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18ß-Glycyrrhetinic Acid Reduces Vasospasm After Aneurysmal Subarachnoid Hemorrhage in an Experimental Model

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Abstract: This study investigated that the beneficial effects of 18ß-Glycyrrhetinic acid (GA) on cerebral vasospasm in subarachnoid hemorrhage (SAH). A total of 28 Spraque-Dawley adult rats were used. Group 1 (Control) (n=7), Group 2 (SAH) (n=7), Group 3 (SAH+GA 50) (n=7), and Group 4 (SAH+GA 100) (n=7). GA performed through gavages in the 30th minute, 12th hour and 24th hour starting from SAH process. The animals sacrificed under intraperitoneal injection anesthesia by taking biochemical and histopathologic samples at the end of 48th hour. TBARS, SOD, CAT, GSH and GPx values were examined in biochemical studies. Brain tissue and basilar arteries were evaluated in histopathological examination. The averages of lumen cross section in histopathologic, morphometric terms and the biochemical values in both groups receiving GA were found different at meaningful levels compare, the biochemical values were found different meaningfully especially in high dose GA group compared to those groups not receiving GA statistically. The results were defined as Mean±Standard Deviation, and $p<0.05$ values were accepted as meaningful statistically. Based on our results, high dose GA found useful in experimental medical treatment of cerebral vasospasm due to SAH. ©2020 NTMS.
Keywords: Subarachnoid Hemorrhage (SAH), Serebral Vasospasm, 18ß Glycyrrhetinic Acid.

1. Introduction

In cranial or spinal region, in the subarachnoid space containing the cerebrospinal fluid between piamater and arachnoid membrane, hemorrhage that occur due to various reasons are called subarachnoid hemorrhage (SAH) (1-3).
Most frequent cause of SAH is traumas. Any SAH with etiologies other than trauma are called primer SAH or spontaneous SAH; and spontaneous SAH may occur because of many different etiologies. Most frequent cause of spontaneous SAH is aneurism ruptures (4). Consensus is not available about its incidence because of different life style, genetic structure, and various risk factors in different populations. Rebleeding and vasospasm in patients with SAH form most significant

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reasons for high mortality and morbidity. Recently, there has been a decrease in the rates of mortality and morbidity based on rebleeding due to early surgical treatment to SAH patients by neurosurgery clinics. In line with such developments, protection, and treatment of vasospasm, being the other most significant cause of mortality and morbidity in SAH patients, gained importance.

Cerebral vasospasm is pathologic stenosis that occurs in cerebral arteries following SAH. Cerebral vasospasm may be diagnosed by the observation of ischemia that occurs in some part of brain following SAH clinically, neurological deterioration that develops in connection with infarcts and stenosis in cerebral artery lumen by radiological means. Cerebral vasospasm is a clinic diagnosis and radiological observation does not make diagnosis alone. It only supports the clinical diagnosis.

Although physiopathology of cerebral vasospasm has been the subject of many studies, it could not been clarified fully. There have been different hypotheses about physiopathology. One of those hypotheses suggested and accepted is the formation of oxyhaemoglobin with deterioration of erythrocytes at subarachnoid distance and that such oxyhaemoglobin is the main spasmogenic responsible in cerebral vasospasm (5-7). Furthermore, it is known that, in cerebral vasospasm, there is reduction in brain perfusion in the distal of artery narrowing in connection with pathologic vasoconstriction and that, as a result, there is oxidative stress in brain tissue due to ischemia/reperfusion. Free oxygen radicals that occur on hypoxic ground are considered as the primary responsible for brain tissue damage during oxidative stress. Antioxidants prevent vasoconstriction making effect of free oxygen radicals that occur during the transition of oxyhaemoglobin into methaemoglobin, which is one of the accepted pathophysiology hypotheses of cerebral vasospasm (5-8).

18β-Glycrrhetinic acid (GA) as we use in our study is a hydrolyzed metabolite of glycyrrhizin. GA is a material known as licorice root extract contained in Glycyrrhiza glabra (licorice) plant that has a wide area of use in traditional Chinese and Japanese alternative medicine (9). Recent studies have shown that GA has strong anticancer, anti-inflammatory, and antioxidant properties (10, 11).

We aimed in this study to determine that GA, having the property of inducing antioxidant defense mechanisms, may prevent cerebral vasospasm in SAH patients and determine how the dose dependent results of its effect reducing the brain tissue damage in connection with ischemia/reperfusion in cerebral vasospasm may be.

