Role of Nrf2 Activator in Ameliorated TLRs Expression in Cerebral Ischemia Reperfusion

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***Abstract*— Background: Ischemic cerebrovascular illness deprives the brain tissue of oxygen. Reperfusion, which can occur naturally through thrombus breakup or through therapeutic recanalization, causes the production of reactive oxygen species (ROS) and inflammation, which further destroys brain tissue.**

Material and Methods: Rats were given general anesthesia and had their bilateral common carotid arteries occluded for half an hour before being reperfused for an hour, both with and without prior treatment with a combination of Bardoxolone 3 mg/kg and DMF 50 mg/kg intraperitoneally (i.p) for 24 hours. Histopathological scoring and quantification of Nrf2, TLR2 and TLR4 levels were performed on brain tissue samples.

Results: In the combination group, the level of nuclear Nrf2 increased significantly (P<0.05). In rats receiving the combination, brain tissue damage scores TLR2 and TLR4 were considerably reduced (P<0.05). The combination group's histological results showed fewer structural lesions.

Conclusions: Combination Nrf2 activators have a neuroprotective effect against cerebral I/R injury by upregulating nuclear Nrf2 protein and decreasing inflammation.

***Index Terms*— bardoxolone, Cerebral I/R, DMF, Nrf2, and TLR.**

I. INTRODUCTION

Cerebral tissues require regular and sufficient oxygenation saturation. Any vascular obstruction, whether transient or continuous, scatters this proportion, causing neurological functions to be distributed and, in certain cases, cerebral cell death. Three areas are affected by global ischemia. an infarct with necrotic cells, a penumbra with viable cells for a varied period of time but non-functional cells, and an exterior non-damaged area (1). During ischemia, hypoxic tissue initiates anaerobic metabolism, resulting in a drop in cell pH and a significant shift in cell electrolyte balance as potassium begins to spill quickly from neurons and glial cells, calcium and sodium ions begin to influx the cells, particularly astrocytes, resulting in a stamped increase in cell water content (2).

To cushion this aggregation of hydrogen ions, the Na+/H+ exchanger discharges abundance hydrogen ions, which creates a vast convergence of sodium ions (3). Ischemia also leads to impairment in ATP production which causes calcium overload through ATPases pumps such as Na+/K+, Ca2+/H+ ATPase pumps, Ca2+ pump which decrease reuptake of calcium by the endoplasmic reticulum (ER) (4). Cells will activate Ca2+ channels leading to cellular membrane depolarization and release of glutamate to the synaptic cleft (5).

When compared to other sections of the body, the cerebrum required 20% of total oxygen, making it the true source of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) during ischemia. When compared to other organs, this trait makes the brain vulnerable to oxidative stress (6). Platelet aggregation by release of leukotrienes, thromboxane, and prostaglandins from arachidonic acid (7) formation of leukocyte–platelet complexes and aggregation induce a prothrombotic response (8), particularly neutrophils, which are the first leukocytes found in the acute ischemic phase , platelet aggregation by release of leukotrienes, and thromboxane (8).

During reperfusion, the production of free radicals increases dramatically, especially in the penumbral zone: (9)

1. Produces O2 through the mitochondrial respiratory chain, xanthine oxidase during hypoxanthine to urate conversion, cyclooxygenase 2 during arachidonic acid breakdown, and NADPH oxidase (NOX) during NADPH oxidation.

2. NOS produces NO, which when combined with O2– produces peroxynitrite anion (ONOO–) when tetrahydrobiopterin (BH4) loses its activity.

Due to its role in drug metabolism, particularly phase II drug metabolism or xenobiotic conjugation, Nrf2 (Nuclear factor erythroid-related factor 2) was dubbed "master regulator of oxidative stress" at first (1).

