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Protective Effect of Cannabinoid Receptor-2 Blockage in Experimental Myoglobinuric Acute Kidney Injury

Efecto protector del bloqueo del receptor cannabinoide 2 en la lesión renal aguda mioglobinúrica experimental

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RESUMEN

Objetivo: La lesión renal aguda es un importante problema de salud pública en todo el mundo debido a la significativa morbilidad y carga económica que provoca. Nuestro objetivo fue investigar los efectos de los agonistas y antagonistas de los receptores cannabinoides sobre los parámetros bioquímicos e histopatológicos de la lesión renal aguda mioglobinúrica (LRA). **Métodos:** Las ratas Wistar se dividieron en siete grupos. Seis grupos se sometieron a una inyección de glicerol al 50% para establecer la lesión renal, mientras que un grupo recibió una inyección de solución salina fisiológica (PS) como control. Se administraron inyecciones intraperitoneales de agonistas (WIN55.212-2) y antagonistas (AM251 o SR144528) a los 60 y 75 minutos de la inyección de glicerol. Se recogieron muestras de suero y orina y se extrajeron tejidos renales. Se analizaron los niveles de glutatión, malondialdehído, urea, creatinina y sodio. Se realizó una evaluación histopatológica semicuantitativa de las secciones teñidas con hematoxilina y eosina. **Resultados:** Los niveles séricos de creatinina y urea fueron significativamente superiores en todos los grupos de lesión renal en comparación con el control. El nivel de creatinina sérica fue significativamente inferior en el grupo WIN+SR144528 en comparación con el grupo AKI. La puntuación del daño histológico fue significativamente inferior en los grupos WIN y WIN+SR144528 en comparación con el grupo AKI. **Conclusiones:** El bloqueo de los receptores cannabinoides 2 mejoró la función y la histología renal frente a la LRA, mientras que el bloqueo de los receptores cannabinoides 1 provocó resultados negativos.

Palabras Clave: lesión renal aguda, receptores cannabinoides, estrés oxidativo, SR144528

ABSTRACT

Aim: Acute kidney injury is an important public health problem worldwide due to the significant morbidities and the economic burden it causes. We aimed to investigate the effects of cannabinoid receptor agonists and antagonists on biochemical and histopathological parameters in myoglobinuric acute kidney injury (MAKI). **Methods:** Wistar rats were divided into seven groups. Six groups underwent a 50% glycerol injection to establish kidney injury, while one group received

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physiological saline (PS) injection to serve as a control. Agonist (WIN55,212-2) and antagonist (AM251 or SR144528) injections were administered intraperitoneally at 60- and 75 minutes following glycerol injection. Serum and urine samples were collected, and kidney tissues were removed. Glutathione, malondialdehyde, urea, creatinine, and sodium levels were assayed. Histopathological evaluation was performed semi-quantitatively on the hematoxylin eosin-stained sections. **Results:** Serum creatinine and urea levels were significantly higher in all kidney injury groups compared to control. Serum creatinine level was significantly lower in the WIN+SR144528 group compared to the AKI group. Histological damage score was significantly lower in the WIN and WIN+SR144528 groups compared to the AKI group. **Conclusions:** Blockade of cannabinoid 2 receptors improved kidney function and histology against MAKI, while blockade of cannabinoid 1 receptors caused negative results.

Keywords: acute kidney injury, cannabinoid receptors, oxidative stress, SR144528

INTRODUCTION

Acute kidney injury (AKI) is a significant public health problem affecting millions of patients worldwide. It affects over 13 million people and causes 1.7 million deaths annually worldwide [1]. Mortality associated with AKI has been reported as 23.9% in adults and 13.8% in children [2]. AKI can progress to chronic kidney injury or end-stage renal disease, affecting the course of cardiovascular and other diseases [3]. An essential consequence of short- and long-term complications is prolonged hospital stay and high medical costs [4]. AKI is characterized by increased blood urea nitrogen and serum creatinine levels due to a sudden decrease in glomerular filtration rate, a decrease in urine volume, and abnormalities in fluid electrolyte and acid-base balance [5]. Rhabdomyolysis and myoglobinuric acute kidney injury (MAKI), mainly due to accidents or earthquakes, are significant public health problems. MAKI is a syndrome that occurs when myoglobin gets into circulation due to damage to skeletal muscles following trauma or non-traumatic causes. Iron, myoglobin, and hemoglobin released as a result of muscle damage plays an essential role in the pathogenesis of MAKI by causing free radicals' production in the kidney, lipid peroxidation, decrease in kidney functions, increase in oxidative stress parameters, and decrease in nitric oxide levels [6,7].