2. Material and Methods

2.1. Animals and experimental protocol

The present study was approved by the Ethics Committee on Animal Research of İnönü University and carried out in accordance with The Guidelines for Animal Research from the National Institutes of Health (NIH). Spraque-Dawley male rats weighing 230-250 g were supplied by the İnönü University Laboratory Animals Research Center (Malatya, Turkey), housed in sterilized polypropylene cages, and given an ad libitum diet of standard commercial food pellets and water. All rats were kept under a 12:12 hours light: dark cycle at 22±1 °C ambient temperature and 55±5% humidity.

In this study, the groups were arranged as Group 1 (Control) (n=7) was the one without any processes, Group 2 (SAH) (n=7) that did not receive any treatment following SAH process, Group 3 (SAH+GA 50) (n=7) that received GA, dissolved in corn oil at an amount of 0.5 ml/12.5 mg on the 30th minute, 12th hour and 24th hour starting from SAH process through gavages, and Group 4 (SAH+GA 100) (n=7) that received 1 ml/25 mg GA dissolved in corn oil through gavages in the 30th minute, 12th hour and 24th our starting from SAH process. Totally 28 Spraque-Dawley adult rats were used. The animals in all groups were sacrificed under intraperitoneal injection anesthesia by taking biochemical and histopathologic samples at the end of 48th hour. GA (Sigma-Aldrich, Product No: G10105) was used in our study. GA was prepared by dissolving in corn oil as 25 mg in 1 ml.

2.2. Subarachnoidal hemorrhage model

Following anesthesia, all the rats were shaved between inion and atlas. After cleaning the field with Batticon, approximately 2 cm of skin region was prepared between inion and atlas. Afterwards, the rats in all groups other than the control group were brought to supine position. Abdominal region was cleaned with Batticon; and following skin incision, abdominal aorta was revealed. Abdominal aorta was catheterized and 0.3 ml nonheparinized arterial blood was taken. Then the rats were brought back to prone position. Head was brought to hyperflexion and cisterna magna was entered using PPD injection at the distance of atlanto-occipinal. Cerebrospinal fluid (CSF) of all the rats other than the control group was drained at equal amounts (0.2 ml). Then with the same rats, non-heparinized blood taken from abdominal aorta was injected slowly at equal amounts (0.2 ml). Following this, the rats were kept in trendelenburg position for 15 minutes on the table prepared before to ensure distribution of blood to prepontine cistern. The rats were taken to their cages after waking up.

2.3. Sacrification

Anesthesia was performed on all rats at the end of 48 hours through intraperitoneal injection of the mixture of Ketamine Hydrochloride (60 mg/kg) (Ketalar, Parke Davis) and Xylazine Hydrochloride (10 mg/kg) (Rompun 2% Bayer) by spontaneous respiration. Thoracotomy was applied to all rats following anesthesia. Following thoracotomy, blood drawing started. Left ventricle was entered and 6 cc of blood in

average was drawn from all subjects. Following blood drawing, intracardiac serum physiologic was applied for 5 minutes and tissue perfusion was performed. Thus, brain tissue was mad free of blood elements. Then all subjects were subjected to bilateral frontoparietooccipital craniectomy. The cerebrum, cerebellum and brain stem remaining on the foramen magnum were removed totally by protecting the anatomic integrity.

Macroscopically, subarachnoid hemorrhage was observed prevalently around the vertebral artery and basilar artery on the basal surface of brain stem in all subjects subjected to SAH. Cross sections of brain tissue involving the basilar artery were taken. Those cross sections were fixed by placing in 10% formaldehyde. The samples taken for the purpose of preparing tissue homogenates were stored inside aluminum foil at -30 °C.

The tissues were weighted and placed in glass tubes. 1.15% of potassium chloride was added thereon as dilution at a rate of 1/10 (g/h) and then they were homogenized for 3 minutes at 16.000 cycles/minute in homogenizers of glass-teflon deepfreeze by maintaining their coolness. Tissue protein determinations were performed on such homogenates prepared. The remaining homogenate was centrifuged at 3500 rpm for 45 minutes at +4 °C and supernatant was obtained.

