



Time-Dependent Neurovascular Effects of Thymoquinone on Basilar Artery Morphometry in a Rat Model of Subarachnoid Haemorrhage

Cengiz ATIS¹, Mehmet Akif ERBAS², Cengiz COKLUK², Mehmet Emin ONGER³, Abdullah Hilmi MARANGOZ²

¹Carsamba State Hospital, Department of Neurosurgery, Samsun, Türkiye

²Ondokuz Mayıs University, Faculty of Medicine, Department of Neurosurgery, Samsun, Türkiye

³Ondokuz Mayıs University, Faculty of Medicine, Department of Histology and Embryology, Samsun, Türkiye

Corresponding author: Abdullah Hilmi MARANGOZ ✉ abdullahbeyin@gmail.com

ABSTRACT

AIM: To investigate the effects of short- and long-term intraperitoneal thymoquinone administration on basilar artery morphometry and cerebral perfusion in a subarachnoid haemorrhage (SAH) model.

MATERIAL and METHODS: Forty-two adult male Wistar rats were randomly allocated to seven experimental groups, including control, sham, untreated SAH, and SAH groups treated with vehicle or thymoquinone (10 mg/kg/day) for 3 or 8 days. SAH was induced by autologous blood injection into the cisterna magna, and animals were sacrificed on days 3 or 8. Basilar artery luminal and tunica media areas were assessed using unbiased stereological methods. Cerebral blood flow was measured by laser Doppler flowmetry, and neurological function was evaluated as a secondary outcome.

RESULTS: Basilar artery luminal area differed significantly among experimental groups ($p < 0.001$), with pronounced luminal narrowing in vehicle-treated SAH animals during the delayed phase (day 8). Prolonged thymoquinone administration significantly attenuated delayed luminal narrowing, whereas short-term treatment did not produce a measurable morphometric effect. Long-term treatment was associated with partial preservation of cerebral blood flow. No significant differences were observed in basilar artery tunica media area. Neurological deficit scores differed among groups, with significant impairment in vehicle-treated SAH animals at day 8 compared with both control and thymoquinone-treated SAH animals at the same time point. This study demonstrates duration-dependent vascular effects of thymoquinone in experimental SAH.

CONCLUSION: Prolonged intraperitoneal thymoquinone administration exerts time-dependent neurovascular effects in experimental SAH by attenuating delayed basilar artery luminal narrowing and partially preserving cerebral perfusion, highlighting the importance of treatment duration in delayed vascular pathology.

KEYWORDS: Subarachnoid haemorrhage, Cerebral vasospasm, Basilar artery, Thymoquinone, Neurovascular pathology

ABBREVIATIONS: CBF: Cerebral blood flow, DMSO: Dimethyl sulfoxide, PEG: Polyethylene glycol, PEG300: Polyethylene glycol 300, SAH: Subarachnoid haemorrhage

Cengiz ATIS

ORCID : 0009-0009-1132-6923

Mehmet Emin ONGER

ORCID : 0000-0001-8922-9642

Mehmet Akif ERBAS

ORCID : 0009-0008-0548-9534

Abdullah Hilmi MARANGOZ

ORCID : 0000-0002-6183-8427

Cengiz COKLUK

ORCID : 0000-0003-2146-3621



This work is licensed by "Creative Commons Attribution-NonCommercial-4.0 International (CC)".

■ INTRODUCTION

Subarachnoid haemorrhage (SAH) induces a complex cascade of neurovascular alterations that extend beyond the initial haemorrhagic insult and evolve over time. Among these secondary processes, cerebral vasospasm represents a hallmark delayed vascular pathology characterized by progressive arterial narrowing and impaired cerebral perfusion. In experimental models, vasospasm typically emerges several days after haemorrhage and reaches peak severity between days 7 and 10, reflecting a delayed and sustained disturbance of cerebrovascular regulation rather than an acute response to bleeding (4,8,18,22,34).

Despite extensive investigation, the mechanisms underlying cerebral vasospasm remain incompletely understood. Accumulating experimental and translational evidence suggests that vasospasm arises from the convergence of multiple inter-related processes, including the presence of oxyhaemoglobin within the subarachnoid space, excessive generation of reactive oxygen species, increased endothelin signaling, reduced nitric oxide bioavailability, inflammatory cell infiltration, and progressive endothelial dysfunction (21,36). Importantly, these mechanisms exhibit a time-dependent evolution, which is considered central to the delayed onset and persistence of vasospasm-associated neurovascular dysfunction.

