# Comparison of the effects of thymoquinone and silymarin on the brain of rats having ischemia-reperfusion in the lower extremities

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**Comparison of the effects of thymoquinone and silymarin on the brain of rats having ischemia-reperfusion in the lower extremities**

AIM: *We aimed to show whether ischemia reperfusion (I/R) injury causes damage on brain or not, and whether thymoquinone and silymarin, as antioxidant and anti-inflammatory herbs, have beneficial effects on this damage or not.*
METHODS: *Forty Wistar albino rats were carried out and were randomized to 4 groups with equal numbers (n=10): sham group, implemented of only anesthesia; control group, implemented of anesthesia and I/R injury; silymarin group, implemented of anesthesia and I/R injury and treated with a dose of 200 milligram/kg silymarin ip and thymoquinone group, implemented of anesthesia and I/R injury and treated with a dose of 20 mg/kg thymoquinone. Serum lipid hydroperoxide (LOOH) and total free sulfhydryl (Sh) levels were determined. Light microscopy was used to evaluate histological changes in brain tissue.*
RESULTS: *Serum LOOH levels (0.21 ± 0.04 for control group, 0.29 ± 0.01 for sham group, 0.23 ± 0.09 for silymarin group, 0.29 ± 0.09 for thymoquinone group) were significantly higher and Sh levels (10.74 ± 1.71 for control group, 6.82 ± 0.24 for sham group, 9.12 ± 1.04 for silymarin group, 8.41 ± 1.12 for thymoquinone group) were significantly lower in control, silymarin and thymoquinone groups compared to control group ($p<0.05$ for all). According to the histopathologic damage score assessment, it was seen that the damage decreased significantly in the silymarin and the thymoquinone groups.*
CONCLUSION: *We showed that tissue damage also occurs in brain following the ischemia reperfusion. It was shown that thymoquinone and silymarin is quite effective in preventing this damage.*

KEY WORDS: Brain, Hydroperoxide levels, Ischemia reperfusion injury, Sulfhydryl levels, Silymarin, Thymoquinone

## Introduction

Ischemia reperfusion injury (I/R) is a complex case that is encountered during many surgical interventions, where surgical interventions are implemented by clamping the aorta, such as lower extremity surgery practiced with tourniquet, or vascular surgery arising from arterial occlusion, traumatic vascular surgery or abdominal aorta aneurism [6]. In such cases, even when the blood supply of the tissue remaining ischemic is promoted again, damages occur in distant organs due to physiopathologic alterations ingenerating in the organ remaining ischemic, and this is called ischemia-reperfusion injury. Ischemia-reperfusion injury hinges upon the reactive oxygen metabolites that occur depending on the hypoxia in tissues remaining in ischemia and inflammatory leucocytes to participate in circulation and the destruction created by these metabolites in distant organs [2].
Distant organ damages were shown in many organs (lungs, heart, kidney and intestinal organs) because of the I/R injury [6]. Furthermore, many bio-active agents were used to prevent the I/R injury. We couldn't find

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any study which shows whether cerebral damage occurs due to I/R injury or not in the literature review we made. Therefore, we aimed at showing whether I/R injury causes damage on brain or not, and if it does, whether thymoquinone and silymarin are influential on this damage or not.

## Material and Method

The study was performed with 7 numbered and 12.02.2013 dated approval of Dicle University Prof. Dr. Sabahattin Payzın Health Sciences Research and Application Center Experimental Animals Local Ethics Committee. The experimental animals were obtained from Dollvet vaccine production center. 40 Wistar albino rats with mean weight of 180-240 g were separated into 4 equal numbers of groups (n=10) randomly.
The rats were divided into 4 groups, which are control, sham, silymarin and thymoquinone. All rats used in the experiment were implemented *ketamine 35mg/kg* + *xylazine 5mg/kg* after 8 hours of fasting. General anesthesia was implemented through (Ketalar; Parke Davis, Eczacibasi, Istanbul, Turkey) and Xylazine (Rompun; Bayer AG, Leverkusen Germany) intraperitoneally.

### Ischemia/Reperfusion Model

Ischemia was created with the implementation of tourniquet during 4 hours to the right rear limbs of the rats in the other groups except sham. The saturation loss and color change were provided after taking the ischemia-reperfusion model made by Yassin et al. as example and wrapping the right rear limbs of the rats with elastic bandage. Reperfusion was implemented for 2 hours after 4 hours of ischemia.

