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Increased SIRT1 Signalling and VEGF Expressions by Dexpanthenol Suppress Oxidant, Inflammatory and Apoptotic Processes in a Rat Experimental Model of Subarachnoid Haemorrhage

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ABSTRACT

AIM: To investigate the effects of dexpanthenol (Dex) on subarachnoid haemorrhage (SAH) induced brain injury via sirtuin 1 (SIRT1) signaling in a rat experimental model.

MATERIAL and METHODS: A total of 46 Wistar Albino rats were divided into 5 groups as control, sham, SAH, SAH+Dex, and Dex. First day; 0.3 ml of saline was given to the cisterna magna of the control, sham, and DEX group animals and 0.3 ml of autologous blood was given to SAH and SAH+Dex. On day 4; brain tissues of the rats were removed under anaesthesia.

Brain tissues were collected for the biochemical analysis as total antioxidant level (TAS), total oxidant level (TOS), and oxidative stress index (OSI) levels; histopathological and immunohistochemical analysis as caspase-3 (Cas-3), vascular endothelial growth factor (VEGF); and genetical anaylsis as SIRT1/p53/B-cell lymphoma (BCL2)/Bcl-2-associated X protein (Bax).

RESULTS: Oxidant TOS, OSI levels, inflammatory TNF-α, apoptotic Cas-3, p53, Bax expressions enhanced and antioxidant TAS, antipoptotic BCL2, angiogenetic marker VEGF and SIRT1, which affects all these biomarkers decreased in the SAH group significantly. Besides significant subarachnoidal and parenchymal hemorrhage areas, edematous cerebral membranes, degenerative and necrotic changes and neuronophagies were observed. With the Dex treatment, a decrease was observed in the elevated oxidative, inflammatory, and apoptotic markers. Additionally, antioxidant, anti-apoptotic, VEGF, and SIRT1 levels, showed an increase.

CONCLUSION: SAH caused inflammation, oxidative stress and apoptosis with decreasing levels of SIRT1 signaling and Dex treatment ameliorated and improved all these pathological conditions.

KEYWORDS: Subarachnoid haemorrhage, inflammation, SIRT-1, BCL2, BAX, rats

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■ INTRODUCTION

Subarachnoid haemorrhage (SAH) occurs in the subarachnoid space in the brain, usually due to arterial and, rarely, venous causes. The incidence varies between 10 and 16 per 100,000 population per year, and these rates are reported to increase with increasing age (1,11).

In SAH, which is mostly caused by aneurysmal rupture and has a high mortality rate, hypoxia-induced inflammation, oxidative stress, and apoptosis occur distal to the injury site. The induction of this damage by various intracellular pathways is the main reason for the progressive pathologic process in the tissue. It is known that an increase in sirtuin 1 (SIRT1), a member of a gene family known as NAD+-dependent class III deacetylase enzymes, inhibits the apoptotic process by increasing the expression of the antiapoptotic genes p53 and B cell lymphoma gene (BCL2)-associated X protein (BAX) (3). SIRT1 has been proven to suppress the expression of nuclear factor kappa beta (NFkβ), which plays a central role in the development of inflammation, and to reduce the formation of reactive oxygen radicals, which play a role in the development of oxidative stress, by increasing antioxidant synthesis. The decrease in SIRT1 levels that occurs in a major injury such as SAH can reverse all these effects (5,9) (Figure 1).

Amongst the studies in which pantothenic acid, from which dexpanthenol (Dex) is derived, was used as a preservative, only one study found that pantothenic acid increased SIRT1-mediated BCL2 levels and decreased p53 levels and showed anti-inflammatory and antioxidant effects by passing into the central nervous system (7,8). In another study Dex treatment prevent the brain tissues against traumatic brain injury by decreasing cytokine levels and histopathological findings as ongestion in the piamater layer, cell infiltration, vascular congestion, hemorrhage, and neuronal degeneration, and enhancing superoxide dismutase, glutathione peroxidase and catalase levels (18).

This study aimed to investigate the protective effect of Dex in reducing oxidative stress, inflammation, and apoptosis in SAH-induced brain injury via SIRT1 signaling and VEGF expression.

■ MATERIAL and METHODS

Animals and Ethical Approval

Experimental procedures were performed under the guidelines of the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines 2.0 and were approved by the local ethical committee on Animal Research (Approval No: 29/03/2023-109/999). Forty-six adult Wistar albino male rats weighing 300-350 g were used in the experiments. The animals were obtained from the Animal Research Facility and were housed at 21-22 °C and 60% ± 5% humidity with a 12-hour light and 12-hour dark cycle and fed with standard commercial feed ad libitum and water during the experiments.

Experimental Protocol

A total of 46 rats to be used in the study divided into 4 groups. Groups were;

Control group (n=8): Only intraperitoneal (ip) 0.5 ml saline (SF) was administered between days 1-4.

Sham group (n=10): 0.3 ml SF in the cisterna magna of the rats on day 1 and only 0.5 ml of SF ip applied on days 1-4.

SAH group (n=10): On day 1, approximately 0.3 ml of autologous blood taken from the tail artery of the rat was applied to the cisterna magna of the animal and only ip 0.5 ml SF was applied between days 1-4 (21).

