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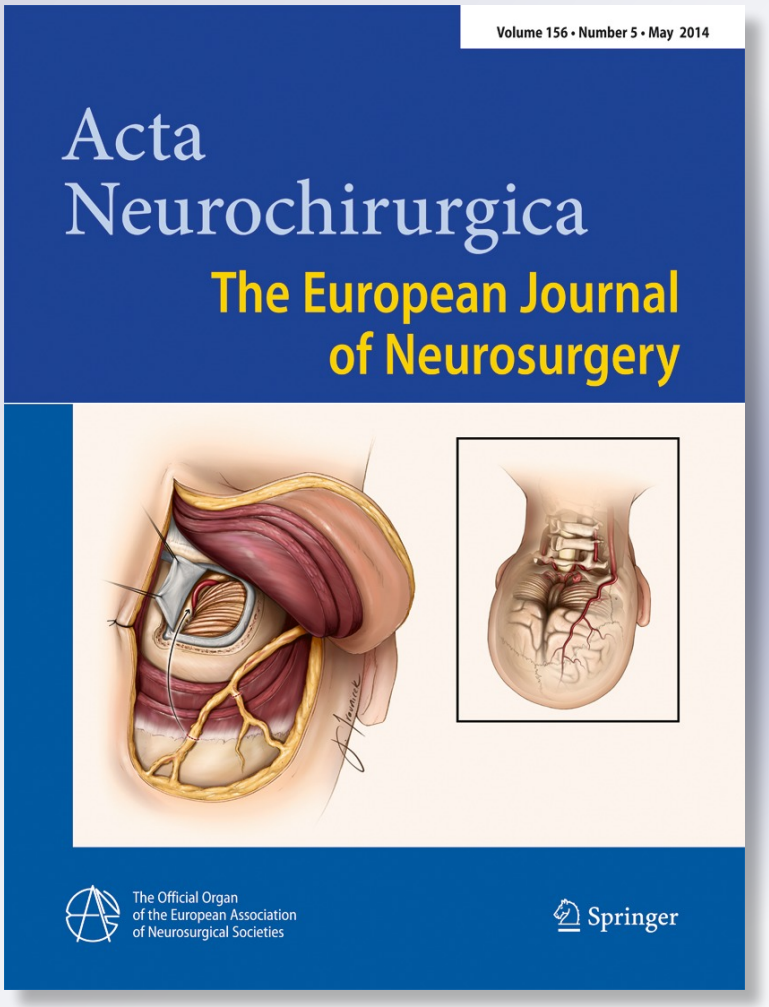

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EXPERIMENTAL RESEARCH - VASCULAR

The comparative effects of recombinant human erythropoietin and darbepoetin-alpha on cerebral vasospasm following experimental subarachnoid hemorrhage in the rabbit

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Abstract
Background Darbepoetin alpha is a hypersialylated analogue of erythropoietin effective for activating erythropoietin-receptors. This study investigated the vasodilator and neuroprotective effects of darbepoetin alpha on an experimental subarachnoid hemorrhage model and compared it with erythropoietin.
Methods Forty adult male New Zealand white rabbits were randomly divided into four groups of ten rabbits each: group 1 (control), group 2 (subarachnoid hemorrhage), group 3 (erythropoietin), and group 4 (darbepoetin alpha). Recombinant human erythropoietin was administered at a dose of 1,000 U/kg intraperitoneally after the induction of subarachnoid hemorrhage and continued every 8 h up to 72 h. Darbepoetin alpha was administered at a single intraperitoneal dose of 30 μg/kg. Animals were killed 72 h after subarachnoid hemorrhage. Basilar artery cross-sectional areas, arterial wall thicknesses, hippocampal degeneration scores and biochemical analyses were measured in all groups.
Results Both erythropoietin and darbepoetin alpha treatments were found to attenuate cerebral vasospasm and provide neuroprotection after subarachnoid hemorrhage in rabbits. Darbepoetin alpha revealed better morphometric and histopathological results than erythropoietin among experimental subarachnoid hemorrhage-induced vasospasm.
Conclusions Our findings, for the first time, showed that darbepoetin alpha can prevent vasospasm and provides neuroprotection following experimental subarachnoid hemorrhage. Moreover, darbepoetin alpha showed better results when compared with erythropoietin.

Keywords Darbepoetin alpha · Erythropoietin · Rabbit · Subarachnoid hemorrhage · Vasospasm

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Introduction

Cerebral vasospasm following subarachnoid hemorrhage (SAH) is characterized by prolonged and reversible contraction of major cerebral arteries, which further leads morbidity and mortality. The exact pathogenesis of this unique entity is still unclear; unless it is that the breakdown products of blood are considered to play an important role in the pathogenesis of post-SAH vasospasm. Other factors in vasospasm: inflammatory processes, free radical formation, an imbalance between vasoconstrictive and vasodilator mediators, neuronal mechanisms that regulate vascular tone, endothelial proliferation and apoptosis have all been accused as causative and pathogenic factors [31, 33].

Erythropoietin (EPO), which is a glycoprotein hormone, is the primary regulator of erythropoiesis [34]. Recombinant human erythropoietin (rhEPO) has been developed to treat anemia associated with chronic renal failure, chemotherapy for cancer patients, and HIV infections [37]. Erythropoietin has been shown to be neuroprotective in a variety of hypoxic/ischemic central nervous system disorders [10, 38, 44, 45, 48]. Furthermore, numerous studies have shown that EPO has neuroprotective and vasodilator effects in experimental SAH models [3, 11, 13, 22–24, 39, 46, 49, 58]. Moreover, some preliminary randomized clinical trials revealed mild to moderate protective effects of rhEPO therapy for aneurysmal SAH [27, 50–52].

Darbepoetin-alpha (DA) is a novel erythropoiesis-stimulating agent with additional sialic acid-containing oligosaccharide when compared with EPO. Darbepoetin-alpha has an extended circulatory half-life and an increased in vivo biological activity greater than EPO [21]. Since DA is a hypersialylated analogue of EPO and activates EPO-receptors, here we hypothesized that DA may have potential vasodilator and neuroprotective effects on SAH-induced cerebral vasospasm when compared with EPO.