Recent studies have shown that arterial endothelial cells exhibit TLR2 and TLR4 multiple folds further than venous endothelium, are responsive to several TLR ligands, and cause pro-inflammatory signals via MyD88 stimulation in turbulent blood flow areas like the carotid artery and heart (10-12). TLR2 is implicated in the pathophysiology of cerebral ischemia in both the early and occlusive phases. The most important ligands of toll-like receptors involved in ischemia include HMGB1, oxLDL, and pro-inflammatory activation via increase of TLR2 and TLR4 expression (13). Figure (1)

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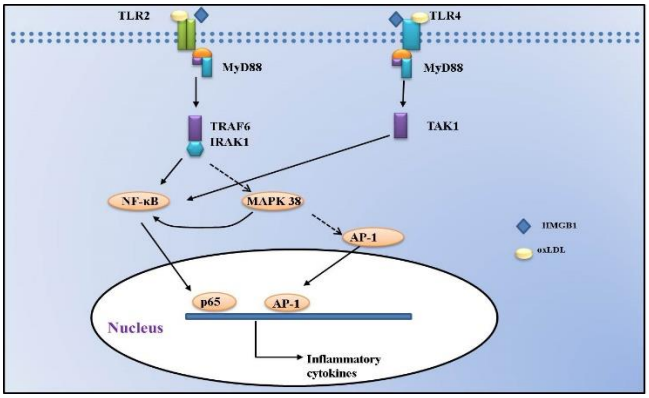

Figure 1: TLR2 and activation of. HMGB1 and ox LDL extracellularly interaction with TLR 2, 4 receptors then through MyD88 induce cascade of released from inflammatory process (13).

Breast and prostate cancer, heart disorders, asthma, COPD, chronic kidney disease, and type 2 diabetes have all been treated with bardoxolone methyl (CDDO-me) and dimethyl fumarate in clinical trials (14-16). Bardoxolone methyl, (CDDO-Me or RTA 402), has anticancer and cancer-preventive activities (17, 18), Dimethyl fumarate (DMF), a fumarate ester, is a recently FDA approved drug for the treatment of multiple sclerosis (MS) and psoriasis under market name Ticfedra (19). Through binding and activation of the IKK at Cys-179, bardoxolone methyl and DMF are a strong inhibitor of the NF-B pathway. This binding prevents NF-B from being released from its cytoplasmic complex (NF-B-IB), hence suppressing downstream proinflammatory signaling pathways (20). Figure (2)

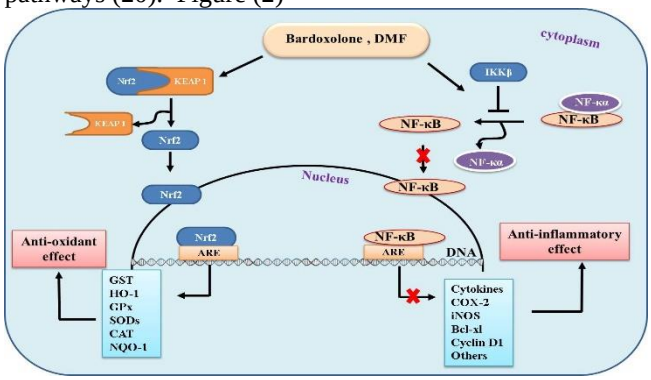

Figure 2: DMF and bardoxolone methyl have similar mechanisms of action. It lowers NF-kB activity by releasing Nrf2 from its Nrf2- keap1 complex, allowing it to translocate to the nucleus and stimulate anti-oxidant enzymes, while also suppressing NF-kB pathway activation by preventing its release from its cytoplasmic complex (2).

A. Review Stage

II. STATISTICAL ANALYSIS

The data are presented as mean ±SEM. A P-value of 0.05 was considered statistically significant using one-way analysis of variance (ANOVA) followed by Tukey's post-test. Kruskal-Wallis nonparametric tests were used to examine histopathological variables. The graphs were created with notepad prism software v8.1 and the analyses were done with SPSS software latest version.