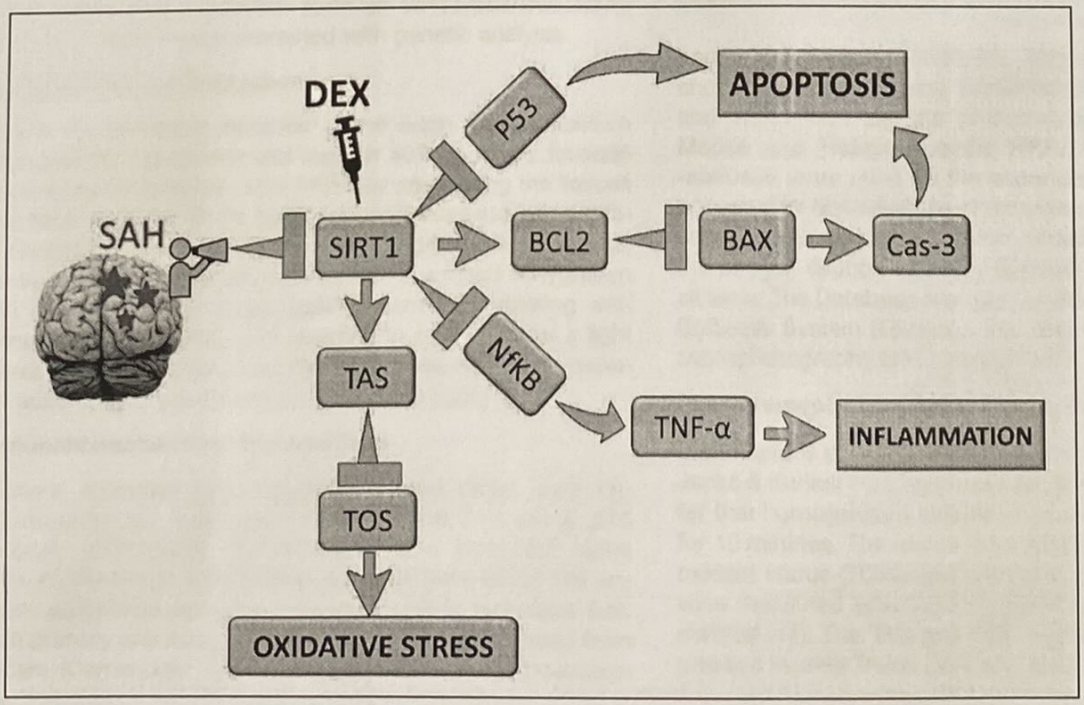


Figure 1: Protective effects dexpanthenol on subarachnoid hemorrhage (SAH) induced brain injury. **SAH:** Subarachnoid hemorrhage, **TOS:** Total oxidant species, **TAS:** Total antioxidant species, Reactive oxygen species, **SIRT1 1:** Sirtuin 1, **NFkβ:** Nuclear factor kappa beta, **TNF-α:** Tumor necrosis factor alpha, **BCL2:** B-cell lymphoma 2, **BAX:** Bcl-2-associated X protein, **Cas-3:** Caspase 3.

Table I: Histopathologic Subarachnoid Hemorrhage Scores (23)

Score	Structure
0	Normal meninges and parenchyma structure
1	No blood in the subarachnoid space, ventricles or brain parenchyma
2	Local or diffuse thin subarachnoid blood, no intraventricular or intraparenchymal blood
3	Diffuse or localized thick layers of subarachnoid blood, no intraventricular or intraparenchymal blood
4	Intraventricular or intraparenchymal blood with subarachnoidal blood regardless of thickness or location

SAH+DEX group (n=10): Approximately 0.3 ml of autologous blood taken from the tail artery of the rat on day 1 was applied to the cisterna magna region of the animals and only 0.5 ml of 500 mg/kg ip DEX was applied between days 1-4 (22).

DEX group (n=8): 0.3 ml SF was given in the cisterna magna of the rats on day 1 and only 0.5 ml of 500 mg/kg ip DEX was administered between days 1-4.

After the last DEX administration (day 4), the experimental animals have euthanized with surgical exsanguination under 80-100 mg/kg ketamine (Keta-control, Doğa İlaç, Türkiye)/8-10 mg/kg xylazine (Xylazinbio %2, Bioveta, Czech Republic) anesthesia by making an abdominal incision and take blood from the inferior vena cava. Following decapitation, the skull was opened and brain tissue was removed (Figure 2).

Three sections of the cerebral cortex tissue were identified. In the first section, the levels of TAS (total antioxidant level), TOS (total oxidant level), and oxidative stress index (OSI) were measured biochemically; in the second section, hematoxylin and eosin staining and immunohistochemical analysis were performed to demonstrate the inflammation and apoptosis in SAH-induced damage and in the third section the expression of intracellular pathways, including those that are markers of inflammation and apoptosis, such as SIRT1/p53/BCL2/BAX gene expressions were examined with genetic analysis.