Materials and methods

Experimental groups

Animal care and all experiments were conducted following the European Communities Council Directive of November 24, 1986 (86/609/EEC) concerning the protection of animals for experimental use. All experimental procedures used in this investigation were reviewed and approved by the ethical committee of the Ministry of Health Ankara Education and Research Hospital Committee of Animal Ethics. Forty adult male New Zealand white rabbits, weighing 2.750–3.900 g were randomly divided into following four groups of ten rabbits in each group:

Group 1: Control group (n=10); was a sham surgery group, in which SAH was not induced. In this group, after induction of anesthesia, the cisterna magna was punctured as described below and 1 ml/kg of physiological saline (0.9 % NaCl) was slowly injected into the cisterna magna after removal of the same amount of cerebrospinal fluid (CSF).

Group 2: SAH group (n=10); the SAH protocol was used to induce vasospasm as described below.

Group 3: EPO group (n=10); cerebral vasospasm was induced by the SAH protocol described below, and the animals received rhEPO (Eporon, Dem Ilac, Turkey) at a dose of 1,000 U/kg, which was administered intraperitoneally 5 min after induction of SAH and continued every 8 h up to 72 h. This dose was chosen according to the previous studies [3, 11, 23, 26].

Group 4: DA group (n=10); as for group 3, but rabbits received a single intraperitoneal dose of 30 μg/kg DA (Aranesp, Amgen Europe, Netherlands) 5 min after the induction of SAH. This dose was chosen according to a previous study [56].

Anesthesia and surgical procedure

The animals were kept at optimal (18–21 °C) room temperature and fed with standard diet where a 12-h light–dark cycle was implemented. Free access to food and water was allowed. The animals were anesthetized bya combination of intramuscular administration of 70 mg/kg ketamine (Ketalar, Parke Davis Eczacıbaşı, Turkey) and 5 mg/kg xylazine (Rompun, Bayer, Turkey) . All animals breathed spontaneously throughout the procedures. Arterial blood samples for PO_2 and PCO_2 were taken from each animal from the catheterized ear arteries for blood gas analysis during the procedures, and only those animals with PO_2 greater than 70 mmHg and PCO_2 lesser than 40 mmHg were included in the study. Heart rate and arterial blood pressure were measured with the use of an ear arterial catheter. Physiological parameters of the experimental groups are summarized in Table 1. Core body temperature was monitored rectally and maintained at 37±0.5 °C with a heater.

Cerebral vasospasm model

The head of the rabbit was extended in the prone position. A midline nuchal incision was made, and dermal and subdermal tissues; fascia and paravertebral muscles were dissected to expose the atlanto-occipital membrane. A 25-gauge needle was inserted through the dura mater and the arachnoid membrane into the cisterna magna; 1 mL/kg of CSF was withdrawn and an equal volume of fresh, non-heparinized autologous arterial blood, which was obtained from the ear artery was injected into the cisterna magna within 2 min. The animals were then placed in a head-down position at 30° for 30 min to hold the blood in the basal cisterns. After the recovery from anesthesia and confirmation of vital signs, rabbits were left to their cages for the establishment of cerebral vasospasm.

Perfusion-fixation

All animals were euthanized by perfusion-fixation 72 h after procedures. The animals were anesthetized as described above. The ear artery was catheterized for monitoring blood pressure and for blood gas analysis. When satisfactory respiratory parameters were obtained, a thoracotomy was

Table 1 Physiological parameters of the experimental groups

Variables	Control (n=10)	SAH (n=10)	EPO (n=10)	DA (n=10)	p-value
pH	7.46 (0.022)	7.45 (0.022)	7.45 (0.022)	7.46 (0.012)	0.368
$PCO_{2\ (mmHg)}$	35.5±0.7	35.7±0.9	36.0±0.9	36.3±0.9	0.209
$PO_{2\ (mmHg)}$	95.7±1.2	94.9±1.1	95.1±1.5	95.4±1.2	0.600
$MABP_{\ (mmHg)}$	104.5±3.9	103.2±2.9	105.6±2.7	103.9±4.0	0.461
$HR_{\ (bpm)}$	166.9±4.9	164.2±3.9	164.3±4.1	164.3±4.5	0.442

Data are shown as the medians (IQR) or means ± standard deviation

SAH subarachnoid hemorrhage, *EPO* erythropoietin, *DA* darbepoetin-alpha, *MABP* mean arterial blood pressure, *HR* heart rate, *bpm* beats per minute

performed, the left ventricle cannulated, the right atrium opened widely and the descending thoracic aorta clamped. After perfusion with 300 mL of physiological saline, a fixative was perfused (10 % formaldehyde, 200 mL). Perfusion was performed at a standard height of 100 cm from the chest. Then the brains were removed and stored in formaldehyde solution at 4 °C overnight. Before the perfusion-fixation procedure, blood samples (10 cm^3) were taken from the left ventricles for biochemical analysis. The blood samples were centrifuged at 1,000 g for 5 min, and the upper clear supernatants were removed for analysis. All serum samples were stored at −80 °C until analyzed.

Histological morphometric analysis of the basilar artery

Each brainstem specimen was embedded in paraffin. The entire basilar artery was sectioned into three segments at 2 mm in length (Fig. 1), and stained with hematoxylin-eosin (H&E). The morphometric measurements on all three segments of the basilar were performed using the BAB-Bs200ProP Image Processing and Analysis System (Ankara, Turkey). The luminal area was calculated as the area contained within the boundaries of the internal elastic lamina. The size of cross-sectional area for each basilar artery was obtained by averaging these measurements. The mean ± SD value obtained from each artery was used as the final value for a particular vessel.

The wall thickness between the lumen and external border of the muscle layer was measured at four quadrants of each segment of basilar artery. If an undulating luminal border was encountered, an extra measurement was done from the internal elastic membrane to the external border of the muscle layer. The vessel wall thickness for each basilar artery segment was obtained by averaging these measurements. The mean ± SD value obtained from each artery was used as the final value for a particular vessel. All measurements were repeated three times for each artery in a blind fashion by two pathologists and the conclusive values were obtained by averaging these measurements. Inter-observer and intra-observer reliability levels are provided in Table 2.

Hippocampal degeneration

Paraffin-embedded hippocampus slices were sectioned to 4 to 6 μm thicknesses and stained by H&E. Under a light microscope, morphological signs of neuronal degeneration such as neuronal shrinkage, hyperchromasia, and nuclear picnosis were evaluated. The presence and extent of neuronal degeneration were scored semiquantatively in the dentate gyrus, CA1 and CA3 regions as follows: 1 = normal appearance, 2 = few degenerated neurons among normal neurons, 3 = large number of degenerated neurons with scattered normal neurons, 4 = complete degeneration with no residual normal neuron [47]. Scoring was done for each of the three regions of the hippocampus. The sum of these three scores was named as the "degeneration score", and the means were used in the statistical analysis.