Thymoquinone is the principal bioactive constituent of the volatile oil of *Nigella sativa* and has attracted considerable interest due to its antioxidant and anti-inflammatory properties (37). Experimental evidence indicates that thymoquinone modulates key pathways relevant to neurovascular regulation, including free radical scavenging, suppression of nuclear factor- κ B activation, attenuation of pro-inflammatory cytokine release, and regulation of nitric oxide and endothelin signaling (12,15). In experimental models of neurological injury—such as cerebral ischaemia–reperfusion, traumatic brain injury, and spinal cord ischaemia—thymoquinone administration has been associated with reduced tissue damage and preservation of vascular structure and function (1,11,17). However, studies directly examining its effects on cerebral vasospasm following subarachnoid haemorrhage, particularly with respect to treatment duration and temporal alignment with vasospasm development, remain limited.

Delayed cerebral vasospasm constitutes a progressive neurovascular process rather than an abrupt post-haemorrhagic event, suggesting that sustained and temporally aligned pharmacological interventions may be required to influence its course. Accordingly, the present study was designed to evaluate the duration-dependent effects of intraperitoneal thymoquinone administration on basilar artery morphometry and cerebral perfusion in an experimental subarachnoid haemorrhage model, with particular emphasis on vascular alterations associated with the delayed phase of vasospasm rather than acute early brain injury.

■ MATERIAL and METHODS

Animals

This experimental study was approved by the Ondokuz Mayıs University Animal Experiments Local Ethics Committee (Ap-

proval No: 2014/35; Approval Date: 29 September 2014). A total of 42 adult male Wistar rats (3.0 $\pm$ 0.3 months old; body weight 280 $\pm$ 12 g) were used in this study and randomly allocated into seven experimental groups (n=6 per group).

Animals were bred and housed in the institution's experimental animal facility and were allowed to acclimatise to the laboratory environment for at least 7 days prior to experimental procedures. Rats were housed in standard polycarbonate cages (three animals per cage) under controlled environmental conditions (temperature 22 $\pm$ 2 °C; relative humidity 50–60%) with a 12-hour light/dark cycle, and had ad libitum access to standard laboratory chow and water.

All experimental procedures were conducted in accordance with the ARRIVE guidelines for reporting animal research (19).

Study Design and Experimental Groups

Animals were randomly assigned to experimental groups using the sealed-envelope method. Group allocation was concealed throughout the experimental period, and all experimental interventions, neurological assessments, cerebral blood flow measurements, and histomorphometric analyses were performed by investigators blinded to group allocation. Based on the well-established temporal profile of cerebral vasospasm, two predefined termination time points were selected to represent the early and delayed phases following subarachnoid haemorrhage (SAH): day 3 and day 8, respectively (23,26,34).

To evaluate baseline and early procedural effects, a control group without SAH and a sham group undergoing all surgical procedures except autologous blood injection into the cisterna magna were assessed on day 3. In addition, untreated SAH animals and SAH groups receiving either vehicle or thymoquinone for three consecutive days were evaluated at this early time point.

To examine treatment effects during the delayed phase of vasospasm, separate SAH groups received either vehicle or thymoquinone for eight consecutive days and were evaluated on day 8. As the primary hypothesis focused on delayed-phase vascular alterations, comparisons at day 8 were performed between thymoquinone-treated and vehicle-treated SAH groups. Each experimental group consisted of six animals (n=6), no animals were excluded from the final analysis, and no unexpected deaths occurred during the experimental period.

Subarachnoid Haemorrhage and Sham Procedures

In animals assigned to subarachnoid haemorrhage (SAH) induction, general anaesthesia was achieved by intraperitoneal administration of ketamine (50 mg/kg; Pfizer, New York, NY, USA) and xylazine (10 mg/kg; Rompun® 2%, Bayer, Germany). Prior to the procedure, prophylactic intraperitoneal cefazolin (50 mg/kg; Sandoz, Kundl, Austria) was administered. Animals were positioned in a stereotaxic frame with the head fixed at 45° flexion, and a midline occipitocervical incision was performed to expose the atlanto-occipital membrane.