III. MATERIALS AND METHODS

The animal house at the University of Zakho's Faculty of Science provided us with 24 Albino male rats. The animals were housed in the animal house of the University of Kufa's Faculty of Medicine. The University of Kufa's Animal Care and Research Committee approved the experiment, and the study was conducted in accordance with the Laboratory Animals Guide Care. Bardoxolone methyl was dissolved in 0.1% DMSO plus enough distill water and Dimethyl fumarate DMF was dissolved in stock solution (29 mg / 1 ml DMSO plus enough distill water) (21, 22)

The rats were separated into four groups after one week of acclimatization: sham group: rats were given general anesthesia with no occlusion of the bilateral common carotid arteries; control group (ischemic-reperfused): rats were given general anesthesia, then occlusion of the common carotid artery for half an hour, followed by reperfusion for one hour, but no drug was given, combination group: rats were subjected to the same surgical procedure as the control group but received bardoxolone at 3 mg/kg plus DMF 50 mg/kg i.p 24 hours before ischemia, vehicle group: rats were subjected to the same surgical procedure as the control group but received the vehicle of drugs, DSMO, intraperitoneally (i.p) 24 hours before ischemia (13, 23).

A. Induction of global brain ischemia

When the rats were unconscious via given ketamine and xylazine i.p. at 80-100 mg/kg and 8-10 mg/kg, respectively. After the rats were punched out, the neck area was shaved and disinfected with 80 percent ethanol, then the paratracheal muscles were pulled out with sterile artery forceps and the connective tissues were removed with spay forceps (24, 25). The carotid arteries were explored, the common carotid arteries were separated from the vagal nerve, and the arteries were blocked on both sides (left and right) at the same time for 30 minutes, and after that the clamps were removed and reperfusion began for 1 hour to induce global cerebral ischemia/reperfusion injury. Rats were decapitated after 1 hour of reperfusion, brains were extracted immediately and cooled in pre-chilled PBS solution, then frozen for 10 minutes, and each brain was sliced into slices for histopathological and immune-histopathological examinations, as well as an ELISA study (26, 27).

B. Assessment of histopathology

Brain tissues were formalin-fixed and embedded in paraffin wax, then cut into 6 mm thick sections and stained with hematoxylin and eosin (H and E). This study's pathological grading scale was as follows (25): Normal (0): edema, RBC, and eosinophilic neurons are absent. (1) Edema or eosinophilic neurons are present. Moderate (2): edema, eosinophilic neurons, and a few quantities of RBC are present. (3) Edema, eosinophilic neurons, RBC, and necrosis are all present in the severe.

C. Measurement levels of TLR2 and TLR4 through immunohistochemistry technique.

The tissues obtained from untreated and treated groups were

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collected to count the cells labeled with TLR2, TLR4, and HO-1 antibodies according to manufacturer protocol.

The immunohistopathological scoring scale was calculated according to following equation (28)

$$Q = P \times I \quad (1)$$

Q: Quick score P: positive cells I: intensity

Score	0	1+	2+	3+	4+
Positive Cells	<10%	10-25%	25-50%	50-75%	>75%
Score	1	2	3		
Intensity of Staining	weak staining	moderate staining	strong staining		

D. ELISA method was used to measure the levels of Nrf2

The tissue samples for Nrf2 were extracted and isolated using a nuclear and cytoplasmic extraction kit (Beyotime, Jiangsu, China). The supernatants from each group were used to measure Nrf2 levels at the cytoplasmic and nucleoplasmic levels using an ELISA (Nanjing Pars Biochem CO., Ltd, China) according to the manufacturer's procedure.

IV. RESULTS

A. Effect of pharmacological Nrf2 activators on cytoplasm and nuclear brain tissue level of Nrf2.

Bilateral common carotid artery ligation for 30 min followed by 60 min reperfusion was caused translocation of Nrf2 from the cytoplasm to the nucleus. we analyzed the Nrf2 protein level in cytoplasm and nucleus in brain tissue of all groups via the ELISA technique. An ELISA examination revealed that the pharmacological Nrf2 activators groups significantly lowered the level of Nrf2 in the cytoplasm ($p<0.05$), with the combination group having the lowest cytoplasmic Nrf2 level (29.01±0.46) compared to the control group (49.23 ± 1.30) and DMSO group (47.54 ± 1.43). table (1) and figure (3).