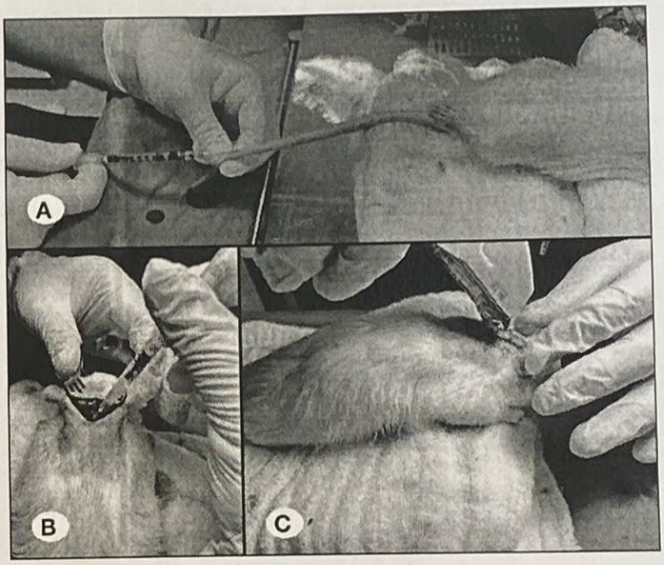


Figure 2: The development of the experimental model. **A)** Blood sampling via percutaneous puncture of the caudal artery; **B)** Insertion of an insulin syringe into the atlanto-occipital membrane in the suboccipital region; **C)** Postoperative suturing with a skin stapler.

Histopathological Evaluation

During the necropsy, samples of the brain and cerebellum were carefully taken out and kept in 10% buffered formalin for histological analysis. After routinely processing the tissues 5µm thick sections of the paraffin blocks were cut using rotary microtomes (Leica RM2155, Leica Microsystems, Wetzlar, Germany). After deparaffinization, we examined rehydration with ethanol graded in decreasing quantities, staining with hematoxylin-eosin (HE), and cleaning in xylene, under a light microscope. Histopathologic findings were scored between 0-4 according to the criteria given in Table I (23).

Immunohistochemical Examinations

Sections collected onto polylysine-coated slides were immunostained for caspase-3 (Cas-3) [Anti-Caspase-3 p12 antibody (EPR16888) (ab179517)], TNF-α [Anti-TNF alpha antibody (RM1005) (ab307164)], an VEGF [Anti-VEGF 164 antibody (ab53465)] using the streptavidin biotin technique (25). Both primary and secondary antibodies were purchased from Abcam (Cambridge, UK). Following a 60-minute incubation period with primary antibodies, sections were immunohistochemically stained using biotinylated secondary antibodies and streptavidin-alkaline phosphatase conjugate. EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit -ab80436 were used as the secondary antibody and diaminobenzidine served as the chromogen (DAB). For the negative controls, the primary antiserum phase was swapped out for the antigen dilution solution. Blinded samples were used for all tests. The Database Manual Cell Sens Life Science Imaging Software System (Olympus Co, Tokyo, Japan) was used for microphotography and morphometric analysis.

Measurement of Oxidative Stress Parameters in the Brain

Brain tissues of rats were homogenized with the Ultra Turrax Janke & Kunkel T-25 homogenizer (IKA® Werke, Germany). After that homogenized tissues were centrifuged at 10000 rpm for 10 minutes. The serum total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) values were measured spectrophotometrically using a modified Erel method (14). The TAS and TOS results in the serum were expressed in µmol Trolox Eq/L and µmol H2O2 Eq/L, respective-

Table II: Primary Sequences, Product Size and Accession Numbers of Genes

Genes	Primary sequence	Product size	Accession number
Rn18s (HouseKeeping)	F: CTCTAGATAACCTCGGGCCG	209 bp	NR_046237.2
	R: GTCGGGAGTGGGTAATTTGC		
SIRT1	F: GGTAGTTCCTCGGTGTCCT	152 bp	NM_001414959.1
	R: ACCCAATAACAATGAGGAGGTC		
p53	F: CTCCTCTCCCCAGCAAAAG	151bp	XM_017597018.2
	R: CCTGCTGTCTCCTGACTCCT		
BCL2	F: CATCTCATGCCAAGGGGGAA	284 bp	NM_016993.2
	R: TATCCCACTCGTAGCCCCTC		
BAX	F. CACGTCTGCGGGGAGTCAC	419 bp	NM_017059.2
	R: TAGAAAAGGGCAACCACCCG		

Rn18s: *18S ribosomal RNA,* ***SIRT1:*** *Sirtuin 1,* ***BCL2:*** *B-cell lymphoma 2,* ***BAX:*** *Bcl-2-associated X protein.*

ly. The TAS and TOS results for the tissues were expressed by dividing them by the protein value. The OSI was calculated using the formula OSI = [(TOS, μmol /l)/(TAS, mmol Trolox eq/l) × 100] (15).