Glutathione (GSH) and protein levels as well as glutation peroxidase (GSH-Px) and catalase (CAT) enzyme activities were measured in those supernatants. The reagent formed of the mixture of chloroform/ethanol (3/5, h/h) was added to the remaining supernatant at a rate of 1/1 (h/h) and was mixed using vortex. Then, it was centrifuged at 3500 rpm for 45 minutes. Tissue superoxide dismutase (SOD) enzyme activity and protein measurements were performed again at the chloroform/ethanol phase on top.

2.4. Biochemical examinations

SOD activity measurement was done based on the method specified by Sun et al. (12). GSH-Px activity determination was done based on the method specified by Beutler (13). CAT enzyme activity determination was done based on the method specified by Aebi. (14). In reduced GSH measurement, the activity was determined based on the method identified by Ellman as dithionitrobenzoic acid recycling method (15). In Tissue Protein Measurement (TBARS), the determination of protein quantity in homogenate and supernatants were done based on the method defined by Lowry at al. (16).

2.5. Histological examinations

The tissue samples were determined for 48 hours inside 10% formaldehyde. Following the determination, histological tissue tracking procedure was applied on the tissue samples and embeded in paraffin blocks. Cross sections of 5μm thickness were taken from paraffin blocks with the help of microtome. Those cross sections were dyed by using Hematoxylin-Eosin dyeing method and were examined and photographed by using Leica DFC 280 light microscope and Leica Q Win Image Analysis System (Leica Microsystems Imaging Solutions, Cambridge, UK).

Transverse sections of basilar arteries in the histological cross sections dyed with Hematoxylin-Eosin were evaluated in histopathologic terms by light microscope. The diameter of basilar arteries, lumen diameter of basilar arteries, and vein wall thickness were measured by using Leica Q Win Image Analysis System.

2.6. Statistical Analysis

Statistical evaluations were performed by using "SPSS for Windows 12.0" package program. Since the data following normality test were in conformity with non-parametric test assumptions, Kruskal-Wallis H variance analysis was used in the comparison of groups. Significances were evaluated by making paired comparisons through Mann-Whitney U test. The results were defined as Mean±Standard Deviation and $p<0.05$ values were accepted as meaningful statistically.

3. Results

3.1. Biochemical findings

The values of TBARS, SOD, CAT, GSH and GPx levels are shown in Table I. As a result of the evaluations, it was determined that the TBARS level, being the indicator of oxidative damage in rats with SAH, increased meaningfully in statistical terms compared to the control and all other groups. Furthermore, it was determined that it caused significant degree of decrease statistically in GSH, SOD, GPx and CAT levels being antioxidant defense system elements in connection with the formation of SAH in the same trial group.

Furthermore, it was determined that there was meaningful level of decrease statistically in the increase of TBARS caused by SAH in GA treatment and that such decrease was shaped in connection with the dosage to the extent that the decrease in TBARS level and the reversal of effects of SAH were more apparent in the group treated with a dosage of 100 mg/kg compared to the group treated with a dosage of 50 mg/kg. It was observed that the TBARS value in the group of high GA dose approached the value of control group and that there was no more a difference between the two groups statistically. In addition, it was determined that GA treatment partially reversed the changes caused by SAH in antioxidant defense systems again in connection with the dosage. It was determined that low dose GA treatment caused meaningful levels of parameters approach normal in GSH, SOD and GPx levels compared to SAH group; however, that there was not any improvement with low dose GA at only CAT level. It was observed that in high dose GA treatment, there were meaningful statistical increases in all parameters compared to SAH group and the values ap-

proached the control group as a result of such increases. It was determined that the changes that occurred when high dose and low dose GA groups were compared changed in all parameters in connection with the dose; and that there were meaningful differences statistically in the groups of high treatment compared to the groups with low dose GA.

3.2. Histopathologic findings:

Brain tissue in the control group was observed with normal histological appearance. In the brain tissue samples of SAH group dyed with Hematoxylin-Eosin, the congestion and cell infiltration (Figure 1A), vascular congestion (Figure 1B), cell infiltration (Figure 1B, 1C, 1D, 1F) and hemorrhage (Figure 1E, 1F) were observed in piamater layer. However, it was determined that 18β-GA application reduced the histopathologic damage in the group of SAH model and remedied such negative effects at meaningful levels. Namely, it was determined that high dose (100 mg/kg) GA application changed the histological damage positively at meaningful level compared to low dose (50 mg/kg) GA application. It was observed that the neurons in brain cortex in the control group had normal histological appearance (Figure 2A). It was determined that, in SAH group (Figure 2B), there were quite a lot degeneration in such neurons; however, 50 mg/kg (Figure 2C) and 100 mg (Figure 2D) GA application caused significant level of decrease in the neuron damage in connection with the dose. In addition to this, it was determined that purkinje cells had normal histological appearance in control group (Figure 3A) when the cerebellum region was examined, and in the group of SAH model (Figure 3B), there were apparent degeneration in purkinje cells.