*Table 1: Nrf2 cytoplasm levels (ng/ml) of 4 experimental groups. The data expressed by one way ANOVA. * vs sham, # vs control*

Groups n=6	Mean ± SEM	Stander Deviation	95% CI		P value
			Lower	Upper	
sham	73.41 ± 2.11	5.16	67.99	78.83	
control	49.23 ± 1.30	3.19	45.88	52.57	# P< 0.05
DMSO (vehicle)	47.54 ± 1.43	3.50	43.87	51.22	# P< 0.05
Combination	29.01 ± 0.46	1.12	27.83	30.19	* P< 0.05

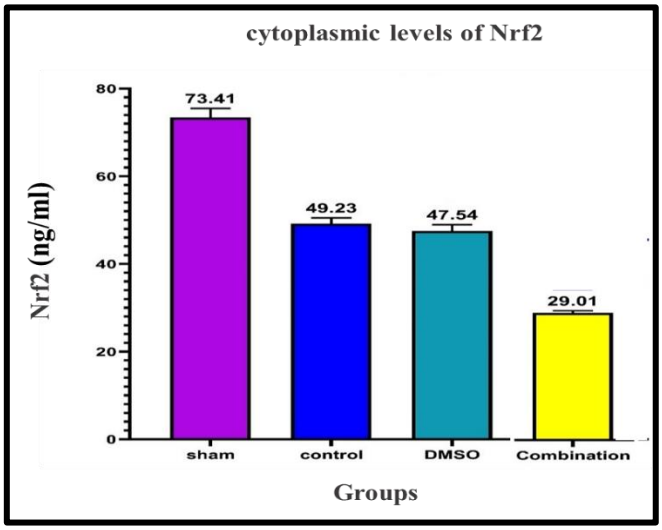

Figure 1: the difference in cytoplasmic levels (ng/ml) of Nrf2 among groups. The control and vehicle groups were reduced in Nrf2 level less than the sham group. Pretreatment rats with combination drugs 24 hr. before ischemia was reduced in cytoplasmic Nrf2 about 25%, 25%, and 41% respectively less than the control group. Combination group was showed a lower level of Nrf2 than two other treated groups (Bardoxolone and DMF).

B. A nuclear level of Nrf2 in brain tissue

An ELISA analysis showed that pharmacological Nrf2 activator groups were elevated level of Nrf2 in nucleus significantly ($p < 0.05$), combination group (46.02 ± 0.50) as compare with control group (31.49 ± 0.43), the combination group was showed the highest nuclear Nrf2 level as compare with control group. The level of Nrf2 in the nucleus of pharmacological activator groups was elevated significantly ($p<0.05$) when compared with a untreated group. While we found insignificant difference ($p>0.05$) between a control group and the vehicle group as shown in table (2) and figure (4).

*Table (2): nuclear levels of Nrf2 of 4 experimental groups. The data expressed by one-way ANOVA. # vs sham, * vs control*

Groups n=6	Mean ± SEM	St. Dv.	95% CI		Pvalue
			Lower	Upper	
sham	22.67 ± 0.72	1.76	20.82	24.52	
control	31.49 ± 0.43	1.04	30.40	32.59	# P< 0.05
DMSO (vehicle)	31.66 ± 0.64	1.57	30.01	33.30	# P< 0.05
Combination	46.02 ± 0.50	1.23	44.73	47.31	* P< 0.05

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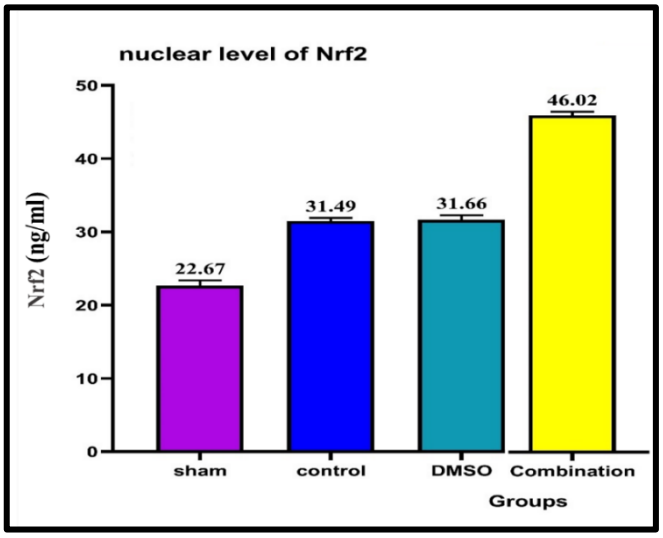

Figure 4: the difference in nuclear levels (ng/ml) of Nrf2 among groups. Administration of combination drugs 24 hr. before ischemia were elevated in nuclear Nrf2 about 32% more than control group. Combination group was shown a higher level of Nrf2 than two other treated groups (Bardoxolone and DMF).