The cannabinoid system plays a vital role in many physiological and pathophysiological processes. The system exerts its effects mainly through two receptors, which are called CB1 and CB2. CB1 and CB2 are transmembrane G-protein coupled receptors (GPCRs) that aid in multiple cellular functions. CB1R binds to Gi/o and inhibits adenylyl cyclase (AC) activity, cyclic adenosine monophosphate (cAMP) formation, and protein kinase A (PKA) activity. Several mitogen-activated protein kinases (MAPKs), including ERK1/2, p38, and JNK, are activated by CB1R. The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway is also activated by CB1R. CB2 receptor signaling mechanisms include inhibition of adenylyl cyclase, activation of mitogen-activated protein kinase (MAPK), and a transient increase in intracellular free calcium levels through modulation of phospholipase C (PLC) [8,9].

CB1 receptors in the kidney are expressed in almost all parts of the nephron and vascular system. CB2 receptors are detected mainly in the renal cortex, especially in mesangial, podocytes, and epithelial cells of proximal tubules [10]. In a previous diabetic nephropathy study, CB1 receptor blockade improved albuminuria [11]. In a study using a renal ischemia-reperfusion mouse model, CB1 and CB2 agonists have been shown to play a protective role against damage [12]. In cisplatin (CP)-induced nephrotoxicity models, it has been shown that CB1 activation has beneficial effects while CB2 activation has detrimental effects [13].

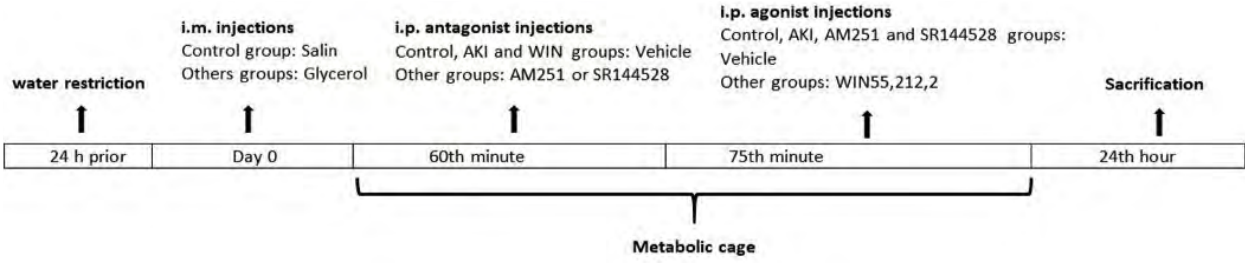
The reported studies mentioned above showed contradictory results. Previous studies have shown that cannabinoid receptors play a role in regulating oxidative stress and lipid peroxidation processes. Considering the critical contribution of oxidative stress in MAKI, we decided to investigate the role of cannabinoid receptors in this model. In our study, the role of the cannabinoid system in the MAKI model has been investigated for the first time.