Again, it was determined that GA meaningfully reversed the cell damage that occurred in 50 mg/kg (Figure 3C) and 100 mg/kg (Figure 3D) doses in connection with the dose.

Basilar artery examination:

It was observed through light microscope examination in control group that basilar artery structure had normal histological appearance. It was observed that intima, media and adventitia layers from the inside out were of normal histological structure. Single layer of flat endothelial cells, circular course smooth muscle cells, and the connective tissue surrounding such formations had normal histological appearance. It was determined that there was meaningful decrease in basilar artery and lumen diameter of basilar artery in SAH group; on the other hand, there was increase in vein wall thicknesses. Decrease in the folds of lamina elastica interna and increase in the contraction of smooth muscle cells of media layer was determined. Furthermore, cytoplasmic vacuolization in smooth muscle cells was distinctive. Moreover, it was determined that GA applications reversed the changes that occurred in basilar artery and caused by SAH meaningfully in connection with the dose (Figure 4).

It was observed through light microscope that the increase in vein wall thicknesses regressed and there was increase in basilar artery diameter and lumen diameter (Table 2). Furthermore, GA application caused minimal contraction in smooth muscle cells and significant decrease in cytoplasmic vacuolization.

Table 1: The values of TBARS, SOD, CAT, GSH and GPx levels in the brain tissue of rats(n=7), (Mean±Standard Deviation).

	Group 1	Group 2	Group 3	Group 4
TBARS nmol/g tissue	8.62 ± 0.46^{a}	18.2 ± 0.93^{b}	14.1 ± 1.21^{c}	9.3 ± 0.96^{d}
GSH nmol/ml	219.8 ± 6.7^{a}	113.9 ± 8.1^{b}	158.8 ± 11^{c}	201.1 ± 9.1^{d}
CAT k/mg protein	0.024 ± 0.001^{a}	0.011 ± 0.001^{b}	0.012 ± 0.001^{c}	0.018 ± 0.001^{d}
SOD U/mg protein	32.60 ± 1.23^{a}	17.16 ± 1.91^{b}	23.81 ± 1.86^{c}	28.15 ± 2.19^{d}
GPx U/mg protein	280.6 ± 12^{a}	165.4 ± 11^{b}	203.6 ± 19^{c}	255.4 ± 17^{d}

Letters a,b,c, and d on the same column show the statistical difference between the groups ($p \leq 0.01$).

Table 2: Basilar artery dimension, basilar artery lumen diameter and basilar artery wall thickness values in groups applied with SAH and GA (n=7), (Mean±Standard Deviation).

	Group 1	Group 2	Group 3	Group 4
Diameter of basilar artery	172.98±35.00[a]	106.57±11.66[b]	120.57±10.91[c]	143.48±3.81[d]
Lumen diameter of basilar artery	102.32±23.45[a]	57.65±7.76[b]	65.69±2.08[c]	71.10±9.84[d]
Wall thickness of basilar artery	25.01±1.50[a]	39.95±9.67[b]	38.40±4.34[c]	36.84±3.87[d]

Letters a,b,c, and d on the same column show the statistical difference between the groups ($p \leq 0.01$).