Reverse Transcription-Polymerase Chain Reaction (RT-qPCR)

Using the GeneAll RiboEx (TM) RNA Isolation Kit (Cat No: 301–001) and the manufacturer's instructions, RNA was extracted from homogenized brain tissues (GeneAll Biotechnology, Seoul, South Korea). A BioSpec-nano NanoDrop UV–Vis spectrophotometer (UV-2600, Shimadzu Ltd. Kyoto, Japan) instrument was used to measure the quantity and purity of the RNAs that were collected. cDNA synthesis was performed using the A.B.T.™ cDNA Synthesis Kit (Cat No: C03-01–05) from Atlas Biotechnology in Türkiye according to the instructions. One microgram of RNA was used for cDNA synthesis. Using the Primer-BLAST tool, NCBI website, specific mRNA sequences were found, and potential primer sequences were then tested. In Table I, the primers' sequences used accession numbers, and the product size of the genes are given. A.B.T.™ SYBR Master Mix (Atlas Biotechnology, Türkiye) (Cat No: Q04-01–05) was used to quantify the expression levels of genes in a Biorad CFX96 real-time PCR equipment (CA, USA). In the study, the Rn18s was used as a housekeeping gene. The reaction mixture was prepared according to the manufacturer's protocol to a final volume of 20 μL. The resultant reaction mixture was put into real-time qPCR equipment with a thermal cycling setup following the manufacturer's protocol for the kit, and each sample was examined in three replications. The PCR protocol was applied as 1 cycle with an initial denaturation at 95 °C for 300 s and 40 cycles with denaturation at 95 °C for 15 s, annealing/extension at 55 °C for 30 s. Relative mRNA levels were calculated by applying the 2–ΔΔCt formula to normalize the results (Table II).

Statistical Analysis

Variables were presented as mean ± standard deviations. One-way ANOVA (posthoc Tukey's test for biochemical and genetic analysis, Duncan test for histopathological and immunohistochemical analysis) was used to compare groups with each other. Statistical calculations were made using the Graphpad Prism 8.0 program package (Graphpad Software. Inc., MA, USA). $p \leq 0.05$ was set as the value for significance.

■ RESULTS

Brain TOS, TAS and OSI Values

The TOS and OSI values, which are indicators of oxidative stress in brain tissue, showed a significant increase in the SAH group compared to the control, sham and Dex-only groups ($p \leq 0.001$ for all). In the treatment group, SAH+Dex group, the TOS and OSI values were significantly decreased compared to the SAH group ($p \leq 0.01$ and $p \leq 0.001$; respectively) (Figure 3). However, compared to the control and sham groups, they were found to be higher in the SAH-Dex group, but this increment was not significant. When the TAS values were analysed, a significant decrease was observed in the SAH group compared to the control ($p \leq 0.001$), sham ($p \leq 0.01$), and Dex ($p \leq 0.001$) groups, while a significant increase was observed in the SAH+Dex group compared to the SAH group. The results were similar between the control, sham, and SAH-Dex groups.

Histopathological and Immunohistochemical Findings

The brain tissue samples of all the rats in the control group were observed to have normal tissue histology. When the brain tissue samples of the rats in the sham group were examined, severe hyperaemia and marked oedema in both the meningeal and parenchymal vessels were observed, and these were not observed in the control group ($p \leq 0.001$). Oedema was observed in the meninges and subarachnoid space.