MATERIAL AND METHODS

Experimental design

The local Ethics Committee approved animal experiments for the study (TÜHADYEK 2019/15). The study used 56 Wistar female rats aged 2-2.5 months and kept in standard laboratory conditions (22 ± 1 °C, 12/12 h light/dark cycle). The rats were divided into seven groups, 8 in each group. In the control group, rats were dehydrated for 24 hours before the injection of physiological saline (PS, 0.9% NaCl). In the other groups (AKI, WIN, AM251, SR144528, WIN+AM251, and WIN+SR144528), rats were dehydrated for 24 hours before hypertonic (50%, in PS) glycerol injection. The injection volume was adjusted to 5 ml/kg and equally divided to the right and left leg muscles for both PS or glycerol solution. Cannabinoid agonists and antagonists were administered intraperitoneally in a vehicle solution (78% PS + 1% Ethanol + 1% Tween 80 + 20% DMSO). In our model, 60 minutes was allowed between the glycerol administration and the start of the cannabinoid administration to resemble real-life hospital admission timing. Cannabinoid receptor antagonists AM251 (CB1) or SR144528 (CB2) were administered at 1 mg/kg following intramuscular glycerol injections at a 60-minute interval. Cannabinoid agonist WIN 55,212-2 (5 mg/kg) was administered at 75th minutes after intramuscular glycerol injection. (**Figure 1**).

Figure 1: Study design



Intramuscular and intraperitoneal injection volumes given to each group were kept equal. After intramuscular injection of PS or glycerol, the rats were placed in the metabolic cage, and urine samples were collected for 24 hours. Rats were sacrificed under general anesthesia (10 mg/kg xylazine and 50 mg/kg ketamine), blood samples were collected by cardiac puncture, and both kidneys were removed and stored for further analysis. Kidneys were bisected by cutting longitudinally, one half was placed in 10% formalin solution for histopathological examination, and the other half was homogenized under cold conditions within phosphate buffer for ELISA, placed in microtubes, and stored at -80°C until analysis. After, blood samples were taken into tubes and cold centrifuged at 1000 g at +4°C for 15 minutes, and urine samples were centrifuged at 1000 g at +4°C for 20 minutes; they were stored at -80°C until assay.

Evaluation of kidney function

Urea, creatinine levels in serum samples, and creatinine and sodium levels in urine samples were measured using an auto-analyzer (AU5800; Beckman Coulter Inc., CA, USA).

Evaluation of oxidative stress

Malondealdehyde (MDA) measurements in kidney homogenate samples were performed using the commercial test kit Bioassay Technology Laboratory Rat Malondealdehyde ELISA Kit (Catalog No: E0156Ra). In order to determine glutathione (GSH) content, the color formed by the free sulfhydryl groups in the tissue homogenates with Ellman's reagent was determined spectrophotometrically [14].

Histological Studies

The kidneys were sliced into 5µm sections after formalin fixation. Histopathological evaluation was carried out on the Hematoxylin and Eosin-stained sections at 200x magnification by Olympus BX51 light microscope. Renal tissue was evaluated in 10 areas, including renal cortex, outer medulla, inner medulla,

and papillae areas, by scoring necrosis, cellular debris, tubular dilatation, and casts deposition between 0-5, semi-quantitatively [15, 16].