Biochemical analysis

Serum nitric oxide (NO) and nitric oxide synthetase (NOS) analysis

The level of NO was estimated by the method based on the diazotization of sulfanilic acid by NO at acid pH and subsequent coupling to N-(1-napthyl-ethylene diamine) (Griess reaction) as described before [17]. Since nitrate anion does not give a diazotization reaction with sulfanilic acid, the samples were treated by cadmium (a reducing agent) to reduce nitrate anions into nitrite anions before the NO estimation [43]. The total NOS activity (IU/ml) method is based on the Griess reaction [17].

Serum arginase analyses

Arginase activity (mIU/ml) was measured by using the spectrophotometric method as described previously in the literature [14]. The enzyme activity was determined by measuring the amount of ornithine produced from the hydrolysis of arginine by arginase. One international unit (IU) of arginase activity was defined as one micromole of produced ornithine per minute at 37 °C.

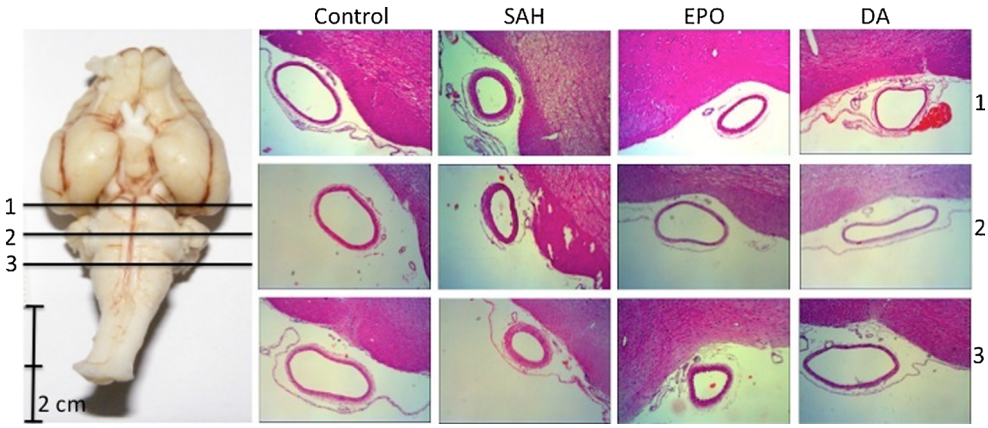

Fig. 1 Macroscopic view of a basis of the rabbit brain from the control group (*left*) and representative histological cross-sections of the basilar artery corresponding to all study groups (*right*, H&E, 40X obj.). *SAH* subarachnoid hemorrhage, *EPO* erythropoietin, *DA* darbepoetin-alpha

Serum glutathione peroxidase (GPx) analyses

GPx activity was measured by following changes in NADPH absorbance at 340 nm as described [41]. In the activity calculations, extinction coefficients of NADPH were used for GPx. The results were expressed as IU/ml.

Serum catalase (CAT) analyses

Catalase activity was determined by measuring the absorbance decrease of hydrogen peroxide (H_2O_2) at 240 nm [1]. In the activity calculations, an extinction coefficient of H_2O_2 was used for CAT. The results were expressed as IU/ml.

Serum malondialdehyde (MDA) analyses

The MDA concentration was determined by using the thiobarbituric acid reaction [16]. MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid to form a colored complex that has maximum absorbance at 532 nm. For this purpose, 0.1 ml of homogenate was suspended in 1 ml of phosphate-buffered saline (pH 6, 100 mmol/l) and then 1 ml 20 % trichloroacetic acid, 1 ml ethyl alcohol (95 %) and 1 ml thiobarbituric acid solution (2 %) were added. After keeping it in boiling water for 30 min, the tube's content was removed and absorbance was read at 532 nm. MDA concentrations were calculated by comparing the absorbance values of the samples with those of standard MDA solutions. Results were expressed as nmol/ml.

Table 2 Intra-class and inter-class correlation coefficients regarding the cross-sectional area and wall thickness measurement

	CSA		AWT	
	ICC	95%CI	ICC	95%CI
Intra-observer				
1st observer	0.997	0.994–0.998	0.996	0.992–0.998
2nd observer	0.998	0.996–0.999	0.998	0.996–0.999
Inter-observer				
1st measurement	0.940	0.889–0.968	0.989	0.979–0.994
2nd measurement	0.931	0.874–0.963	0.991	0.982–0.995

CSA cross-sectional area, *AWT* arterial wall thickness, *ICC* intraclass correlation coefficient, *CI* confidence interval

Statistical analysis

Data analysis was performed by using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, United States). Whether the distributions of continuous variables were normal or not were determined by the Shapiro Wilk test. The Levene test was used for the evaluation of homogeneity of variances. Data were shown as mean ± standard deviation or, where applicable. the median (IQR). While the mean differences among groups were analyzed by using One-Way ANOVA; otherwise, the Kruskal Wallis test was applied for comparisons of the median values. When the p value from One-Way ANOVA or the Kruskal Wallis test statistics was statistically significant then the post hoc Tukey HSD or Conover's non-parametric multiple comparison test was used to know which group differed from which others. A p value less than 0.05 was considered statistically significant.

Results

Morphometric analysis of the basilar artery

The mean basilar artery cross-sectional area in the control group was 314,974.3±45,237.8 μm^2. In the SAH group, the mean basilar artery cross-sectional area decreased to 159,640.5±15,888.7 μm^2. This decrease was statistically significant ($p<0.001$). In the EPO and the DA groups, the basilar artery cross-sectional areas were 234,729.2±15,156.5 and 279,702.2±36,382 μm^2, respectively. Both the EPO and the DA treatments were statistically significantly and increased

the cross-sectional area of the basilar artery when compared to the SAH group (p=0.003 and p<0.001, respectively). Also, when the statistical results of DA and EPO treatments were compared, the cross-sectional area of the basilar artery was found to be significantly increased with the DA treatment (p=0.003) (Fig. 2). The mean value of the basilar artery wall thickness was 25.3±2.8 μm in the control group, and 46.1±1.1 μm in the SAH group. The EPO and the DA groups had a mean value of 32.5±1.2 and 27.8±1.3 μm of the basilar artery wall thicknesses, respectively. When compared to the control group, after SAH, basilar artery wall thickness was increased significantly statistically (p<0.001). Basilar artery wall thicknesses in the EPO and the DA groups were both smaller than the SAH group and these comparisons were both statistically significant (p=0.008 and p<0.001, respectively). When the EPO group was compared with the DA group, the DA group had a statistically significant smaller basilar artery wall thickness (p=0.003) (Fig. 3).