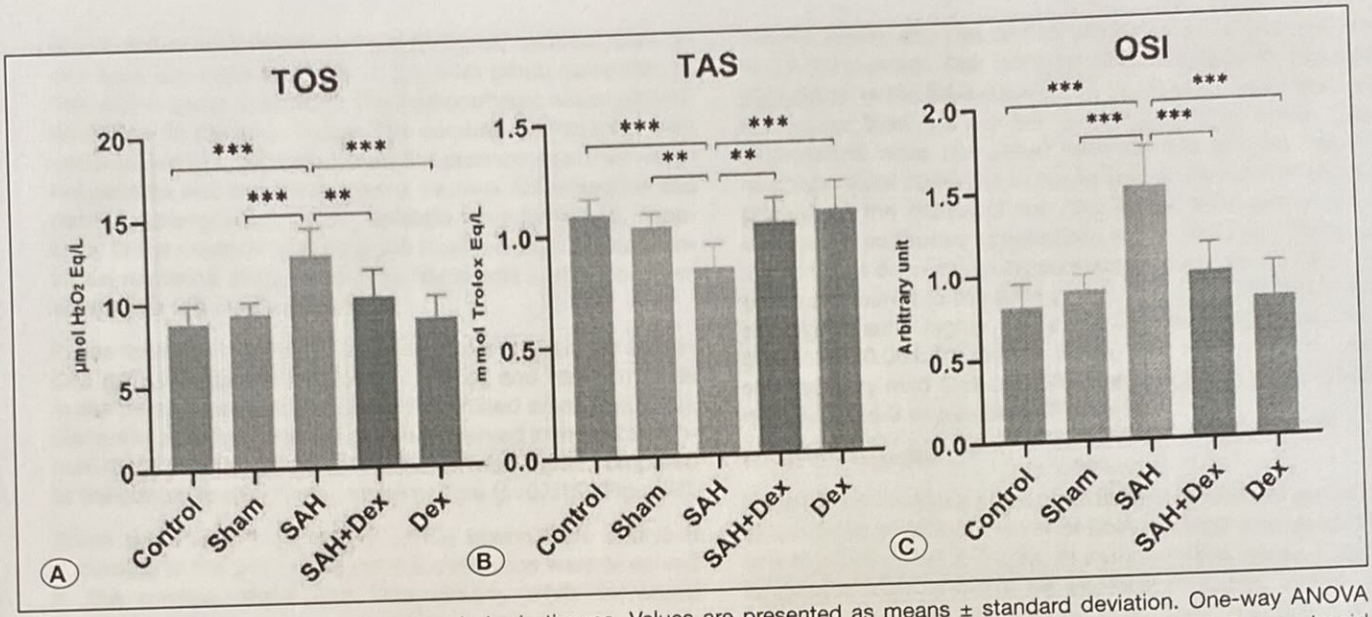


Figure 3A-C: Oxidative stress parameters in brain tissues. Values are presented as means ± standard deviation. One-way ANOVA posthoc Tukey's test was used. **TAS:** Total antioxidant status, **TOS:** Total oxidant status, **OSI:** Oxidative stress index, **SAH:** Subarachnoid hemorrhage, **dex:** Dexpanthenol *p≤0.05, **p≤0.01, ***p≤0.001

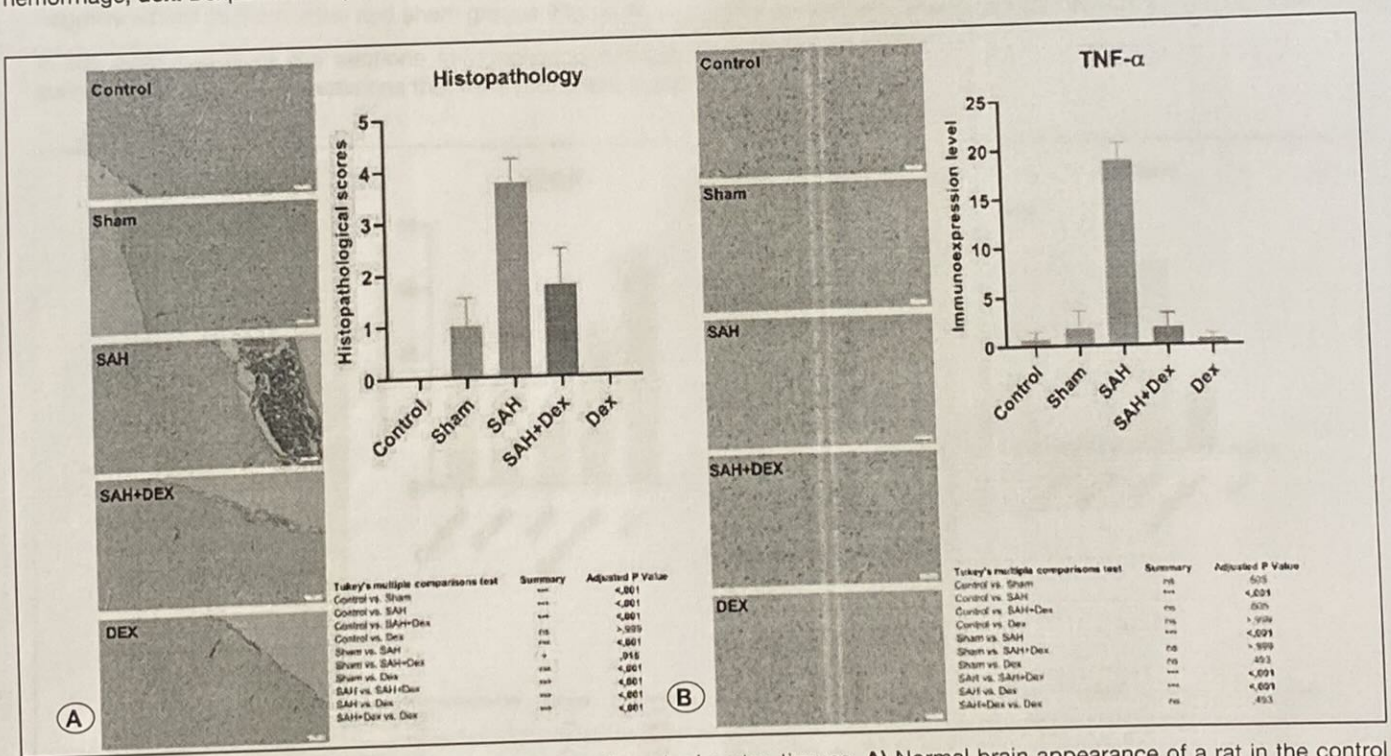


Figure 4: Histopathological and TNF-α immunostaining of cerebral cortex tissues. **A)** Normal brain appearance of a rat in the control group, (Score 0), marked hyperemia in the vessels and edematous subarachnoid space in the sham group, (Score 2), marked hemorrhage area in the subarachnoidal region, edema in the meninges and meningeal reaction in a rat in the SAH group, (Score 3), View of the subarachnoidal space of a rat in the SAH+Dex group, meningeal reaction with hyperemia of vessels and mild hemorrhage, (Score 2), Normal looking brain tissue of a rat in the Dex group, (Score 0); **B)** Negative TNF-α immunoreaction in the brain of a rat in the control and sham group, significantly increased TNF-α expression in neurons of a rat in SAH group, Negative TNF-α expressions in brain neurons of a rat in SAH+Dex group and Dex groups, streptavidin biotin peroxidase method, Scale bars=50μm.

