TBARS. Values represent means ± SE; \*p<0.05, \*\*p<0.01.

#### Index of lipid peroxidation (TBARS)

The administration of 10 µM Aliskiren induced a statistically significant decrease in lipid peroxidation at CPP = 120 cmH<sub>2</sub>O. There were no statistically significant changes in TBARS values during the application of 0.1 µM Aliskiren or 1 µM Aliskiren over the entire CPP range (Figure 2A, 2B, 2C).

#### Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)

The administration of 10 µM Aliskiren induced a statistically significant decrease in H<sub>2</sub>O<sub>2</sub> release at CPP = 100 cmH<sub>2</sub>O. There were no statistically significant changes in H<sub>2</sub>O<sub>2</sub> values during the application of 0.1 µM Aliskiren or 1 µM Aliskiren over the entire CPP range (Figure 3A, 3B, 3C).



**Figure 3A-C.** The effects of 0.1 µM Aliskiren (3A), 1 µM Aliskiren (3B) and 10 µM Aliskiren (3C) on the oxidative stress parameter H<sub>2</sub>O<sub>2</sub>. Values represent means ± SE; \*p<0.05, \*\*p<0.01.

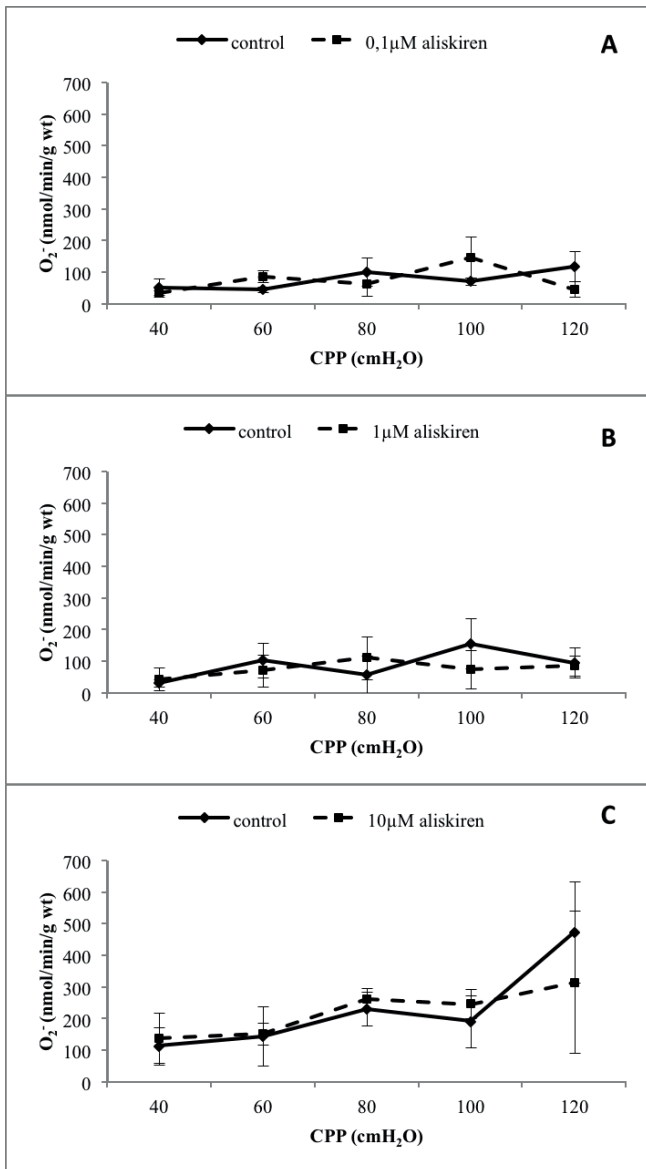
#### Superoxide anion radical (O<sub>2</sub><sup>-</sup>)

No statistically significant changes in O<sub>2</sub><sup>-</sup> release were observed after 0.1 µM, 1 µM and 10 µM Aliskiren administration over the entire CPP range (Figure 4A, 4B, 4C).