C. Pharmacological Nrf2 activators AND BOTH TLR2 AND TLR4

TLR2, TLR4 levels were unaffected by a 30-minute ligation followed by a 60-minute reperfusion. Using immunohistochemistry, we looked at the level of TLRs in all of the groups' brain tissue. An IHC investigation found that pharmacological Nrf2 activators did not cause TLR expression in brain tissues, and that neither the control nor the vehicle groups did, while control positive tissue (normal spleen tissue) did. (see figures 5-7)

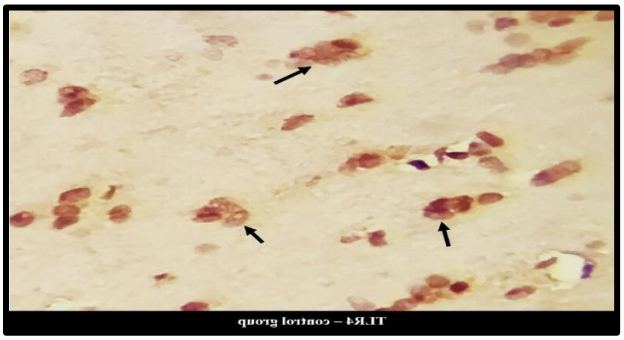

Figure 2: TLR 4 control group. moderate intensity with positive cytoplasmic stain (X 10×40)

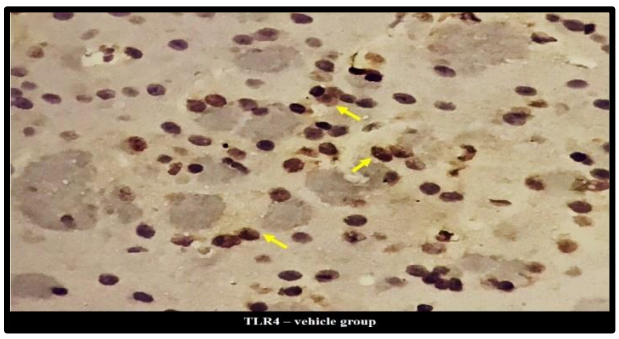

Figure 3: TLR 4 control group. *moderate intensity with positive cytoplasmic stain (X 10×40)*

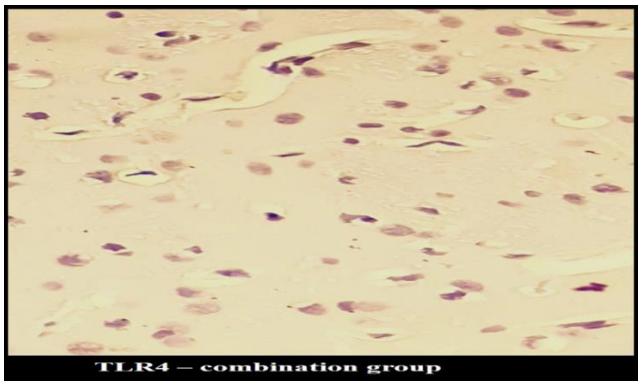

Figure 4: negative TLR 4 for combination group. *photograph represents TLR4 combination group that showed <10% positive cells with moderate intensity (X 10×40).*

D. Pharmacological Nrf2 inducers reduced necrosis, hemorrhage, dark eosinophilic neurons, and edema that caused by BCCAO.

Carotid arteries occlusion for 30 min followed by 60 min reperfusion was caused damage to brain tissue which appeared under the microscope as normal tissue, mild, moderate, or severe damage depend on the present of edema, dark neurons, hemorrhagic area, or necrosis. At the end of the experiment, examination of the brain tissue of all groups by a specialist pathologist. A histopathological examination showed that significant difference ($p<0.05$) in brain tissue damage score between pharmacological Nrf2 activator groups and the control group.