### Test Groups and Protocol

Four groups were formed. Ischemia and reperfusion were implemented with tourniquet respectively for 4 and 2 hours to the right rear extremities of all groups except for sham.

*Group-1:* (Sham, n=10):
Any operation was not carried out except for anesthesia.
*Group-2*: (Control, I/R, n=10):
Ischemia and reperfusion were implemented following the implementation of anesthesia. Any antioxidant agent was not used.
*Group-3*: (I/R,Silymarin, =10):
Ischemia and reperfusion were implemented with tourniquet respectively for 4 and 2 hours to the left lower extremity after anesthesia, 200 milligram/kg silymarin was given as ip 5 minutes before the release of tourniquet.
*Group-4*: (I/R,Thymoquinone, n=10):
Ischemia and reperfusion were implemented with tourniquet respectively for 4 and 2 hours to the left lower extremity after anesthesia, 20 mg/kg thymoquinone was given as ip 5 minutes before the release of tourniquet.

At the end of reperfusion period, laparotomy was implemented with midline incision after disinfecting the abdomen, and blood samples were taken from vena kava inferior for the biochemical study in all groups and sent to biochemical examination on performing serum LOOH level and free sulfhydryl measurements in order to analyze the relationship between the ischemia reperfusion and oxidative stress. Sample was taken from the brain tissue for histopathological examination. The brain samples taken from each of the four groups formed were

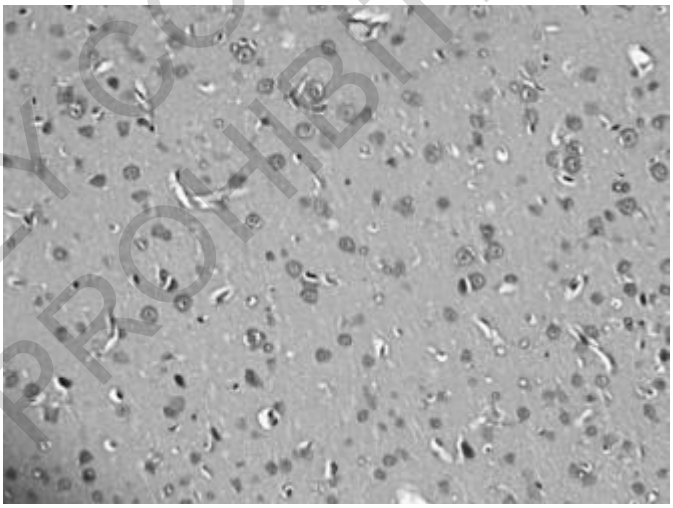

Fig. 1: Normal neurons are shown by black arrow in the Sham cortex section (H&E x400 magnification).

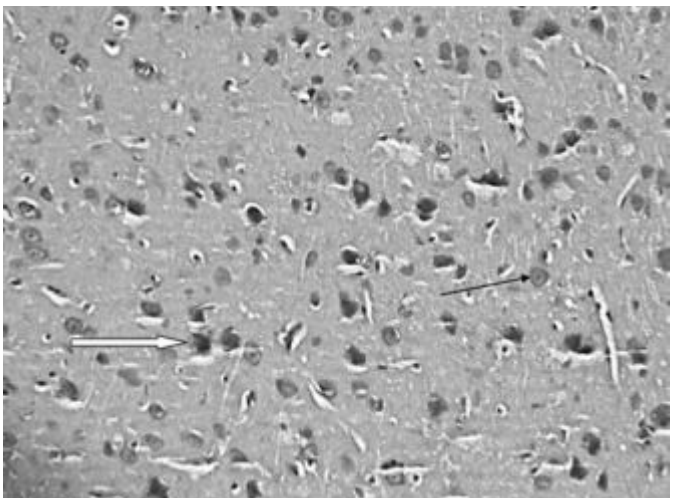

Fig. 2: Normal neurons are shown by black arrow and degenerated neurons are shown by white arrow in the control cortex section (H&E x400 magnification).

fixated in 10% of formaldehyde solution for 24 hours and embedded in paraffin blocks. 5 micron meters thick sections obtained from the blocks were stained with hematoxylin (H&E x400 magnification). The sections were evaluated under microscope. The rats were implemented decapitation after the samples were taken. The total working times were equated in all groups. Normal neurons in the sham cortex section (Fig. 1), normal neurons and degenerated neurons in the control cortex section (Fig. 2) normal neurons and degenerated neurons in the thymoquinone cortex section (Fig. 3) and normal neurons and the degenerated neurons showing pathological indications in the silymarin cortex section (Fig. 4) were shown in the same pathologic frame in order to determine the damage in each brain sample.