Significant subarachnoid and parenchymal haemorrhage areas were observed in all rats in the SAH group compared to the control group (p≤0.001). The haemorrhagic areas extended deep into the brain tissue. The cerebral membranes were oedematous and separated from the parenchyma. Perineuronal oedema was observed in some neurons. Degenerative and necrotic changes and neuronophagia were observed, especially in the neurons located in the haemorrhagic areas. Meningeal reactions characterized by fibroplasia in the meninges were observed in many areas.

It was noteworthy that the SAH areas decreased in the SAH+-Dex group compared to the SAH group, and haemorrhages in the parenchyma were observed in limited areas (p≤0.001). Generally, mild haemorrhages were observed in the subarachnoid region. On the contrary, SAH areas were higher compared to the control (p≤0.001) and sham groups (p=0.015) (Figure 4).

When the TNF-α expressions of the brains were analysed according to the groups, negative expressions were observed in the control, sham and Dex groups, while increased expressions were observed in the neurons of the rats in the SAH group (p≤0.001 for all). In many rats in the SAH+Dex group, TNF-α expressions in the brain were found to be negative similar to the control and sham groups (Figure 4).

In the examination of the sections immunohistochemically stained with VEGF, the expressions that were prominent in the control, sham, and Dex groups exhibited a considerable drop in the SAH group. Dex administration significantly increased expression in the SAH+Dex group similarly to sham (p=0.987) and lower than the control group (p≤0.001). When Cas-3 expressions were compared between the groups, negative reactions were observed in the brains of the control and Dex groups. In the brains of the rats in the SAH group, Cas-3 expression increased significantly in the neurons. There was a significant decrease in immunoexpressions in the SAH+Dex group compared to the SAH group (p≤0.001), but levels were still significantly higher compared to the control and sham groups (p≤0.001 for both). Some rats in the sham group showed very mild Cas-3 expressions, but most rats showed negative Cas-3 expressions (Figure 5).

RT-qPCR Results

In the genetic analysis of the brain tissues of the SAH group, the expressions of p53, a marker of DNA damage and apoptosis, and the BAX gene, a marker of mitochondrial stress-induced apoptosis, were found to be significantly higher in the SAH group compared to the control, sham and Dex-only groups (p≤0.001 for all). In the SAH+Dex group receiving the Dex treatment, both values were significantly lower, similar to the control and sham groups, compared to the SAH group (p≤0.001 for both).