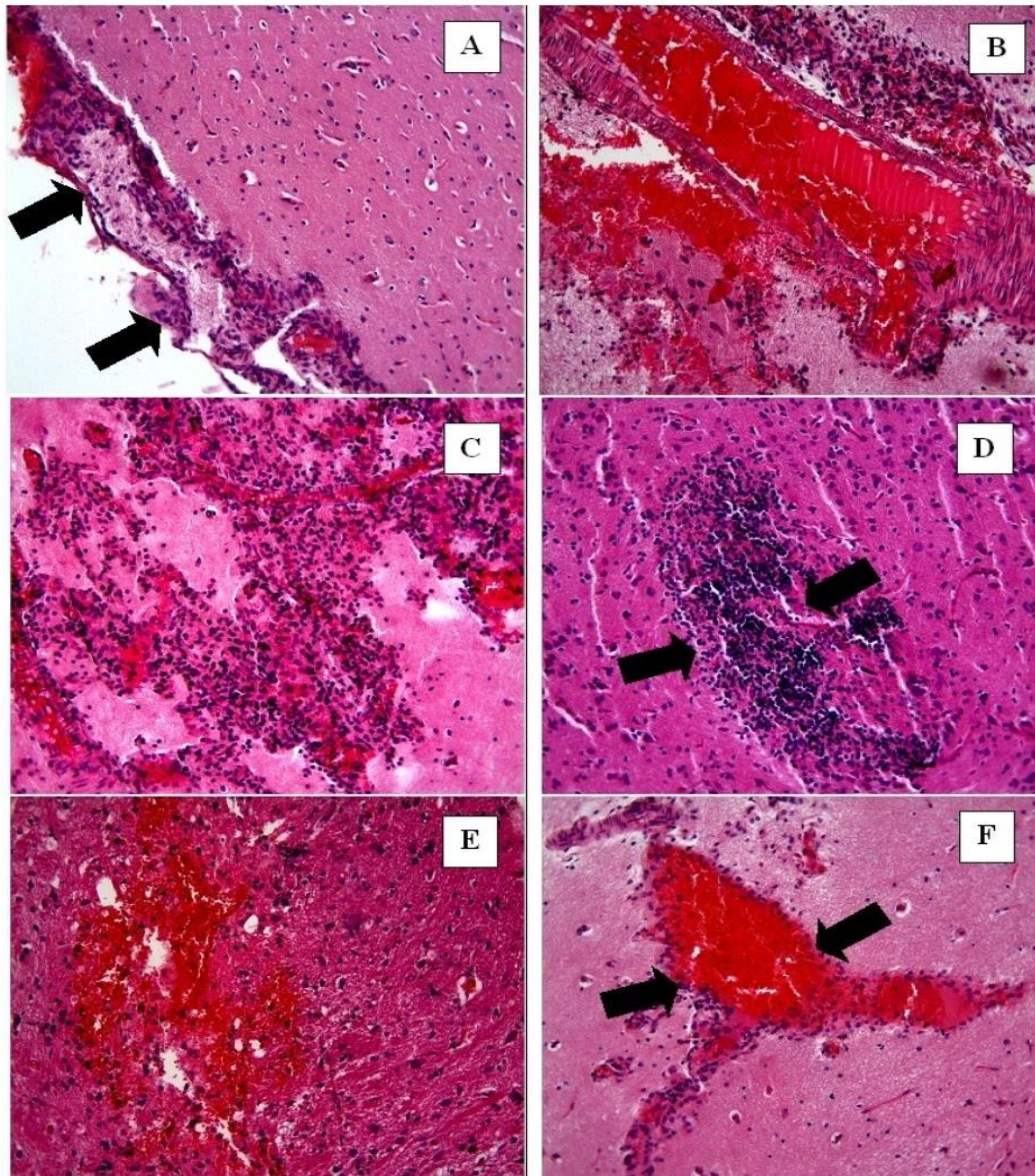

Figure 1: Group 1; In piamater layer, cell infiltration and congestion (arrows) (A), in brain tissue, vascular congestion (B) and cell infiltration (B, C, D, F), hemorrhage (E, F). A, B, C, E, F: H- E; x20, D: Hematoxylin Eosin; x40.