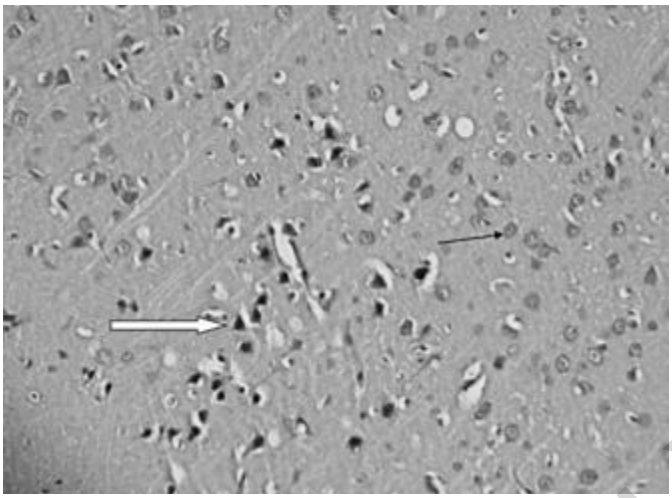

Fig. 3: Normal neurons are shown by black arrow and degenerated neurons are shown by white arrow in the thymoquinone cortex section (H&E x400 magnification).

The material and method section in this study was created by taking sample from the article below.
Perillyl alcohol improves functional and histological outcomes against ischemia-reperfusion injury by attenuation of oxidative stress and repression of COX-2, NOS-2 and NF-κB in middle cerebral artery occlusion rats.

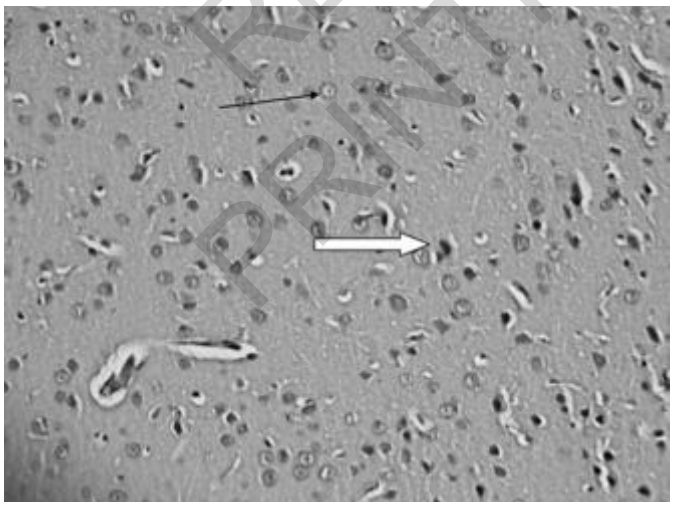

Fig. 4: Normal neurons are shown by black arrow and degenerated neurons are shown by white arrow in the silymarin cortex section (H&E x400 magnification)

### Measurement of lipid hydroperoxide (LOOH) levels

They were calculated with ferrous ion oxidation-xylenol orange method, the Serum LOOH levels of which were defined previously [1].

### Measurement of total free sulfhydryl (Sh) groups

Serum Sh levels were calculated by Ellman[2] method, which was modified by Hu[3]. According to modified Ellman method, 1 ml of buffer containing 0.1 M Tris, 10 mmol/l EDTA, pH 8.2, and 50 ml serum was added to cuvettes, followed by 50 ml of 10 mmol/l 5,5-dithiobis 2-nitrobenzoic acid in methanol. Blanks were run for each sample as a test. After incubation for 15 min at room temperature, sample absorbance was interpreted at 412 nm on a spectrophotometer. Sample and reagent blanks were subtracted. The concentration of Sh groups was calculated using reduced glutathione as the free Sh group standard and the results were expressed as millimolars/liter. Coefficients of variation for measurement of serum Sh levels were 3.6%.