The histopathological score of both control and the vehicle groups were elevated significantly ($p<0.05$) when compared with a sham group, while Nrf2 activator groups were reduced the score significantly ($p < 0.05$) 94% respectively less than the control group. We noticed insignificant difference ($p>0.05$) in the score between a combination group and sham group and also between a vehicle group and control group. See figures 8 and 9.

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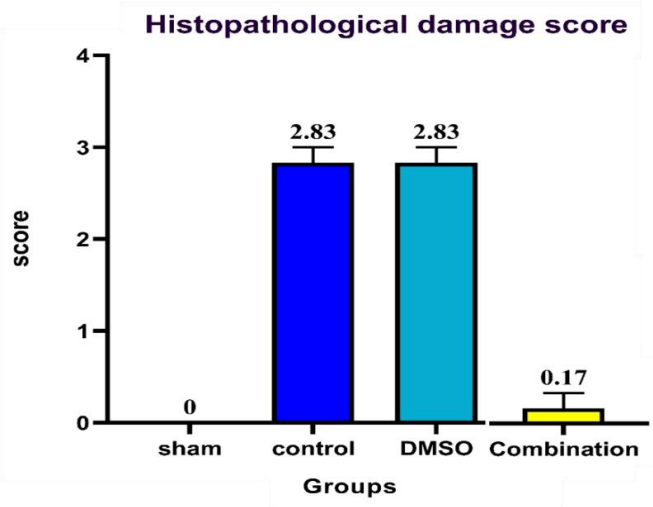
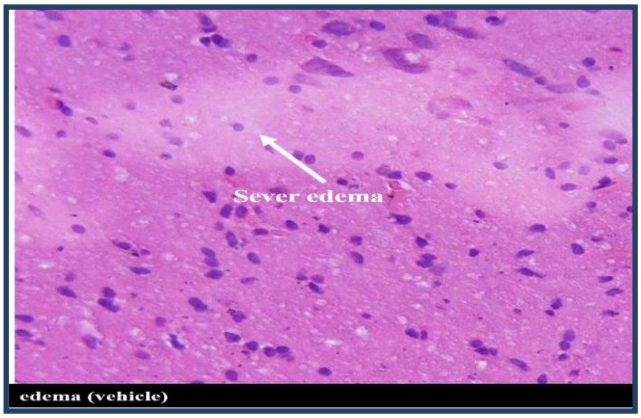
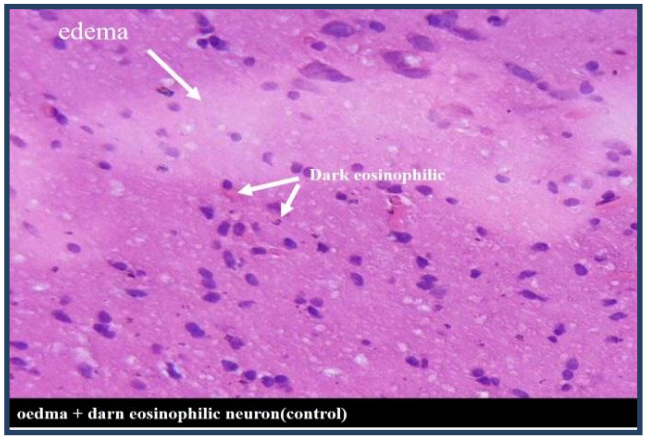
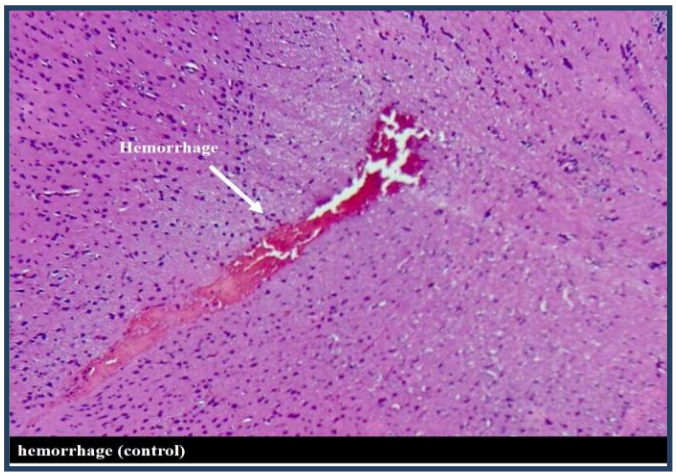
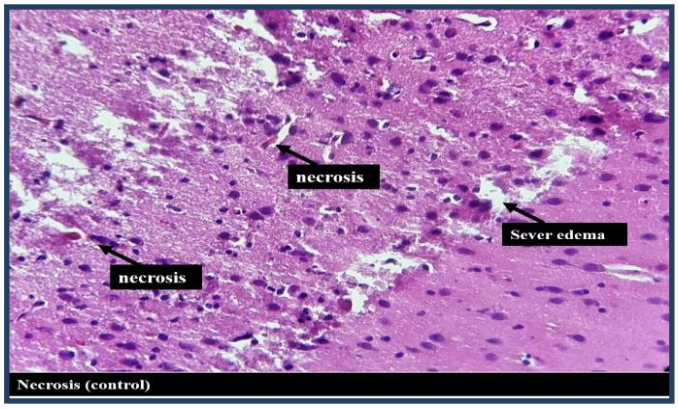