The cisterna magna was accessed using a 27-gauge needle, 0.1 mL of cerebrospinal fluid was gently aspirated, and an equal volume of fresh autologous arterial blood was slowly injected to induce SAH. Following wound closure, animals were

maintained in a supine position with the head tilted downward at 45° for 15 minutes to standardise clot distribution within the cisternal space. After recovery from anaesthesia, animals were returned to their cages and monitored daily throughout the postoperative period (Figure 1). Supportive care was provided as needed, and animals were evaluated for predefined humane endpoints.

In sham-operated animals, the same anaesthesia, positioning, surgical exposure, and cisterna magna access procedures were performed; however, no blood was injected into the cisterna magna. All remaining procedural steps, including wound closure and postoperative positioning, were identical to those used in the SAH group. Successful induction of subarachnoid haemorrhage was confirmed by visualisation of blood within the basal cisterns during dissection and by the presence of early neurological deficits and reductions in cerebral blood flow following the procedure.

Preparation and Administration of Thymoquinone and Vehicle

The thymoquinone dose was selected based on previous experimental studies reporting vascular and neuroprotective

effects within a comparable dosage range (9,14,25,29); dose-response evaluation was beyond the scope of the present study.

Thymoquinone ($\geq 98\%$ purity; Sigma-Aldrich, St. Louis, MO, USA) was prepared immediately before administration by dissolution in a co-solvent vehicle consisting of 5% dimethyl sulfoxide (DMSO), 40% polyethylene glycol 300 (PEG300), and 55% 0.9% saline (v/v), according to formulations previously reported to be well tolerated for intraperitoneal administration of hydrophobic compounds (6,25). All formulations were freshly prepared on the day of administration and visually inspected for precipitation.

Thymoquinone was administered intraperitoneally once daily at a dose of 10 mg/kg/day using a fixed injection volume of 10 mL/kg/day, consistent with dosage ranges previously shown to exert vascular and neuroprotective effects (9,14, 29). This dose has been reported to be well tolerated and not associated with significant toxicity following intraperitoneal administration (14). However, the present study was not designed to evaluate dose-response relationships or long-term safety outcomes. The intraperitoneal route was selected over