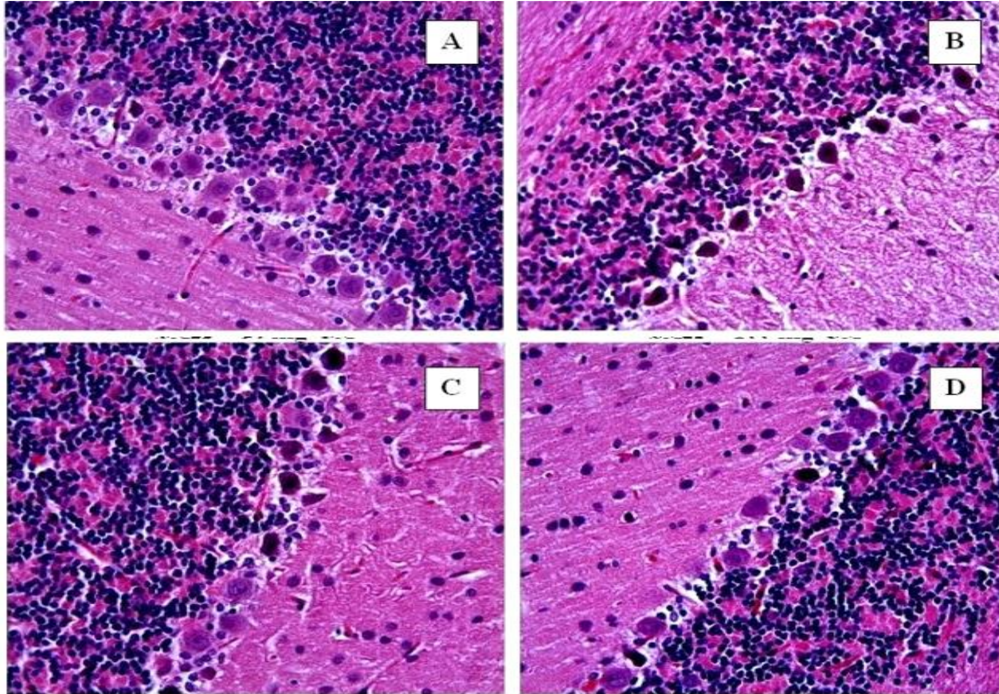

Figure 2: Group 1; (A) Purkinje cells with normal histological appearance. Group 2; (B) degenerated purkinje cells, group 3 (C) and group 4 (D); decrease in degenerated cells. A, B, C, D: H-E; x40.

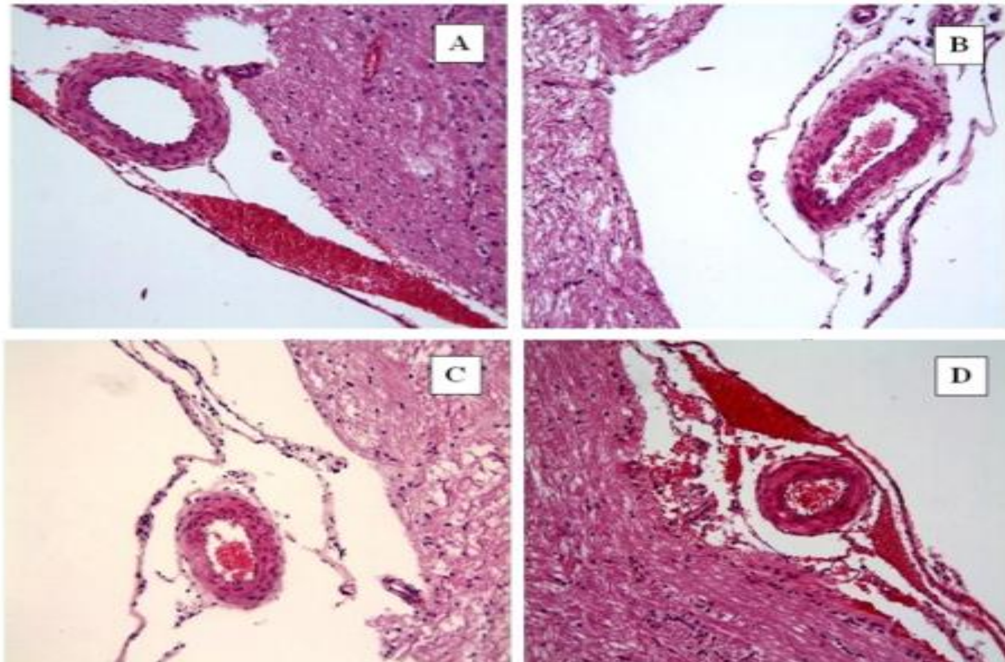

Figure 3: Basilar artery cross sections in group 1 (A), group 2 (B), group 3 (C), group 4 (D) groups. H-E; x20.