Mean basilar artery cross-sectional areas and arterial wall thicknesses values are provided in Table 3.

Pathological examination of the hippocampus

Light microscopic examination of the CA1, CA3 and the dentate gyrus samples of the hippocampus were normal (Fig. 4). In the SAH group, almost complete degeneration of the neurons was observed (Fig. 5). In the EPO group, mild to moderate degeneration was observed in the CA1, CA3 and the dentate gyrus samples of the hippocampus (Fig. 6). The DA group revealed a better pathological appearance of the CA1, CA3 and the dentate gyrus, where minimal degenerated neurons with hyperchromasia and nuclear picnosis were noticed (Fig. 7).

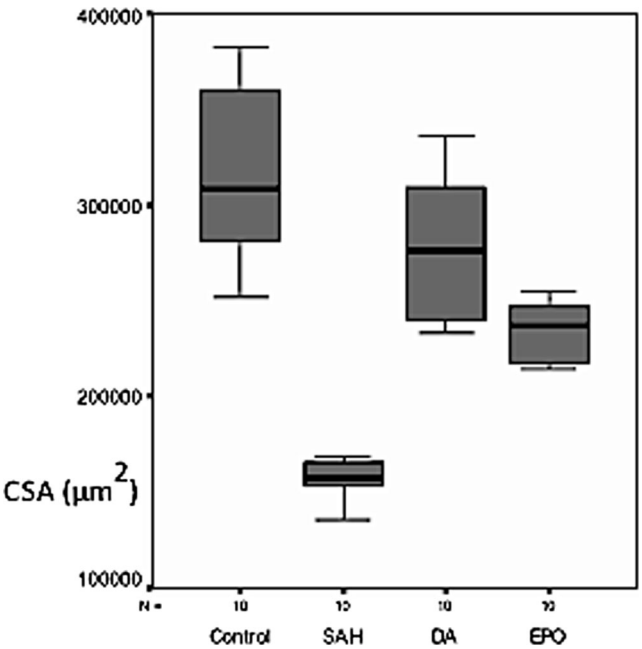

Fig. 2 Mean basilar artery cross-sectional areas of the study groups. The *horizontal lines* in the middle of each *box* indicate the median, while the *top* and *bottom* borders of the *box* mark the 25th and 75th percentiles, respectively. The whiskers above and below the *box mark* indicate the maximum and minimum levels. *CSA* cross-sectional area, *SAH* subarachnoid hemorrhage, *EPO* erythropoietin, *DA* darbepoetin-alpha

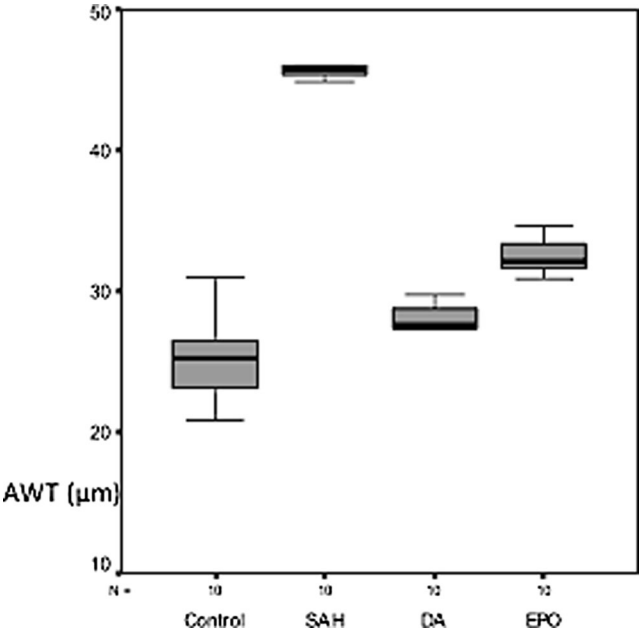

Fig. 3 Mean basilar artery wall thicknesses of the study groups. The *horizontal lines* in the middle of each *box* indicates the median, while the *top* and *bottom* borders of the *box* mark the 25th and 75th percentiles, respectively. The whiskers above and below the box mark indicate the maximum and minimum levels. *AWT* arterial wall thickness, *SAH* subarachnoid hemorrhage, *EPO* erythropoietin, *DA* darbepoetin-alpha

The mean degeneration score for the control group was 3.8±0.7; the mean degeneration score for the SAH group was 11.2±1; and the mean degeneration scores for the EPO and the DA groups were 9.1±1.4 and 6.9±0.8, respectively. The difference between the control and the SAH group was statistically significant (p<0.001). The mean degeneration scores were statistically significantly lower in both the EPO and the DA groups when compared with the SAH group (p=0.023 and p<0.001, respectively). Also, the DA group showed better mean degeneration scores when compared to the EPO group (p=0.019). Pathological examinations of the hippocampus are summarized in Table 4.

Biochemical results

Serum nitric oxide (NO) levels

Serum NO levels were linked to a statistically significant decrease in the SAH group compared with the control group (p<0.001). In both the EPO and the DA groups, serum NO levels were significantly increased when compared to the SAH group (p=0.028 and p=0.001, respectively). There

Table 3 Mean basilar artery cross-sectional areas and wall thicknesses values

Groups	CSA (μm^2)	AWT (μm)
Control (n=10)	314,974.3±45,237.8[a,b]	25.3±2.8[a,b]
SAH (n=10)	159,640.5±15,888.7[a,c,d]	46.1±1.1[a,c,d]
EPO (n=10)	234,729.2±15,156.5[b,d,e]	32.5±1.2[b,d,e]
DA (n=10)	279,702.3±36,382[c,e]	27.8±1.3[c,e]

CSA cross-sectional area, *AWT* arterial wall thickness, *SAH* subarachnoid hemorrhage, *EPO* erythropoietin, *DA* darbepoetin-alpha

[a] Control vs SAH (p<0.001)

[b] Control vs EPO (p<0.001)

[c] SAH vs DA (p<0.001)

[d] SAH vs EPO (p<0.01)

[e] DA vs EPO (p<0.05)

was no statistically significant difference between the EPO and the DA groups (p=0.691).