Figure (8): the histopathological score among groups. The control and vehicle groups were increased damage score of 100% than the sham group. Pretreatment rats with combination drugs 24 hr. before ischemia reduced the score 94 percent than the control group. Combination group was showed a lower score than two other treated groups (Bardoxolone and DMF).









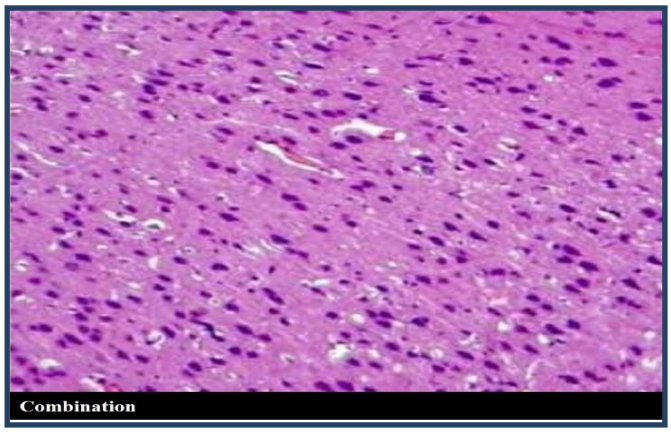


Figure (9) a brain tissue for the combination, control and vehicle groups. The sections showed edema, hemorrhage, and necrosis. The section stained with Hematoxylin and Eosin (X 400).

V. DISCUSSION

In our study, we found that the sham group had the highest cytoplasmic and lowest nuclear levels of Nrf2, indicating a basal level of protein in non-ischemic brain slices, and that the cytoplasmic level decreased while the nuclear level increased significantly in untreated groups after induction of BCCAO when compared to the sham group, indicating that Nrf2 was released from its complex and translocated to the nucleus due to increased stress and inflammation.

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A. Nrf2 level

Nrf2 is a transcription factor that protects against cerebral ischemia-reperfusion injury by upregulating antioxidant genes. Many studies revealed that Nrf2 was elevated at the protein level 24 hours after activators were administered, whereas others found that Nrf2 upregulation began at eight hours and lasted for 72–300 hours. When compared to untreated groups, the administration of Bardoxolone (3 mg/kg), DMF (50 mg/kg), and their combination (3 mg plus 50 mg/kg) 24 hours before ischemia significantly reduced cytoplasm level and significantly elevated nuclear level of Nrf2. The combination group also significantly reduced cytoplasm level and elevated nuclear level significantly more than the other two treated groups, indicating that more Nrf2 protein translocation from cytoplasm to nucleus to induce anti-oxidant activity.