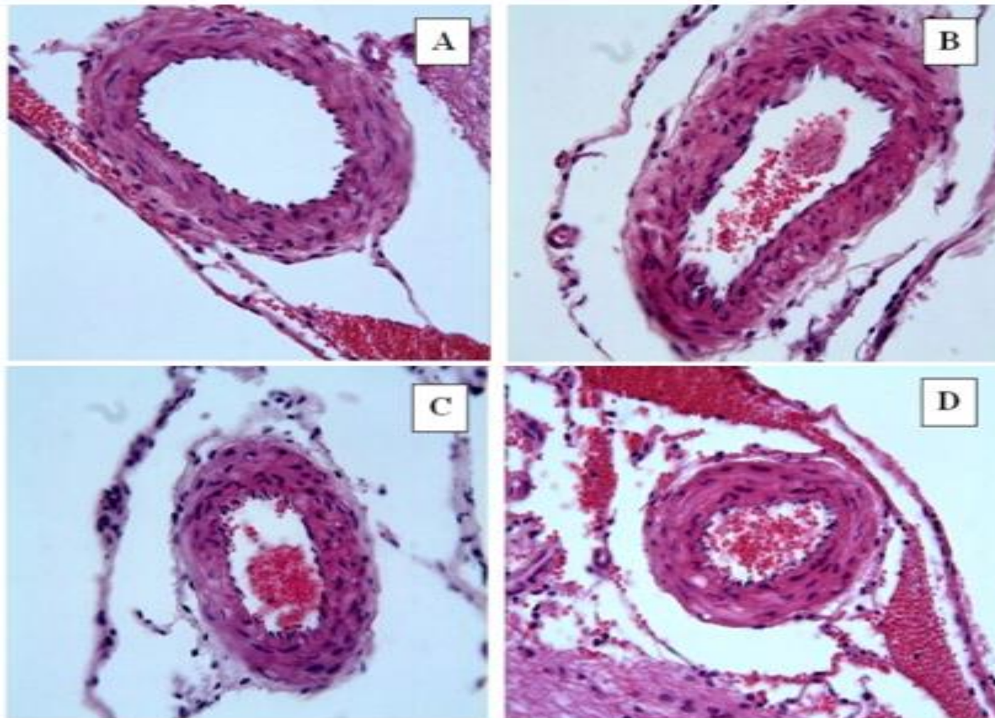

Figure 4: Basilar artery cross sections in group 1(A), group 2 (B), group 3 (C), group 4 (D) groups. Hematoxylin-Eosin; x40.

In addition, it was determined that there was endothelial structure of normal appearance and minimal vacuolization in smooth muscle cells in the group provided with 100 mg/kg GA.

4. Discussion

Cerebral vasospasm is late term recyclable pathological stenosis of large diameter arteries in brain basis. Such pathologic stenosis causes perfusion decrease in brain tissue in the distal of artery of stenosis and consequently ischemic brain damage. Cerebral vasospasm that develops following the subarachnoid hemorrhage in connection with the aneurism rupture is responsible at first degree of late ischemic neurological deficit in aneurism patients and it is the most importance cause of mortality and morbidity (17-19).

The existence of cerebral vasospasm in the first two week following SAH increases mortality from 1.5 times up to 3 times (20). Vasospasm is observed in 70% of aneurismal subarachnoid hemorrhage patients and symptomatic cerebral vasospasm and ischemia is seen in 30-40% of the same. It was notified in joint aneurism study that angiographic vasospasm incidence was in more than 50% of patients and symptomatic vasospasm was in 32% of the patients (17, 20, 21).

Since cerebral vasospasm is considered as the primary responsible of mortality and morbidity in SAH patients that develop in connection with the aneurism rupture, the pathophysiology and treatment of cerebral vasospasm has been the subject of many researches from the description of angiographic vasospasm until today.

Although many studies were performed, the physiopathology and medical treatment of cerebral vasospasm has not been revealed fully yet because of being multifactorial etiology. Clinical and experimental studies are still continued because it can not be revealed. This experimental study was planned for the purpose of developing a pharmacological treatment alternative for vasospasm.

The studies are performed on animal models because of the impossibility to make experimental study on cerebral arteries in human. Ideal subject should be resistant against the efficiency and toxicity of the treatment applied. The applied SAH model should be realized close to the subarachnoid hemorrhage that develops following the aneurism rupture in human. Rats are ideal choice for SAH models that are inexpensive and much easy to use compared to large laboratory animals.