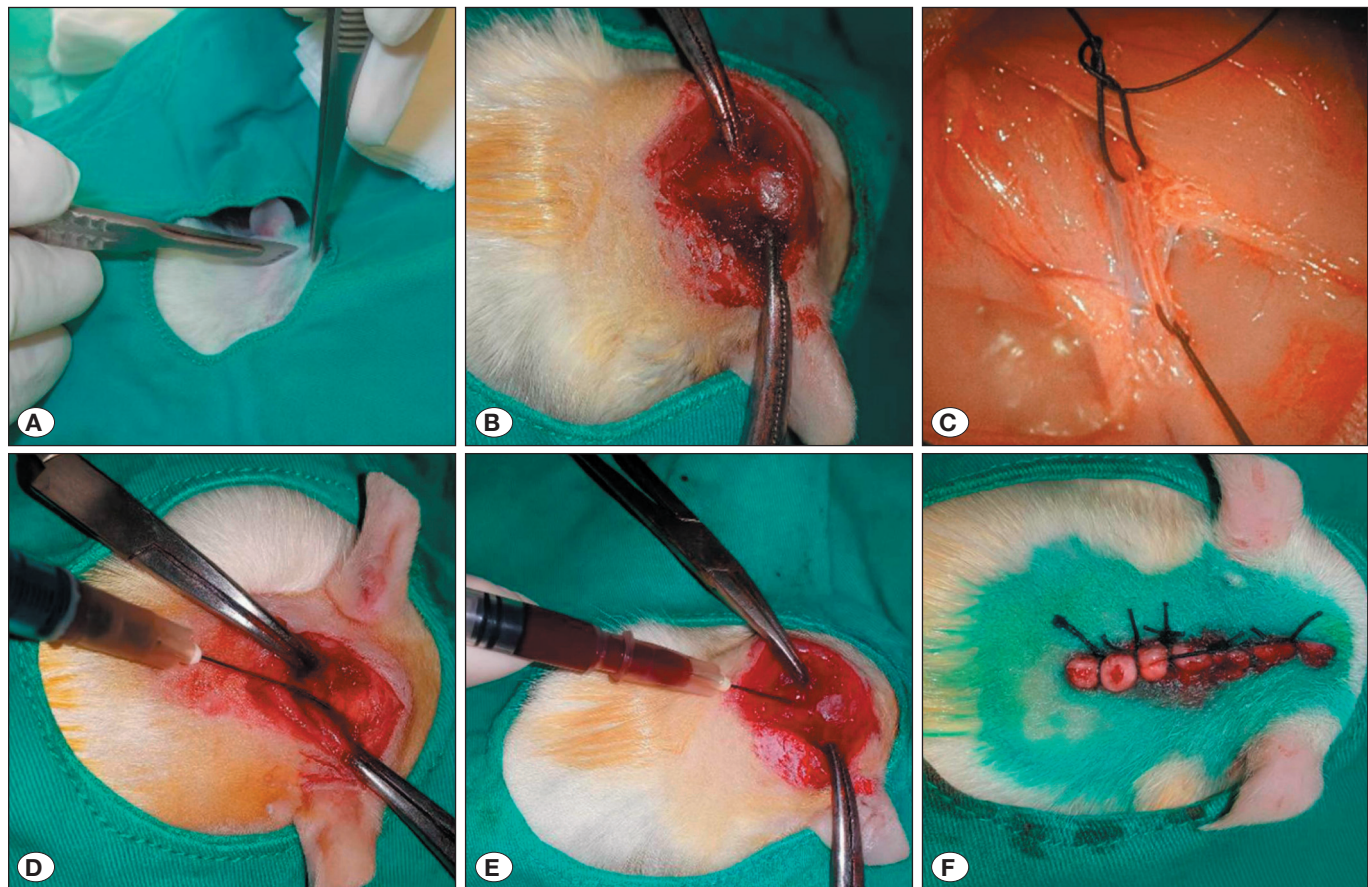


Figure 1: Establishment of the experimental subarachnoid haemorrhage (SAH) model in rats. **A)** Midline occipitocervical incision performed under general anaesthesia; **B)** exposure of the atlanto-occipital membrane; **C)** femoral artery cannulation for collection of fresh autologous arterial blood; **D)** gentle aspiration of cerebrospinal fluid from the cisterna magna; **E)** slow injection of an equal volume of autologous arterial blood into the cisterna magna to induce SAH; and **F)** wound closure following completion of the surgical procedure. Representative images illustrating the stepwise induction of experimental SAH are shown.

oral gavage to minimise variability related to oral bioavailability during the post-subarachnoid haemorrhage period, which may confound assessment of duration-dependent treatment effects (3,31).

Vehicle-treated SAH groups received the identical vehicle formulation without thymoquinone, administered intraperitoneally at the same volume and schedule for the corresponding treatment duration (3 or 8 days). Animals in the sham group received the same vehicle formulation via the same route and administration schedule. Thus, each animal received a fixed intraperitoneal injection volume of 2.8–3.0 mL per dose, corresponding to its body weight.

Neurological Evaluation

Neurological status was assessed using a standardized neurological deficit scoring system adapted from previously described experimental subarachnoid haemorrhage models (10). All assessments were performed by an investigator blinded to group allocation and were conducted daily until sacrifice. Neurological evaluations were analysed primarily at days 3 and 8, corresponding to the early and delayed phases of vasospasm, respectively.

The scoring system comprised five components: spontaneous activity, symmetry of limb movements, forelimb outstretching, climbing ability, and response to tactile stimulation. Each component was scored on a scale from 0 to 2, with higher scores indicating better neurological function and a maximum total score of 10.

Animals exhibiting severe neurological impairment or distress unrelated to the experimental protocol were predefined to be excluded according to established humane endpoints; no animals met these exclusion criteria. Detailed neurological scoring criteria are provided in Table I.

Cerebral Blood Flow Measurement

Cerebral blood flow (CBF) was assessed using laser Doppler flowmetry to evaluate haemodynamic changes associated with experimental cerebral vasospasm. Measurements were performed under standardised anaesthesia to minimise physiological variability. Under light anaesthesia, the Doppler probe was positioned over the parietal cortex, and relative cortical perfusion values were continuously recorded.

Baseline CBF measurements were obtained prior to subarachnoid haemorrhage induction. Subsequent assessments

were conducted on days 3 and 8, corresponding to the early and delayed phases of vasospasm, respectively. To enable inter-individual comparison, CBF values were normalised to baseline and expressed as a percentage of each animal's pre-SAH value. Cerebral blood flow measurements were obtained using a laser Doppler flowmetry system (PeriFlux System 5000; Perimed AB, Järfälla, Sweden). Each recording was performed for at least 5 minutes after stabilisation of the signal, and mean values were used for analysis.