According to one study, after global ischemia, the level of nuclear Nrf2 increased considerably in untreated groups compared to the sham group, whereas it increased thrice in the 5-hydroxymethyl-2-furfural (5-HMF) treatment group compared to the untreated group (29). Another study found that BCCAO did not enhance nuclear Nrf2 levels and that endogenous Nrf2 protein was insufficient to protect neurons against I/R, whereas Rifampicin treatment significantly decreased cytoplasm and increased nuclear Nrf2 levels in the nucleus (30). Other studies focus on the role of Nrf2 activators in focal ischemia-reperfusion, Takahiko Imai et al. (2016) who study the effect of Bardoxolone in reducing hemorrhage induced by focal cerebral ischemia by an increased level of Nrf2 (31). Keita Yamauchi et al. (2016) found that treatment of mice with RS9 (derivative of Bardoxolone) elevated the level of Nrf2 after focal ischemia (32). Jun Zeng et al. (2017) found that Is liquiritigenin (Nrf2 activator) reduced the cytoplasmic level of Nrf2 and elevated its nuclear level (33). Wang H et al. (2019) who study the effect of Swertiamain on same our model, they found that Swertiamain reduced the cytoplasmic level of Nrf2 and elevated its nuclear level (34).

B. Effect on TLR2 and TLR4

In our study, we found that TLR2 and TLR4 do not express after 30 min ischemia and 1 hr. reperfusion according to immunohistochemistry staining while the positive control (normal human spleen, normal human placenta respectively) shown high expression of TLR2 and TLR4. Our suggestion that the time of administration of Nrf2 activators and I/R was not enough to induce expression of TLR2 and TLR4.

Hyakkoku K et al. (2010) who study the expression of TLR4 in MCAO, they found that the number of positive cell increase after 2 hr ischemia to 22 hr reperfusion(35). Ying Wang et al. (2013) shown cerebral I/R was caused elevation of TLR2 or TLR4 after 2 hr. of ischemia when a study by immunohistochemistry(36). Wei Sun et al. (2017) who showed that the TLR2 at mRNA level reaches a maximum at 12 hr. reperfusion(37).

Nrf2 knockout increased expression the TLR4 as compared with the wild type mice after cerebral I/R for 1 hr. ischemia and 24 hr. reperfusion(38). Tissue kallikrein (Nrf2 activator) reduced TLR4 after 2 hr. ischemia and 24 hr. reperfusion and this reduction were better when tissue kallikrein administration immediately than that of 12 hr. after reperfusion(39). Yanzhe Wang et al. (2018) who observed that ursolic Acid treatment decrease number TLR-positive cells compared with control when measured by immunohistochemistry(40).

C. Lower damage score

In our study, we discovered that the sham group had a low damage score, indicating a baseline sore in non-ischemic brain slices, and that damage scores increased dramatically in untreated groups following induction of BCCAO, compared to the sham group, indicating that I/R causes brain tissue damage. When compared to untreated groups, administration of Bardoxolone, DMF, or their combination 24 hours before ischemia significantly reduced brain damage scores; additionally, the combination group reduced its score more than the other two treated groups, indicating that activation of Nrf2 protein reduces brain damages such as hemorrhage and necrosis, and this result supports our hypothesis.

Chandrashekhar et al. (2010) who notice that microscopic examination of control group brain tissue has congestion of blood vessels and neurotic necrosis features after BCCAO(25). Beibei Chen et al. (2016) shown I/R caused marked neuronal damage and pyknotic nuclei in I/R group, while Nrf2 activator (rifampicin) group there are normal neurons notes(30). Ya et al. (2017) found I/R induce neurons damage which appears as pyknosis and shrunken but administration of Nrf2 activator (5-HMF) reduced significantly as compared with the untreated group(29). BH Clausen et al. (2017) who used monomethyl-fumarate as Nrf2 activator in middle cerebral artery occlusion model, they found elevation of Nrf2 reduced edema significantly in the treated group when compared with untreated (41)

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CONCLUSION

We can concluded the following conclusions based on our research findings:

Combination of Bardoxolone plus DMF have a neuro-cytoprotective effect against cerebral I/R injury, manifested as an anti-inflammatory effect that preserved cell structure and viability, which could be mediated by the Nrf2 signaling pathway. The combination reduces the levels of TLRs (2 and 4) in the brain, which could provide a molecular explanation for their protective effects.

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