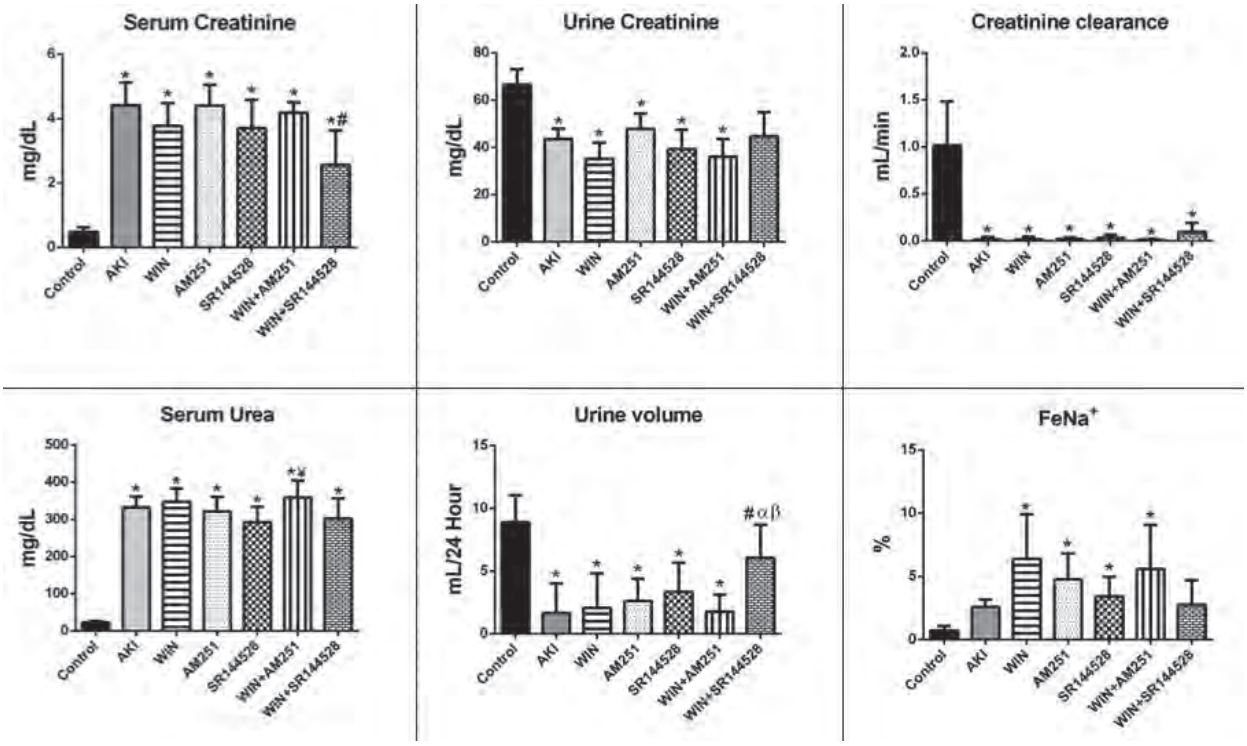
Statistical Analysis

The results were presented as mean ± standard deviation. The suitability of the data for normal distribution was evaluated with a one-sample Kolmogorov-Smirnov test. One-Way Analysis of Variance (ANOVA) was used to compare the groups' mean serum urea and sodium levels. Tukey's HSD test was used for post-hoc between-group comparisons. For non-parametric comparisons, Kruskal-Wallis and Dunnett T3 tests were used. A p-value lower than 0.05 was accepted as significant.

RESULTS

Comparison of renal function markers among the groups is given in **Figure 2**.

Figure 2: Comparison of renal function markers among the experimental groups.



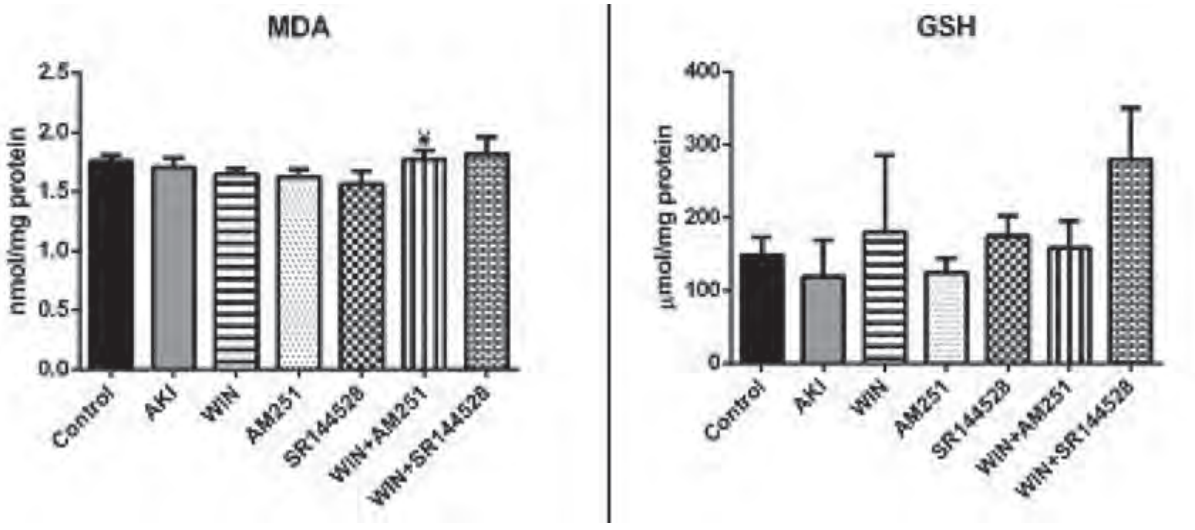
Results are expressed as mean ± SD, n=8, and statistical analysis for serum urea and sodium data was performed by one-way analysis of variance (ANOVA) and post hoc turkey test. Other data were evaluated by Kruskal-Wallis and Dunnett T3 test.
** Compared to the control group p<0.05, # Compared to AKI group p<0.05, **α** Compared to WIN group p<0.05, **β** Compared to WIN+AM251 group p<0.05, ¥ Compared to SR144528 group p<0.05.*
***FeNa+:** Fractional sodium excretion*