Serum nitric oxide synthetase (NOS) activity

SAH serum NOS activity decreased significantly statistically when the control group was compared with the SAH group (p<0.001). Both treatment with the EPO and the DA caused an increase in the serum NOS activity significantly when compared to the SAH group (p=0.031 and p=0.003, respectively). When the EPO group was compared with the DA group, there was no statistically significant difference observed (p=0.319).

Serum arginase activity

Statistically significant differences were observed between the control and the SAH groups with regard to mean serum arginase activity (p<0.001). However, this data showed that, after SAH, serum arginase activity is elevated. Treatment, both with the EPO and the DA led to a statistically significant decrease in the arginase activity when compared to the SAH group (p<0.001 for both). There were no statistically significant differences between the EPO and the DA groups (p=0.306).

Serum glutathione peroxidase (GPx) activity

When mean GPx activity was compared between the control and the SAH groups, there was a statistically significant difference observed (p<0.001); therefore, we concluded that after SAH, due to highly elevated oxidative stress, serum GPx

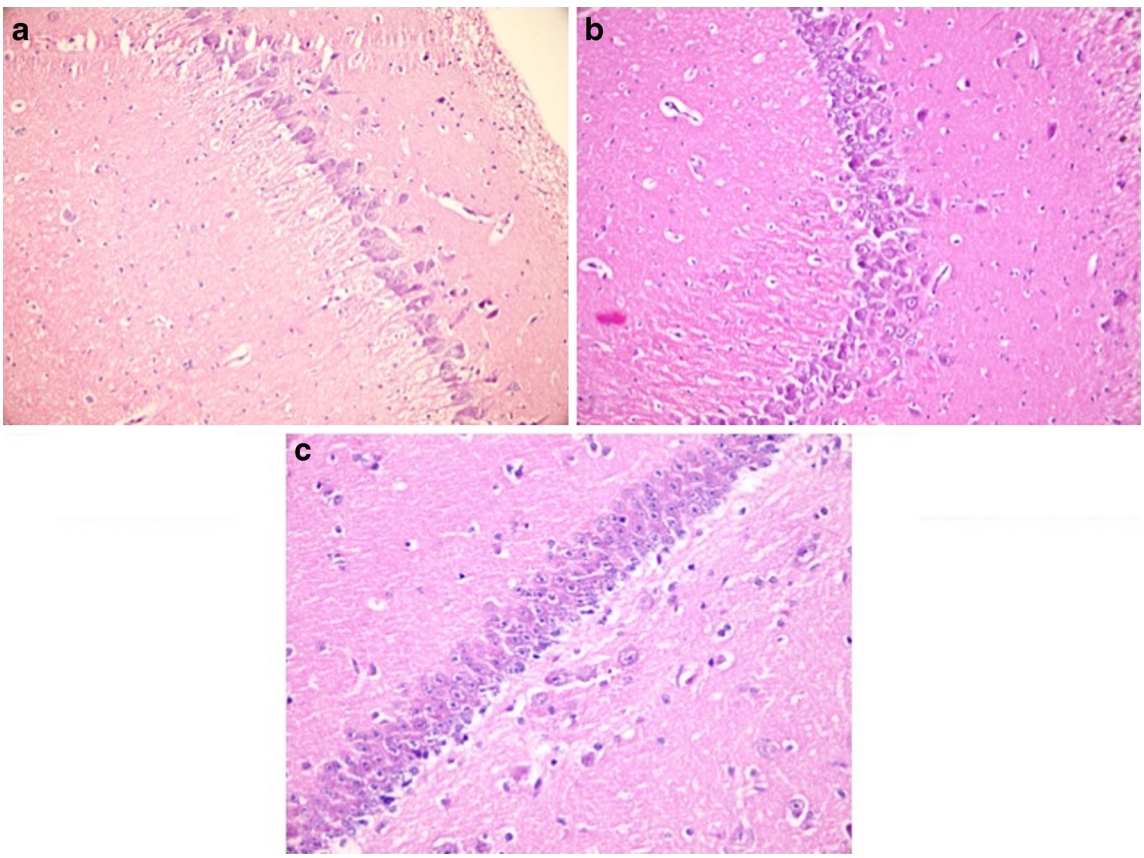

Fig. 4 Photomicrographs showing the normal appearing slices from the CA1 (**a**), CA3 (**b**) and dentate gyrus (**c**) of the hippocampus of the control group (H&E, 40X obj.)