Physiological Monitoring and Perfusion Conditions

During anaesthesia, sacrifice, and transcatheter perfusion, physiological variables known to influence vascular tone were monitored and maintained within predefined ranges. Depth of anaesthesia, respiratory pattern, and core body temperature were regularly assessed, with core temperature continuously maintained at 37±0.5 °C using a rectal probe.

Transcatheter perfusion was performed under standardised pressure and duration conditions to minimise variability in vascular diameter attributable to systemic haemodynamic fluctuations.

Tissue Processing and Histology

Upon completion of the experimental procedures, animals were sacrificed under general anaesthesia induced by intraperitoneal ketamine (50 mg/kg; Pfizer, New York, NY, USA) and xylazine (10 mg/kg; Rompun® 2%, Bayer, Germany), followed by transcatheter perfusion with 200 mL of heparinised 0.9% saline (10 IU/mL) and fixation with 200 mL of 10% neutral buffered formalin.

After craniotomy, the entire brain was removed and post-fixed overnight in 10% neutral buffered formalin. In all experimental groups, the brainstem—preserving the pons and basilar artery—was separated from the cerebral hemispheres and maintained in the same fixative (Figure 2).

Following routine tissue processing, basilar artery specimens were embedded in paraffin, and 5-µm-thick sections were obtained using a rotary microtome (Leica RM 2135, Leica Instruments, Nussloch, Germany) according to systematic random sampling principles. Sections were stained with haematoxylin and eosin and examined under light microscopy; representative histological images are presented in Figure 3.

Morphometric measurements of basilar artery luminal area and tunica media area were performed using standard stereo-

Table I: Neurological Deficit Scoring System

Parameter	Score 0	Score 1	Score 2
Spontaneous activity	No spontaneous movement	Reduced activity	Normal activity
Symmetry of limb movement	Marked asymmetry	Mild asymmetry	Symmetric movement
Forelimb extension	No extension	Partial extension	Full symmetric extension
Climbing ability	Unable to climb	Impaired climbing	Normal climbing
Response to tactile stimulation	No response	Delayed response	Normal response

Total score range: 0–10, with higher scores indicating better neurological function.



Figure 2: Tissue harvesting procedure. **A)** Transcatheter perfusion performed under deep anaesthesia during sacrifice; **B)** excised brain and brainstem, including the pons and basilar artery, obtained from a control animal; and **C)** excised brain and brainstem following experimental subarachnoid haemorrhage induction. Representative gross anatomical images demonstrating tissue collection are presented.