Serum creatinine level was higher in all kidney injury groups compared to the control. The WIN+SR144528 group showed significantly lower serum creatinine than the AKI group (2.79±1.18 vs 4.75±0.40, p=0.02). Serum urea level was higher in all kidney injury groups compared to the control. The WIN+AM251 group showed significantly higher serum urea than the SR144528 group (358.60±45.59 vs 291.80±41.65, P=0.01). Urinary creatinine level was similar in the control and WIN+SR144528 groups, while it was lower in other kidney injury groups compared to the control. Urine volume was similar in the control and WIN+SR144528 groups, while it was lower in other kidney injury groups compared to the control. Creatinine clearance was lower in all kidney injury groups compared to the control. Fractional sodium

excretion was similar in control, AKI, and WIN+SR144528 groups, while it was higher in other groups compared to control.

Oxidative stress markers are given in **Figure 3**.

Figure. 3 Oxidative stress markers of the experimental groups.



Results are expressed as mean ± SD, n=8, and statistical analysis was performed by Kruskal-Wallis and Dunnett T3 test.
***¥** Compared to SR144528 group p<0.05.*
***MDA:** Malondialdehyde, **GSH:** Glutathione*

MDA level was higher in WIN+AM251 compared to SR144528 group (1.786±0.10 vs 1.56±0.12, P=0.02). All other groups showed similar MDA levels. GSH levels were also comparable.

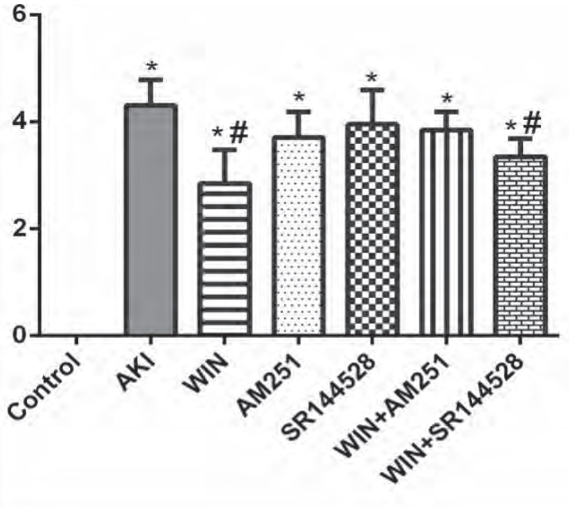
Kidney sections from the control group renal corpuscle, proximal and distal tubules showed normal histology in the cortical areas (**Figure 4 A**).

Tubule structures in the medulla and papillary tips also have a normal histological appearance (**Figure 4 B-D**). In the renal cortex of the glycerol-administered groups, peritubular edema was marked. Microvilli and cellular debris were lost in the proximal tubule lumen. Except for partly mild congestion, no changes were detected in the glomeruli (**Figure 4 E**). Tubular necrosis, dilatation, and cellular vacuolization were observed in the renal cortex and medulla. In addition to tubular dilatation and cellular debris in the inner medulla (**Figure 4 F**), severe tubular necrosis and cast accumulation were seen in the outer medulla (**Figure 1 G**). WIN treatment improved glycerol-induced histopathological damage compared to the AKI group (**Figure 4 I-L**).