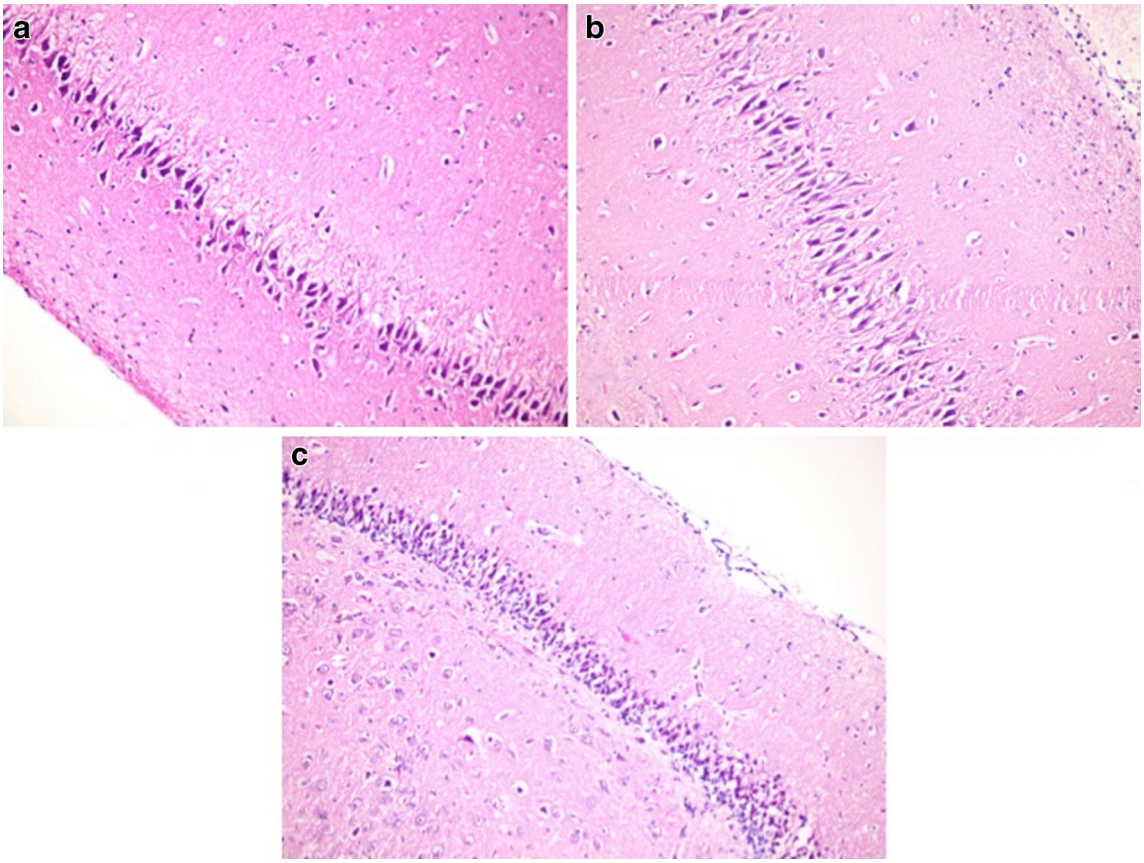

Fig. 5 Photomicrographs of the slides from the SAH group showing the completely degenerated neurons in the CA1 (**a**), CA3 (**b**), and dentate gyrus (**c**) of the hippocampus (H&E, 20X obj.)

activity was decreasing. When we compared the EPO group with the SAH group, there was no statistically significant difference observed ($p=0.08$). On the other hand, in the DA group, serum GPx activity was increased significantly when compared with the SAH group ($p=0.007$). Furthermore, there was no statistically significant difference observed between the EPO and the DA groups ($p=0.294$).

Serum catalase (CAT) activity

When the mean CAT activity of the control group was compared with the SAH group, there was a statistically significant difference observed ($p<0.001$); this data showed after SAH, serum CAT activity was decreased. Treatment, both with the EPO and the DA, was associated with a statistically significant increase in the CAT activity ($p=0.023$ and $p<0.001$, respectively). Also, in the DA group, the increase of the serum CAT activity was statistically significantly higher when compared with the EPO group ($p=0.004$).

Serum malondialdehyde (MDA) levels

When the mean serum MDA levels were compared between the control and the SAH groups, a statistically significant difference was observed ($p<0.001$). These data showed that following SAH, serum MDA levels were increased. When we compared both the EPO and the DA groups with the SAH group, there were statistically significant differences observed for both comparisons ($p<0.001$ for both). Both treatment with the EPO and the DA decreased the serum MDA levels. When the EPO group was compared with the DA group, no statistically significant difference was found ($p=0.713$).

All biochemical results are summarized in Table 5.

Discussion

Cerebral vasospasm is one of the most important clinical problems of the SAH and may cause significant morbidity and mortality. Vasospasm is reported to cause symptoms only in 20–30 % of the patients suffering from SAH; on the other hand, up to 70 % of the patients were reported to have radiological vasospasm [30]. The most catastrophic consequence due to cerebral vasospasm following SAH is ischemic neurological deficit. Current treatment strategies and research are both focused on the question of how to prevent and treat SAH-induced vasospasm. However, the pathogenesis of

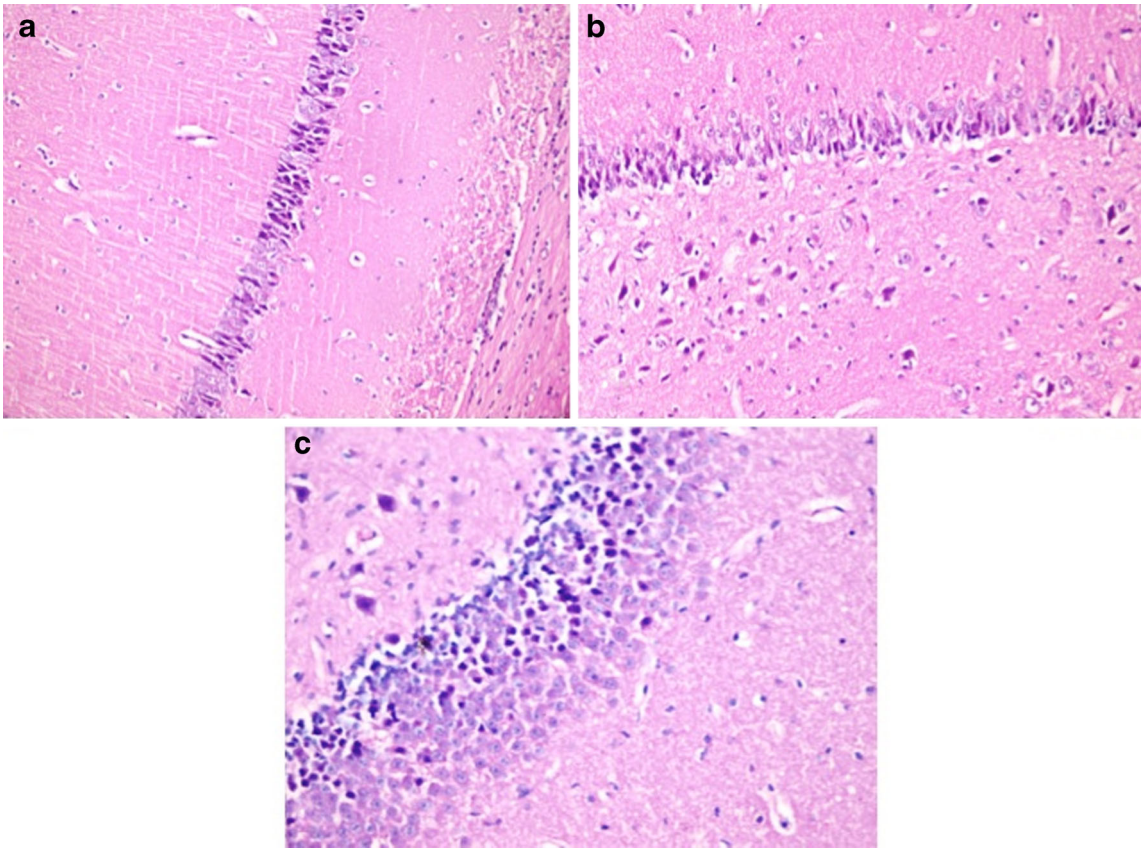

Fig. 6 Photomicrographs of the slides from the EPO group showing a mild to moderate degeneration of the neurons in the CA1 (**a**), CA3 (**b**), and dentate gyrus (**c**) of the hippocampus (H&E, 20X obj.)

cerebral vasospasm is complex, multifactorial and still is not fully understood.