## Results

A total of 40 rats were included in this study and they were divided into 4 groups each having 10 rats. All rats were applied the study protocol and sacrificed after their tissue and blood samples were taken. Serum lipid hydroperoxide (LOOH) level and free sulfhydryl (Sh) parameters were measured in the blood samples taken from the rats and compared statistically among the groups (Table I). The scores obtained as a result of the histopathologic evaluations made by a senior pathologist were compared statistically. The Light Microscope specimens belonging to all groups were shown in the Images 1-4. The histopathologic scoring of the groups was shown in Table II.
According to the histopathologic damage score assessment, as expected, the least score was in the Sham group, while the most damage was in the control group. It was seen that the damage decreased significantly in the thymoquinone and the silymarin groups.

TABLE I - *Statistical comparison of the serum lipid hydroperoxide (LOOH) and free sulfhydryl (Sh) levels in the blood samples taken from the rats.*

| | Control | Sham | Silymarin | Thymoquinone | P ANOVA |
|---|---|---|---|---|---|
| SH | 0.21 ± 0.04 | 0.29 ± 0.011 | 0.27 ± 0.091 | 0.29 ± 0.091 | P< 0.05 |
| LOOH | 10.74 ± 1.71 | 6.82 ± 0.241 | 7.12 ± 1.041 | 7.41 ± 1.121 | P< 0.05 |

1: p< 0.05 ( Comparison with Control group)

TABLE II - *Histopathologic scorings of groups*

| (I) Group | (J) Group | Mean Difference (I-J) | Std. Error | P value | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| sham | control | -9.3472(*) | 1.06638 | <0.001 | -12.7099 | -5.9845 |
| | thymoquinone | -9.5725(*) | 1.06638 | <0.001 | -12.9353 | -6.2098 |
| | silymarin | -9.1539(*) | 1.06638 | <0.001 | -12.5166 | -5.7912 |
| control | sham | 9.3472(*) | 1.06638 | <0.001 | 5.9845 | 12.7099 |
| | thymoquinone | -.2254 | 1.06638 | 1.000 | -3.5881 | 3.1373 |
| | silymarin | .1932 | 1.06638 | 1.000 | -3.1695 | 3.5560 |
| thymoquinone | sham | 9.5725(*) | 1.06638 | <0.001 | 6.2098 | 12.9353 |
| | control | .2254 | 1.06638 | 1.000 | -3.1373 | 3.5881 |
| | silymarin | .4186 | 1.06638 | 1.000 | -2.9441 | 3.7813 |
| silymarin | sham | 9.1539(*) | 1.06638 | <0.001 | 5.7912 | 12.5166 |
| | control | -.1932 | 1.06638 | 1.000 | -3.5560 | 3.1695 |
| | thymoquinone | -.4186 | 1.06638 | 1.000 | -3.7813 | 2.9441 |

*The mean difference is significant at the 0.05 level.



## Discussion

Ischemia is the state where blood flow is under the level required to meet the cellular functions [4]. And reperfusion is the reaching of oxygen again to the tissue, the structure, functions and metabolism of which were impaired by ischemia. While energy is provided to the ischemic tissue with the resumption of blood flow, toxic metabolites are removed from the medium as well. The re-establishment of blood flow to ischemic tissues is needed to stop the growth of cellular damage. However, toxic metabolites comprising in the ischemic area with the reperfusion of the ischemic tissues turn to the systemic circulation and these toxic metabolites lead to serious metabolic results and tissue damage in distant organs [5]. There are many mechanisms to explain the pathogenesis of ischemia-reperfusion injury. Reactive oxygen metabolites and inflammatory leucocytes are the most important ones among these mechanisms [5]. Superoxide radical ($O_2$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical (OH) are formed in the reperfusion. These radicals lead to mitochondrial swelling in cells at ultrastructural level, loss of mitochondrial integrity and destruction in the enzymatic system primarily. As a result of the reaction with unsaturated fatty acids, lipid peroxidation increases and membrane enzymes are inhibited and mitochondrial damage increases with the increase of peroxidation [6].