(ver Fig. 4 en pág. 204)

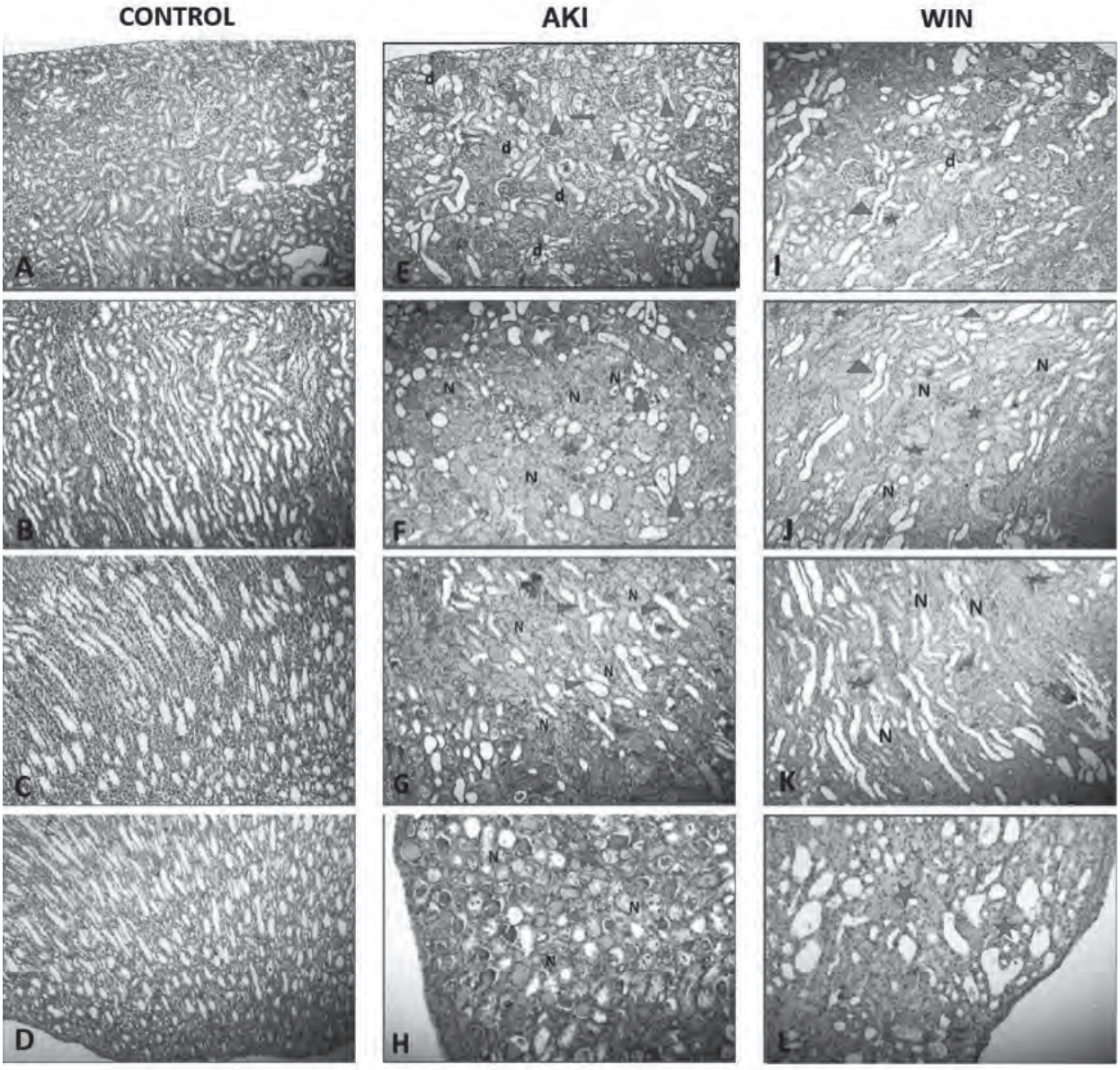
Histological evaluation scores of the cortex, outer medulla, inner medulla, and papilla areas of the experimental groups are given in **Figure 5**.