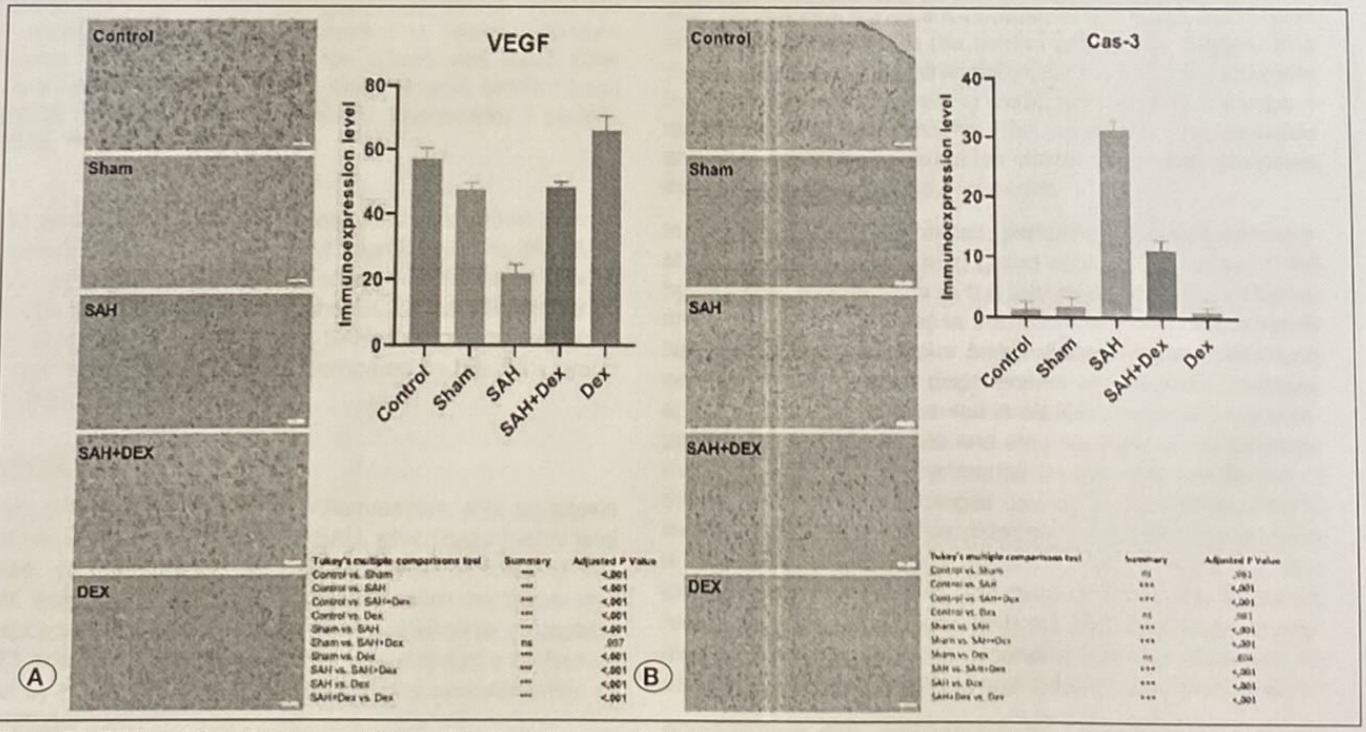


Figure 5: VEGF and Cas-3 immunostaining of the brain tissues. **A)** Image of prominent VEGF expressions in brain neurons in the control group, prominent VEGF expressions in sham group, markedly decreased VEGF expressions in brain neurons in SAH group, significantly increased VEGF expressions in SAH+Dex group compared to SAH group, prominent VEGF expressions in brain neurons in Dex group; **B)** Negative Cas-3 immunoreaction in brain tissue of a rat in the control and sham group, significantly increased Cas-3 expression in brain neurons of a rat in SAH group, significantly decreased Cas-3 expressions in brain neurons of a rat in SAH+Dex group compared to SAH, negative Cas-3 immunoexpression in a rat in Dex group, Streptavidin biotin peroxidase method, Bar=50μm.

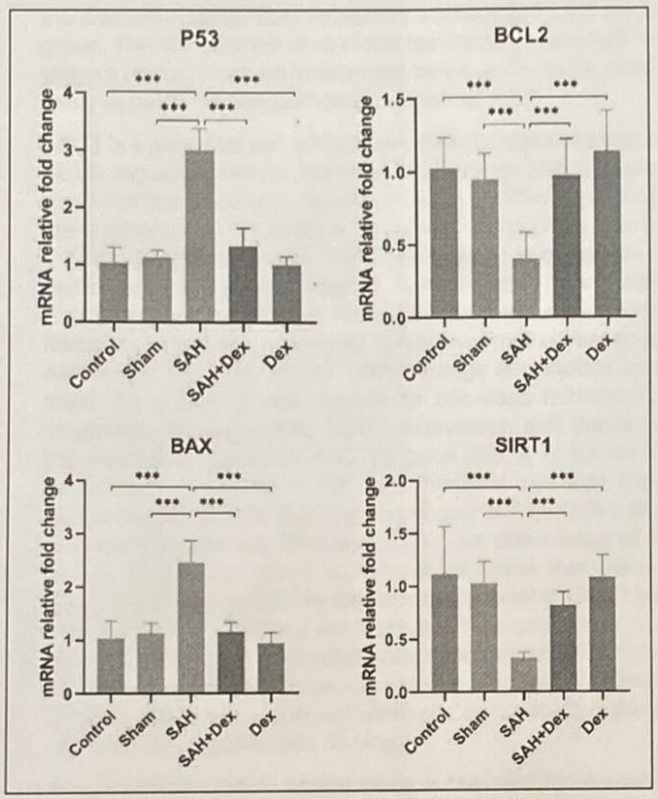


Figure 6: mRNA relative fold changes of p53, SIRT-1, BCL2 and BAX genes in brain. Values are presented as means ± standard deviation. One-way ANOVA posthoc Tukey's was used. **SAH:** Subarachnoidal hemorrhage, **Dex:** Dexpanthenol, **SIRT1:** Sirtuin 1, **BCL2:** B-cell lymphoma 2, **BAX:** Bcl-2-associated X protein, *p≤0.05, **p≤0.01, ***p≤0.001.

SIRT1 and BCL2 gene expressions, which are known to have protective properties, decreased significantly in the SAH group compared to the control, sham, and Dex-only groups (p≤0.001 for all). The SIRT1 and BCL2 gene expressions were found to be higher in the SAH+Dex group, similar to the control and sham groups, compared to the SAH group (p≤0.001) (Figure 6).

■ DISCUSSION

In this study, oxidative stress, inflammation, and apoptosis occurring in brain damage due to SAH, which has a highly fatal course, were regressed with Dex treatment. In creating this effect, apoptotic p53, Cas-3 expressions were decreased and antiapoptotic BCL2 expressions were increased by increasing SIRT1 signaling. On the other hand, Dex showed a protective effect by increasing VEGF expressions and vascularization of the tissue.

SAH is a highly aggressive and often fatal disease state that can cause widespread brain damage. In this disease, the oxygen deprivation behind the haemorrhage site can induce inflammation in the brain tissue through various intracellular mechanisms such as hypoxia-induced factor 1 alpha, and other intracellular pathways such as mitochondrial stress-induced apoptosis can induce apoptosis (12,17). These two important damage states are also accompanied by oxidative stress and accelerate progression (16). Fan et al. demonstrated that both oxidative stress and apoptosis increase simultaneously in brain tissue following SAH, and that these two mechanisms are interrelated (9).