Oxidative stress period is based on the cytotoxic effects that involve contact with reactive oxygen species (ROS) that contain superoxide anion ($O_2$), hydroxyl radical (OH) and hydrogen peroxide ($H_2O_2$), which are formed as by-products during both normal and abnormal metabolism by using the molecular oxygen. Occurring in the course of ischemic reperfusion, ROS was shown to lead to cerebral edema and changes in vascular permeability [7]. Silymarin is a bioflavonoid complex extract derived from dry thistle seed (Silybum marianum). Silymarin, by balancing antioxidant status and regulating inflammatory mediators reduces oxidative damage [8]. Silymarin pharmacologically is an active flavinoids its cardioprotective [9,10], renoprotective [11], neuroprotektif [12], lungprotective [13], anti-inflammatory, antioxidant, immunomodulator [14], anti-proliferative [15], hepatoprotective [16], and antidiabetic[17] effects have been shown in clinical and experimental studies. It has been reported that silymarin reduces ischemia reperfusion damage in heart, lung and kidney

Thymoquinone, the active constituent of *Nigella sativa* seeds, is a pharmacologically active quinone, which possesses several properties including analgesic and anti-inflammatory actions [18]. It has been reported that thymoquinone prevents oxidative injury in various *in vitro* and *in vivo* studies in rats [19,20]. It has been suggested that thymoquinone may act as an antioxidant agent and prevents membrane lipid peroxidation in tissues [21].

In our study, it was shown that IR/I was formed in

brain tissue following the hind limb ischemia, and thymoquinone and silymarin were effective in preventing this damage.
In the literature review we made while starting to this study, ischemia-reperfusion induced brain damage was formed by connecting middle cerebral artery, external carotid artery, internal carotid artery or common carotid artery [22-24]. But it is controversial whether the damage in this model is ischemic or it is damage due to reperfusion. We could not find any study, which states that the I/R induced organ damage affected the brain, in the literature review we made.
This damage was shown both biochemically and pathologically in our study. The bio-active agents used on the purpose of preventing or reducing the changes induced by I/R formed in the other organs were also included in our study and it was shown that these materials have protective effects against the damage occurring in brain as well.

## Conclusion

This study showed that tissue damage also occurs in brain, like the case in many distant organs, following the ischemia reperfusion. It was shown that thymoquinone and silymarin are quite effective in preventing this damage.
ln the IR / I model, brain tissue is also affected as distant organ damage. It has also been shown experimentally that Thymoquinone and Slymarin can prevent this damage. There is a need for further studies to be conducted on human metabolism by investigating the presence of clinical conditions leading to IR / I damage of our findings.

## Riassunto

Questo studio sperimentale è finalizzato alla dimostrazione se la lesione da riperfusione di ischemia (I / R) causa danni cerebrali o meno, e se il thymoquinone e la silimarina, come antiossidanti e anti-infiammatori, hanno effetti benefici su questo danno o meno.
Sono stati utilizzati ratti albini di Forty Wistar randomizzati a 4 gruppi di eguale numero (n = 10): un gruppo di controllo, utilizzato solo per l'anestesia; un secondo gruppo di controllo, trattato con anestesia e lesioni I / R; un gruppo della silimarina, impiegato per anestesia e lesioni I / R e quindi trattato con una dose di 200 milligrammi / kg di silimarina ip; un quarto gruppo del timochinone, impiegato per anestesia e lesioni I / R e trattato con una dose di 20 mg / kg di chemochinone. Sono stati controllati il siero idroperossido lipidico (LOOH) ed i livelli totali di solfidrile libero (Sh). La microscopia ottica è stata utilizzata per valutare i cambiamenti istologici nel tessuto cerebrale.
Risultati: i livelli sierici di LOOH (0,21 ± 0,04 per il gruppo di controllo; 0,29 ± 0,01 per il secondo gruppo; 0,23 ± 0,09 per il gruppo silimarina; 0,29 ± 0,09 per il gruppo timoquinone sono risultati significativamente più elevati, ed i livelli di Sh (10,74 ± 1,71 per il gruppo di controllo; 6,82 ± 0,24 per il secondo gruppo; 9,12 ± 1,04 per il gruppo silimarina; 8,41 ± 1,12 per il gruppo timochinone) sono risultati significativamente più bassi nei controlli e nei gruppi silymarine e timochinone rispetto al gruppo di controllo (p<0.05 for tutti).
Secondo la valutazione del punteggio del danno istopatologico, si è visto che il danno diminuiva significativamente nei gruppi silimarina e timochinone.
Si è così dimostrato che il danno tissutale si verifica anche nel cervello dopo la riperfusione da ischemia. È stato anche dimostrato che il timochinone e la silimarina sono abbastanza efficaci nel prevenire questo danno.

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