Figure 5: Renal tissue damage score of the experimental groups.



Results are expressed as mean ± SD, n=8, and statistical analysis was performed by Kruskal-Wallis and Dunnett T3 test.
** Compared to the control group p<0,05, # Compared to AKI group p<0,05*

Figure 4: Histological microphotographs of renal in control, acute kidney injury (AKI) and AKI+WIN treated rats.



Renal histopathology was presented using hematoxylin and eosin staining at x200 magnifications. ***(A-D)*** *microphotographs show normal renal histology in the control group.* ***(E-H)*** *microphotographs are sections of glycerol treated acute kidney injury group.* ***(I-L)*** *microphotographs are sections of the glycerol and WIN treated group. A-E-I Cortex,* ***(B-F-J)*** *outer medulla, (****C- G-K****) inner medulla, x100,* ***(D-H-L)*** *papillary tip x200. (arrowheads) tubular dilatation, (arrow) loss of microvilli, (d) cellular debris in the tubular lumen, (star) cast formation and (N) necrosis.*

All the glycerol-treated groups showed higher renal damage scores when compared to the control group. WIN treatment caused mild histological alterations in the kidney. WIN and WIN+SR144528 treated groups showed lower tissue damage scores when compared to AKI, AM251, SR144528, and WIN+AM251 groups.

DISCUSSION

Sudden traumatic events frequently cause myoglobinuric acute kidney injury. The development time of the traumatic event cannot be predicted. Therefore, we preferred therapeutic rather than preventive interventions. In our model, 60 minutes were taken into

account between the development of the event, hospitalization, and the start of treatment to mimic real life. To the best of our knowledge, our study is the first to investigate the role of the cannabinoid system in the myoglobinuric acute kidney injury model.

The main finding of this study is that exogenous cannabinoid (WIN55,212-2) administration led to partial protective effects on kidney function indicated by lower serum creatinine levels in the myoglobinuric acute kidney injury model. Blocking CB2 receptors by SR144528 augmented these effects, whereas blocking CB1 receptors worsened injury indices. These findings collectively suggest that activation of CB1 receptors mediates the protective effects of cannabinoids, while activation of CB2 receptors may lead to a higher injury in this model. On the other hand, administration of CB2 blocker SR144528 alone was not associated with a decrease in serum creatinine. Deleterious effects of CB2 activation were apparent in serum urea levels. Blocking CB1 receptors by AM251 in the presence of WIN55,212-2 led to higher serum urea compared to the SR144528 group.

Previous studies with different kidney injury models reported contradictory results of cannabinoid receptor antagonists. Li et al. showed that increased 2-arachidonoylglycerol (2-AG), a full agonist that binds to both CB1 and CB2, was associated with serum urea and creatinine levels in a model of renal ischemia-reperfusion injury. Besides, in the same study, serum urea and creatinine levels were found to be lower in the CB1 antagonist AM251 administered group together with ischemia-reperfusion compared to the group suffering only ischemia-reperfusion, and the CB2 antagonist AM630 administered group with ischemia-reperfusion remained similar to the group in which only ischemia-reperfusion was administered [17]. These results were interpreted as the positive effects on kidney functions developed due to exogenous cannabinoid administration in the renal ischemia-reperfusion model mediated by CB2 receptors. Another study, using a cisplatin-induced kidney injury model, showed lower levels of urea and creatinine in the CB1 receptor antagonists (AM281 and SR141716) administered groups compared to the kidney injury group [18]. In another study, the same team showed that the CB2 receptor agonist LEI-101 reduced urea and creatinine levels in a cisplatin-induced kidney injury model in a dose-dependent manner [19]. These studies collectively report the beneficial effects of CB2 activation and CB1 blockade, which contradict our results. The fact that the kidney injury models used in the studies, as mentioned earlier, were different from the model we used may cause the expression levels of CB1 and CB2 receptors to differ. The extent of renal injury is not the same in these models. Cisplatin-induced kidney injury and ischemia-reperfusion mainly affect the S3 segment of the proximal tubule, whereas myoglobinuric injury results are more widespread, affecting most cortex and medulla regions [20]. In addition, the difference between the selectivities of the used receptor agonists and antagonists and the agents we used may be the reason for the discrepancy between the study results. Moreover, the high heterogeneity of the cannabinoid system may have caused the same agent to exert different biological effects (such as GRP55, TRPV1) by affecting defined different receptors other than classical cannabinoid receptors. CB receptor expression varies throughout the nephron. CB1 receptors are present in almost all nephron segments, whereas CB2 receptors are expressed mainly in glomerulus and proximal tubules [21, 22]. Thus, the blockade of CB1 or CB2 receptors in the presence of agonist results in supraphysiologic activation of unblocked receptors in different parts of the nephron. On the other hand, CB1 blockers may not have found any target receptor, as kidney injury affects mainly renal regions where CB1 receptors are expressed in our model.