In the case of inflammation, the cytokines released from the damaged cells in contact with the surrounding tissues, and the spread of the damage cannot yet be completely stopped by the current treatment modalities. Many agents have been tried to reduce cellular responses such as oxidative stress, inflammation, and apoptosis in tissues (20,21,27).

During the oxidative stress, it is known that there is an increase in oxidant substances that cannot be overcome by antioxidant enzymes. Over time, antioxidant enzymes used to reverse the damage are stretched, leading to an increase in oxidant molecules (10,24). Considering the oxidative stress markers of this study, the increased TOS and OSI values and decreased TAS levels, which are indicators of antioxidant activity in the SAH group indicate a considerable occurrence of SAH-induced oxidative stress. In parallel with these results, Zhang et al. and Huang et al. also confirmed the development of oxidative stress after SAH (13,28). The reversal of this highly destructive picture with Dex treatment and its reversal compared to the SAH group can be interpreted as the antioxidant effect of the drug itself. As a definitive proof of this, the lack of a significant increase in TAS levels in the Dex-only group compared to the control group may suggest that the drug itself does not have antioxidant activity, but prevents oxidative stress secondary to these mechanisms because it regresses other mechanisms. The reverse is also possible and the decrease in oxidative stress may also suppress inflammatory or apoptotic processes.

In the histopathologic analysis, performing the surgical model but giving SF in the sham group may be the cause of the hyperaemia and oedema in the meningeal and parenchymal areas. However, the intense subarachnoid and parenchymal haemorrhage, oedematous cerebral membranes, perineural oedema in the neurons, degenerative and necrotic changes, and neuronophagies observed in the SAH group are not comparable to the sham group and are indicative of the development of SAH as an experimental model. The parallelism of these histopathologic changes caused by an intense inflammatory picture with TNF-α detected immunohistochemically is more evidence of inflammation. Consistent with this, Cao et al. reported that TNF-α also plays a role in the increased neuroinflammation following SAH (4). Histopathologic changes and immunoexpressions decreased with Dex treatment, indicating that the drug utilizes anti-inflammatory mechanisms.

Angiogenesis and cerebrovascular integrity play a crucial role in the pathophysiology of SAH as reported by Barak et al. (2). The immunohistochemical findings suggest that the decreased VEGF status found in the SAH group indicates impaired angiogenesis capacity and supports hypoxia. It can be seen that this situation is prevented with Dex treatment. One of the most important results here is that VEGF levels in

the Dex-only group also increased compared to the control group. The fact that the drug alone can increase angiogenesis without damage can be interpreted as an ability to be used in many hypoxia-related pathologies such as SAH.

SIRT1 is a gene that can affect many cellular pathways and basically regresses cellular damage mechanisms (30). Downregulation of this gene and increased levels of NFkβ may trigger many inflammatory reactions, as proven by the histopathologic findings and increased TNF-α expression in our study. As demonstrated in recent studies, SIRT1-related mechanisms also play a crucial role in mitigating SAH-induced neuroinflammation (19,26). Conversely, it is known that increased p53 expression can both trigger DNA damage and induce apoptosis. Apoptosis is also known to decrease mitochondrial biogenesis by decreasing SIRT1 expression and decreasing the deacetylation of the PGC-1α gene (29). It is known that negativities occurring in the mitochondrial pathway trigger Cas-3-mediated apoptosis by decreasing antiapoptotic BCL2 levels and increasing BAX levels (6). The attenuation of this picture in the SAH group by Dex could mean that the drug prevented this progressive damage by increasing SIRT1 gene expression and reversed the damage. This could also mean that it can be used in mitochondrial stress-induced apoptotic processes, especially those caused by hypoxia. The immunohistochemically decreased levels of Cas-3, which is the end product, also support this finding.

A significant limitation of this study is the inability to examine the protein expression of genes analyzed through PCR using the Western blot method. Additionally, the absence of both male and female rats prevented the investigation of potential sex-specific differences in the outcomes. Furthermore, the lack of cognitive tests in rats to demonstrate the clinical efficacy of Dex treatment highlights another area for improvement. To address these shortcomings, we aim to conduct more comprehensive studies in the future by utilizing national and international research funds, advancing the current findings further.

■ CONCLUSION

With Dex treatment SIRT1 signalling decreases in SAH and oxidative stress, inflammation and apoptosis regress. Increased SIRT1 gene stimulation and VEGF gene expression with Dex treatment may regress these and similar hypoxia-induced damages. Despite these findings, research at the protein expression level is needed to elucidate these issues.

Declarations

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Availability of data and materials: The datasets generated and/or analyzed during the current study are available from the corresponding author by reasonable request.

Disclosure: The authors declare no competing interests. Of note, they have no personal, financial or institutional interest in any of the drugs, materials or devices described in this article.

AUTHORSHIP CONTRIBUTION

Study conception and design: ASO, HA
Data collection: ASO, HA, AD, OO
Analysis and interpretation of results: HA, MYT, II
Draft manuscript preparation: ASO, HA, NS
Critical revision of the article: ASO, HA, NS, RT
Other (study supervision, fundings, materials, etc...): HA, AD, MYT, II, OO
All authors (ASO, HA, MYT, II, NS, AD, RT, OO) reviewed the results and approved the final version of the manuscript.

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