Human EPO is a 30-kD glycoprotein growth factor, which is composed of 165 amino acids with four carbohydrate side chains; and known as the main regulator of erythropoiesis [29]. Despite the neuroprotective effects of EPO that had been widely studied [10, 38, 44, 45, 48]; it has been shown to have vasodilator and neuroprotective effects following SAH [3, 11, 13, 22–24, 26, 39, 46, 49, 58]. In particular, it has been reported that systemic administration of rhEPO significantly attenuates acute cerebral vasoconstriction of the basilar artery following experimental SAH [26, 58]. Furthermore, it has been shown that rhEPO also prevented delayed vasospasm following SAH [13, 22]. However, the exact mechanisms of the vasodilator and neuroprotective effects of the EPO are not known.

After the vasodilator and neuroprotective effects of the EPO had been widely studied in experimental models of SAH, some clinical trials were also conducted [27, 50–52]. In a preliminary phase II randomized, double-blind, placebo-controlled trial, Tseng et al., reported that systemic administration of the rhEPO after SAH had not shown significant effects on overall incidence of cerebral vasospasm, but they concluded that rhEPO therapy following acute SAH seemed to accelerate early recovery by reducing adverse physiological and clinical events associated with delayed ischemic brain injury [51]. Recently, Helbok et al., reported that EPO increases cerebral metabolism and brain tissue oxygen tension in poor grade SAH patients with severe cerebral vasospasm [27].

The EPO analogue, DA, is an erythropoiesis-stimulating agent that exerts similar physiological responses by effecting EPO-receptors [20]. Convincing evidence is available that DA, as well as EPO, acts as a neurothrophic and neuroprotective agent [56]. Banks et al., reported that DA crosses the blood–brain-barrier by way of extracellular pathways in amounts that could account for the neuroprotective effect [6]. As an EPO-derivated agent, we hypothesized that DA may have vasodilator and neuroprotective effects on SAH-induced vasospasm.

In the present study, for the first time, we investigated the effects of DA on SAH-induced cerebral vasospasm, and compared the results with EPO. We used the single-SAH model in the rabbit. Due to past literature, injection of fresh blood into the cisterna magna is one of the most commonly used protocols to establish SAH in rabbits [7, 31]. Also, it has been shown that cerebral vasospasm reaches its maximum level during the third day of SAH in rabbits [55]. Thus, in the

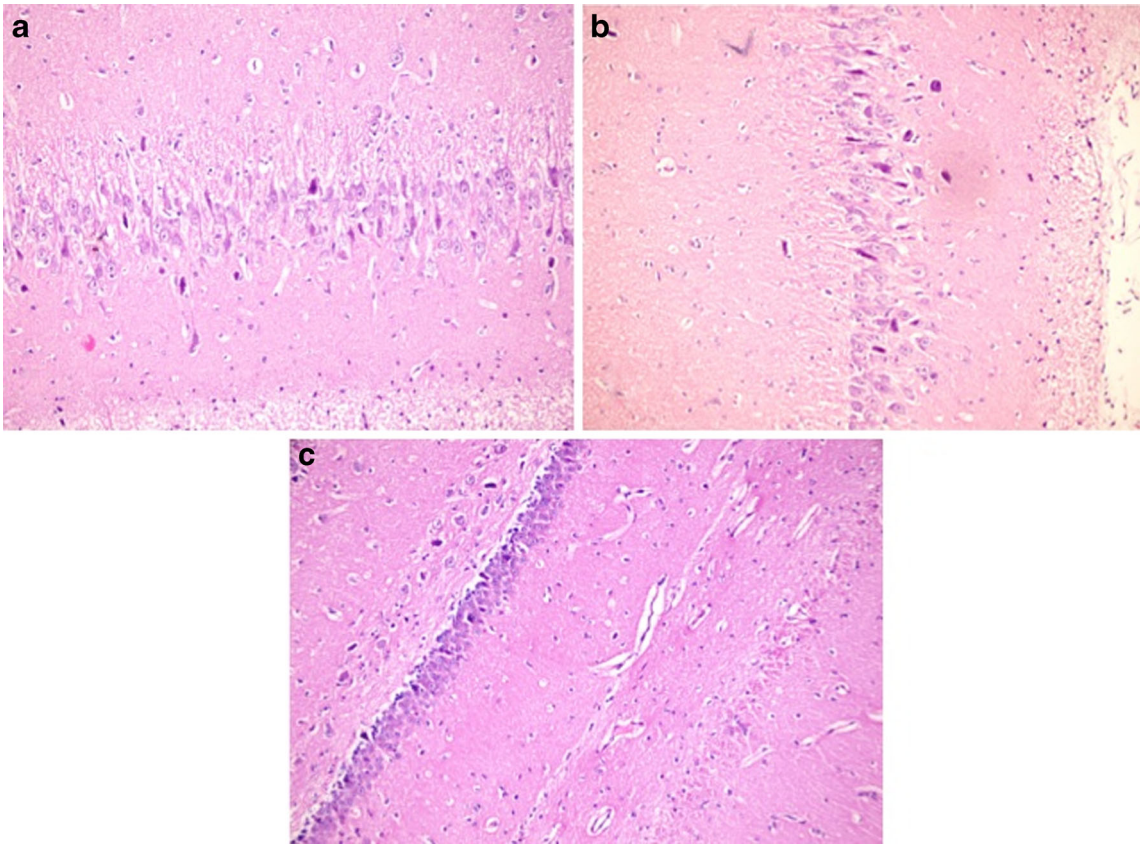

Fig. 7 Photomicrographs of the slides from the DA group showing only mild degeneration of the neurons in the CA1 (**a**), CA3 (**b**) and dentate gyrus (**c**) of the hippocampus (H&E, 20X obj.)

present study, the treatment was stopped and the animals were sacrificed 72 h after the induction of SAH. Due to past studies, the measurements were done in the basilar artery [31, 36].

The results of our study showed that both EPO and DA had vasodilator and neuroprotective effects following experimental SAH. Furthermore, morphometric measurements of the basilar artery and the pathological examinations of the hippocampus revealed that DA had better results in attenuating vasospasm and protecting the brain from ischemic damage than EPO.