## DISCUSSION

Hypertension is the disease that is most responsible for mechanical stress on the heart because of increased arterial pressure and structural and functional alterations of its target organs (22). Long-term hypertension often results in left ventricular hypertrophy, but antihypertensive drugs, including ACEI, ARB and calcium channel antagonists,





**Figure 4A-C.** The effects of 0.1 μM Aliskiren (4A), 1 μM Aliskiren (4B) and 10 μM Aliskiren (4C) on the oxidative stress parameter  $O_2^{\cdot-}$ . Values represent means  $\pm$  SE; \* $p < 0.05$ , \*\* $p < 0.01$ .

exert inhibitory effects on cardiac hypertrophy. However, disease progression and subsequent cardiac dysfunction remain a significant problem in hypertensive subjects (23). Prolonged hypertension also causes structural alterations of the vascular wall, which are characterized by endothelial dysfunction, extracellular matrix deposition, medial layer thickening due to hypertrophy/hyperplasia, and vascular smooth muscle cell migration. There is increasing interest in the development of new therapeutic possibilities against hypertension. One recent addition to the family of RAAS blockers was Aliskiren, which is a DRI that is indicated for the treatment of hypertension. Several studies demonstrated that Aliskiren, as first orally active DRI, could be used as a monotherapy and in combination with other agents

to lower blood pressure (24). Some studies estimated that once daily Aliskiren administration reduced hypertension compared with ACEI, ARB, and diuretics (25-29). Aliskiren is the newest antihypertensive drug, and only a few studies evaluated the influence of Aliskiren on oxidative stress markers.

Several studies investigated Aliskiren and found positive antihypertensive effects (30-32), but no studies investigated the influence of acute Aliskiren administration on the production of oxygen-free radicals in an isolated rat heart.

Yamamoto et al. used a mouse model of renal and cardiac tissue and demonstrated that Aliskiren and valsartan were associated with significant reductions in oxidative stress in these tissues and the combination of these two drugs improved cardiovascular and renal injuries in endothelial NO synthase-deficient mice, which are associated with a greater attenuation of tissue oxidative stress markers (33). Imanishi et al. noted that Aliskiren treatment exhibited protective effects on endothelial function and atherosclerotic changes and co-treatment with an angiotensin II receptor blocker exhibited additive protective effects on both conditions (34).

The present study examined the effects of acute Aliskiren administration (0.1 μM, 1 μM, and 10 μM) on oxidative stress biomarkers in isolated rat hearts.

Our study investigated  $NO_2^-$  and demonstrated statistically significant changes, especially at the highest Aliskiren concentration.  $NO_2^-$  levels were lower than control values (Fig 1C). Luis et al. investigated chronic Aliskiren administration and Aliskiren in combination with Amlodipine in diabetic rats and demonstrated that NO levels increased after Aliskiren administration individually and as a combined therapy (16).

Additionally, TBARS values decreased significantly after 10 μM Aliskiren administration compared to control (Fig 2C). Kamal investigated the effect of 4 weeks Aliskiren administration on TBARS in rat liver tissue homogenates and demonstrated increased TBARS levels, which is similar to our result (35). Lipid peroxidation was also increased in an experimental study using malondialdehyde (MDA) as an end product of polyunsaturated fatty acid oxygenation, which also correlates with our findings (15).

Administration of the highest dose of Aliskiren (10 μM) slightly increased the measured values at lower CPP, but a significant decrease in parameter values was observed at CPP = 100 cmH<sub>2</sub>O (Fig 3C).

Superoxide anion radical levels were similar to Zang et al. who investigated chronic effects of Aliskiren on myocardial ischaemia/reperfusion injury in spontaneously hypertensive rats. This study recorded increased values of oxidative stress parameters in a control group of rats that did not receive Aliskiren. Aliskiren abolished the increased superoxide anion production in the experimental group (17). However, our results did not demonstrate any statistically significant differences, but the Figure for this parameter shows that the highest dose exhibited the lowest values (Fig 4C).



Our data may be significant because few studies examined the influence of acute Aliskiren administration on the production oxidative stress parameters, especially of H<sub>2</sub>O<sub>2</sub>, in isolated rat hearts.

## CONCLUSIONS

The results of the present study provide important insights into the acute and direct effects of Aliskiren on oxidative stress biomarkers in isolated rat hearts. Our results demonstrated that acute Aliskiren effects do not promote the production of reactive oxygen species. Aliskiren may exert beneficial effects on the balance of pro- and anti-oxidants. However, further research on chronic Aliskiren administration is needed to provide a clearer explanation of this phenomenon.

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