Solomon et al. compared cerebral blood flow before and after SAH in rats and they noted that rates are a potential experimental model for SAH studies. Again according to the same study, they informed that the vasospasm in basilar artery following SAH showed same characteristics with the vasospasm in humans (22). Rats were preferred in this study because of finding them easily, because they are inexpensive, they are easy to maintain, and they are still a current model. Acute vasospasm in rats reach maximum in hour 48. Therefore, the rats were sacrificed in 48th hour in our study. It is reached in 7th day maximum in humans. The rate of neurological deficit in connection with vasospasm is low in rats compared to humans. This is because of the collateral circulation in rat brains.

There are three main methods as experimental SAH

development technique. The technique in which congestion is enabled around by perforating a basilar artery or a large artery; the technique in which the artery is dissected surgically and autologous blood taken from another artery is placed around the artery; the technique in which autologous arterial blood is injected to the subarachnoid space form the three main techniques (23). On the basis of these techniques, many different methods were developed. The SAH formation technique in which the autologous blood is injected to subarachnoid space is the most frequently used techniques in the studies. Our study also developed SAH using this technique.

Previously in our study, the method used by Yu-shu Dong et al., in which medication was performed in 30th minute, 12th hour, 24th hour and sacrification was performed on 48th hour following the development of SAH, was used (24).

It was determined in our experimental examination that GA application reduced the histopathologic damage in the group of SAH model and that remedied the negative effects meaningfully. It was determined that high dose (100 mg/kg) GA application changed the histological damage at more meaningful level and positively compared to low dose (50 mg/kg) GA application. In our study, it was determined through the light microscopic examination that GA application reversed the changes, which occurred in basilar artery and caused by SAH, meaningfully in connection with the dose.

Free oxygen radicals are developed in connection with oxidative stress in brain tissue in biochemical terms in cerebral vasospasm. It is known that, with those free oxygen radicals, lipid peroxidation developed and tissue TBARS level increased, causing decrease in antioxidant defense system elements such as SOD, CAT, GPx and GSH (25).

Based on the results obtained in our study, TBARS level, being the indication of oxidative damage in rats with SAH, was increased meaningfully in statistical terms compared to control and all other groups. Furthermore, it caused significant level of decrease statistically in levels of GSH, SOD, GPx and CAT, being antioxidant defense system elements, in connection with the development of SAH in the same test group. It was determined that GA treatment decreased meaningfully in statistical terms in the increase of TBARS caused by SAH and that such decrease was shaped in connection with the dose. The decrease in TBARS level and the reversal of effects of SAH were more apparent in the group treated with a dosage of 100 mg/kg compared to the group treated with a dosage of 50 mg/kg. It was observed that the TBARS value in the group of high GA dose approached the value of control group and that there was no more a difference between the two groups statistically. In addition, it was determined that GA treatment partially reversed the changes caused by SAH in antioxidant defense systems again in connection with the dosage. It was determined that low dose GA treatment caused meaningful levels of parameters approach normal in GSH, SOD and GPx levels compared to SAH group; however, that there was not any improvement with low dose GA at only CAT level. It was observed that in high dose GA treatment, there were meaningful statistical increases in all parameters compared to SAH group and the values approached the control group as a result of such increases.

Moreover, it was determined that the changes that occurred when high dose and low dose GA groups were compared changed in all parameters in connection with the dose; and that there were meaningful differences statistically in the groups of high treatment compared to the groups with low dose GA.

In our study, we wished to examine whether GA, being an antioxidant, had an effect to settle vasospasm and to reduce brain tissue damage in connection with vasospasm. The averages of lumen cross section in histopathologic and morphometric terms in both groups receiving GA were found different at meaningful levels compared to other groups. Likewise, the biochemical values were found different meaningfully especially in high dose GA group compared to those groups not receiving GA statistically. Based on our results, GA was found beneficial in the experimental medical treatment of cerebral vasospasm.

5. Conclusions

Although there have been many studies performed on this issue, medical treatment of cerebral vasospasm has not been revealed fully yet because of having multifactorial etiology. GA has an effect to reduce brain tissue damage in connection with the ischemia/reperfusion that develops in cerebral vasospasm through the induction of antioxidant defense system and to prevent the development of vasoconstriction. Therefore, we consider that GA may be tried as an alternative agent against a situation of high mortality and morbidity such as vasospasm.

Conflict of interest statement

All authors declared that there is no conflict of interest.

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None

Compliance with ethical standards

The study was carried out in accordance with ethical standards in all aspects.

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