In the pathogenesis of SAH-induced vasospasm, the balance between endogenous vasoconstrictors and the primary endogenous vasodilator NO is important [15]. Previous experimental data indicate that potent vasoconstrictors such as endothelin, are increased following SAH [32], and NO is depleted at the very same time [28, 42]. According to several

Table 4 Pathological examinations of the hippocampus relevant to the study groups

Variables	Control (n=10)	SAH (n=10)	EPO (n=10)	DA (n=10)	p-value
CA 1	1.0 (0.25)[a,b,c]	4.0 (1.0)[a,d,e]	3.0 (1.25)[c,e]	2.0 (1.0)[b,d]	<0.001
CA 3	1.0 (1.0)[a,b,c]	4.0 (1.0)[a,d]	3.0 (1.0)[c,f]	2.0 (1.0)[b,d,f]	<0.001
Dentate gyrus	1.0 (0.25)[a,b,c]	4.0 (0.25)[a,d,e]	3.0 (1.25)[c,e]	2.0 (1.0)[b,d]	<0.001
Mean deg. score	4.0 (1.25)[a,b,c]	11.5 (1.25)[a,d,e]	9.0 (2.5)[c,e,f]	7.0 (1.0)[b,d,f]	<0.001

Data are shown as the medians (IQR)

SAH subarachnoid hemorrhage, *EPO* erythropoietin, *DA* darbepoetin-alpha, *Deg.* degeneration

[a] Control vs SAH (p<0.001)

[b] Control vs DA (p<0.05)

[c] Control vs EPO (p<0.001)

[d] SAH vs DA (p<0.001)

[e] SAH vs EPO (p<0.05)

[f] DA vs EPO (p<0.05)

Table 5 Biochemical alterations among the study groups

Variables	Control (n=10)	SAH (n=10)	EPO (n=10)	DA (n=10)	p-value
NO (μmol/ml)	36.9±7.1[a]	18.4±3.9[a,b,c]	28.4±10.4[c]	32.1±7.6[b]	<0.001
NOS (IU/ml)	18.6 (2.1)[a,d,e]	9.6 (3.0)[a,b,c]	12.5 (2.4)[c,d]	13.0 (2.7)[b,e]	<0.001
Arginase (mIU/ml)	51.3 (13.8)[a,d]	186.0 (73.5)[a,b,c]	65.2 (31.4)[c,d]	56.5 (18.4)[b]	<0.001
GPx (IU/ml)	0.16 (0.04)[a,d,e]	0.05 (0.03)[a,b]	0.06 (0.04)[d]	0.09 (0.07)[b,e]	<0.001
CAT (IU/ml)	94.4 (8.3)[a,d,e]	21.6 (5.2)[a,b,c]	35.1 (16.5)[c,d,f]	75.4 (12.4)[b,e,f]	<0.001
MDA (nmol/ml)	2.0±0.7[a,d,e]	4.7±0.7[a,b,c]	3.1±0.4[c,d]	2.8±0.7[b,e]	<0.001

Data are shown as the medians (IQR) or means ± standard deviation

SAH subarachnoid hemorrhage, *EPO* erythropoietin, *DA* darbepoetin-alpha, *NO* nitric oxide, *NOS* nitric oxide synthetase, *GPx* glutathione peroxidase, *CAT* catalase, *MDA* malondialdehyde

[a] Control vs SAH (p<0.001)

[b] SAH vs DA (p<0.01)

[c] SAH vs EPO (p<0.05)

[d] Control vs EPO (p<0.01)

[e] Control vs DA (p<0.05)

[f] DA vs EPO (p=0.004)

studies, depletion of NO is one of the most important mechanisms underlying the SAH-induced cerebral vasospasm [19, 25, 40]. Also, neuroprotective effects of EPO obtained in animal models of ischemic brain injury and SAH had been explained by the augmented basal production of NO in the endothelium [46, 53, 56]. Also, EPO had been shown to have ability to increase NOS activity in endothelial cells [5, 8]. Our results revealed that following SAH both NO and NOS levels were decreased significantly. Administration of the EPO and the DA clearly exhibited antioxidant activity and elevated both the NO and NOS levels significantly.

Arginase is a key enzyme in nitrogen metabolism, which is activated by oxidative stress and regulates NO biosynthesis via competition for the NOS-substrate, L-arginine [9, 35]. Previously, it has been shown that arginase counteracts with NO-mediated vasodilation [12, 57]. Furthermore, it has been reported that arginase activity was increased in the brain after SAH contributes the reduction of serum levels of NO [2]. In this study, we also showed that after SAH arginase activity is increased, both EPO and DA decreased the arginase activity, which further indicates the antioxidant activities of both drugs.

Inhibition of intrinsic antioxidant systems such as GPx and CAT occur after SAH and leads to brain oxidative damage [4]. Antioxidant enzyme activities are shown to be diminished under highly elevated oxidative stress conditions because of molecular damage [54]. The present study showed that, due to oxidative stress, both GPx and CAT levels were decreased following SAH. The DA treatment significantly increased the GPx levels, but no such increase was observed in the EPO treated group. Furthermore, both EPO and DA administration increased the CAT activity following experimental SAH. Moreover, DA elevated CAT levels were statistically significantly more than EPO.

Due to excessive free radicals generated by oxyhemoglobin and enzymatic reactions, lipid peroxidation can occur after SAH [4, 18]. Malondialdehyde is formed from the breakdown of polyunsaturated fatty acids, and serves as an important and reliable index for determining the extent of peroxidation reactions [56]. Our study showed that after SAH, levels of MDA had dramatically increased. When compared with the SAH group, statistically significant effects of both EPO and DA on lowering elevated MDA levels after SAH have been shown.

The results of this study suggest that both EPO and DA have beneficial effects on preserving neuronal function and protecting vessels from SAH-induced vasospasm. Moreover, as DA has an extended circulatory half-life and increased in vivo biological activity more than EPO, DA revealed better morphometric and histopathological results than EPO among experimental SAH-induced vasospasm.

On the other hand, there were some limitations of this study Dose dependent results with delayed histopathological assessment of the vasospasm after SAH may increase the value of the study. As it is common knowledge that SAH-induced vasospasm is a long lasting and complicated process, the prolonged intervals between the induction of the SAH and the harvesting of the tissues would be of utmost interest. Also, functional outcome measures are lacking in this study.

Conclusion

Our findings, for the first time, showed that DA can prevent vasospasm and provides neuroprotection following experimental SAH. Moreover, DA showed better results when compared with EPO. More studies based on these findings may be

helpful for further evaluating this promising medication for SAH-induced vasospasm.

Conflicts of interest None.

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