Another important finding of our study is that the MDA level, an indicator of oxidative stress, was significantly higher in the WIN+AM251 group compared to the SR144528 group. Besides, when the GSH levels, an element of the antioxidant system, were evaluated, the upward tendency in the SR144528 group did not reach statistical significance. We suggest that the cannabinoid system may increase oxidative stress through the CB2 receptors in myoglobinuric acute kidney injury. Lie et al. showed lower MDA levels in the group whose 2 AG level was increased compared to the ischemia-reperfusion group.

Furthermore, the MDA level was comparable in the CB1 antagonist group but higher in the CB2 antagonist group [17]. Mukhopadhyay et al. showed that genetic deletion or pharmacological inhibition of CB1 receptors in mice ameliorated cisplatin-induced oxidative/nitrosative stress [18]. In another study conducted by the same group, CB2 receptor agonist LEI-101 has been shown to attenuate cisplatin-induced renal lipid peroxidation and nitrotyrosine formation [19]. The oxidative stress findings of our study failed to support previous studies. The relationship between the cannabinoid system and oxidative stress parameters may depend on the affected cell type and the severity of the injury [23]. MAKI is characterized by intense iron release and lipid peroxidation. It also severely affects all parts of the nephron, as mentioned above. These features of MAKI may be the reason for the difference in the data of the studies.

Histological changes were evaluated by hematoxylin-eosin staining. Compared to the control group, cortical peritubular edema with varying severity of tubular necrosis and cast cumulation was observed in all glycerol-administered groups. Histological damage score was significantly lower in WIN and WIN+SR144528 groups compared to the AKI group. There was no statistically significant difference between the other groups administered glycerol. It was assumed that exogenous cannabinoid agonists revealed their beneficial histologic effects, mainly affecting CB1 receptors. Zhou et al. inflicted renal damage on BALB-c mice in three different models: unilateral ureteral obstruction, adriamycin administration, and ischemia-reperfusion, and they showed that genetic ablation or pharmacological inhibition of CB2 receptors reduced renal fibrosis; intraperitoneal injections of XL-001, the reverse agonist of CB2, improved kidney injury, fibrosis, and inflammation in both obstruction and ischemia-reperfusion models [24]. In a renal ischemia-reperfusion study, Pressly et al. showed that the use of SMM-295, the selective agonist of CB2 receptors, protects renal tubular epithelial cell structure and function [10]. In the cisplatin-stimulated AKI study conducted by Mukhopadhyay et al., the CB2 agonist HU-308 treatment reduced cisplatin-induced deep histopathological kidney injury, protein cast formation, and epithelial cell desquamation in the renal tubules [25]. The histological evaluation also showed that CB2 receptor blockade led to beneficial effects in terms of morphologic damage.

This study had some limitations. In our study, the possible roles of receptors, such as GRP55 and TRPV1, closely related to the cannabinoid system, were not evaluated. In addition, the used types of cannabinoid agonists and antagonists may produce different findings.

In our study, exogenously administered cannabinoids showed to affect kidney functions and histology in the MAKI model. Our study data showed that CB2 receptor blockade and/or CB1 receptor activation had beneficial effects on kidney function and histopathology.

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