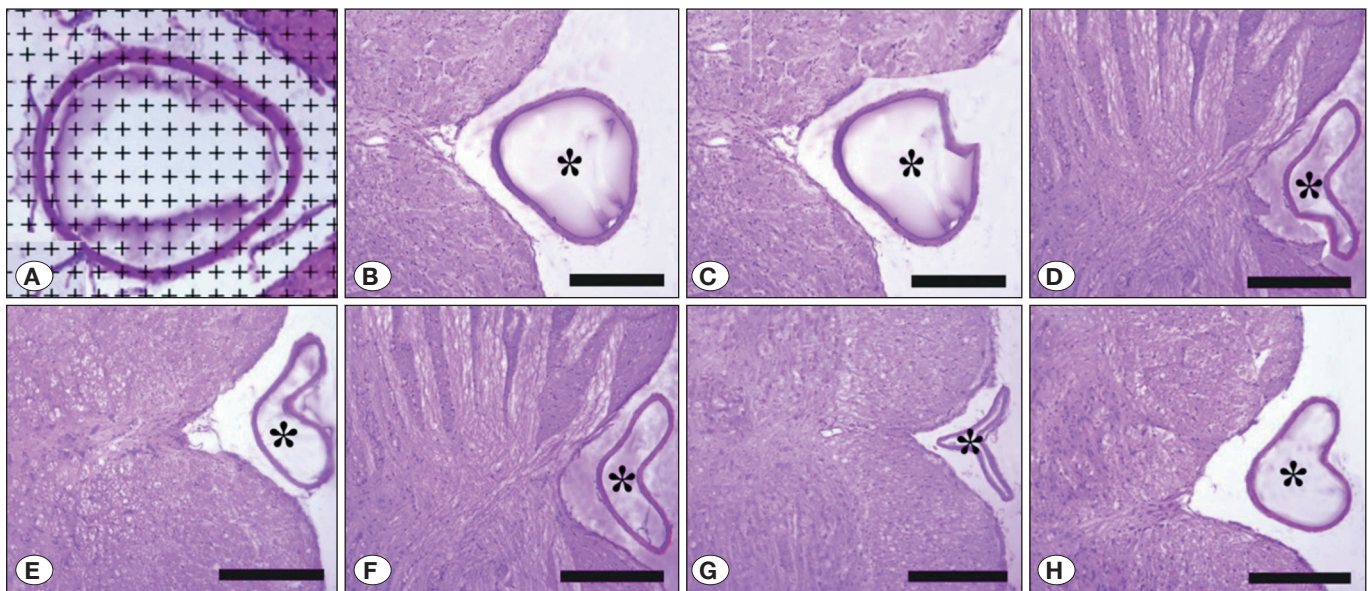


Figure 3: Stereological assessment and representative histological sections of the basilar artery. **A)** Schematic illustration of luminal area and tunica media area estimation using the Cavalieri principle with a point-counting grid. Representative haematoxylin and eosin-stained basilar artery sections from the experimental groups: **B)** Control; **C)** Sham + Vehicle (3 d); **D)** SAH; **E)** SAH + Vehicle (3 d); **F)** SAH + Thymoquinone (3 d); **G)** SAH + Vehicle (8 d); and **H)** SAH + Thymoquinone (8 d). Each group consisted of six animals ($n=6$). The asterisk indicates the basilar artery lumen. Scale bar=250 μm .

logical techniques by investigators blinded to group allocation. To minimise measurement bias, all stereological assessments were performed by the same investigator, and repeated measurements were conducted on randomly selected sections to confirm intra-observer consistency.

Stereology

The section sampling fraction for the basilar artery was set at 1/4. The first section was selected at random, and every fourth consecutive section was included in the analysis. Stereological evaluations were performed using a dedicated analysis workstation (Stereoinvestigator 9.0; MicroBrightField, Colchester, USA).

Basilar artery luminal area and tunica media area were estimated in the x-y plane using a point-counting method based on the Cavalieri principle (2,13). Systematic random sampling was applied throughout the analysis, and all measurements were performed by an investigator blinded to group allocation, as illustrated in Figure 3.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics software (version 21.0; IBM Corp., Armonk, NY, USA). Continuous variables are presented as mean $\pm$ standard deviation (SD). Data normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated using Levene's test.

For variables meeting the assumptions of normality and homogeneity of variance, intergroup comparisons were performed using one-way analysis of variance (ANOVA), followed by appropriate post hoc analyses when a significant overall effect was detected. For variables not satisfying these assumptions, intergroup comparisons were conducted using the Kruskal–Wallis test. When a significant difference was identified, pairwise comparisons were performed using the Mann–Whitney U test with Bonferroni correction for multiple comparisons.

Given the 21 pairwise comparisons among the seven experimental groups, the adjusted threshold for statistical significance was set at $p < 0.00238$. For analyses not involving multiple comparisons, a p value < 0.05 was considered statistically significant. The sample size per group was selected based on previous experimental studies using similar subarachnoid haemorrhage models and morphometric endpoints. A formal a priori power calculation was not performed because this was an exploratory stereology-based study, and group sizes were determined based on precedent in comparable experimental SAH morphometric designs and ethical/practical constraints inherent to animal research. In addition to the global Kruskal–Wallis analysis, a limited set of pre-specified primary comparisons focused on the delayed-phase hypothesis (SAH + Vehicle day 8 vs SAH + TQ day 8). These comparisons were evaluated separately from the full set of exploratory pairwise tests, which were adjusted using Bonferroni correction.

■ RESULTS

Baseline Characteristics

No statistically significant differences were observed among the experimental groups with respect to baseline age or body weight. All animals included in the study were male. Evaluation of the control and sham groups at the early time point showed no statistically significant differences in baseline physiological or morphometric parameters (Table II).

Neurological Deficit Scores

The distribution of neurological deficit scores differed significantly among the experimental groups (Kruskal–Wallis test, $p < 0.001$), as shown in Figure 4. Post hoc pairwise comparisons performed using the Mann–Whitney U test with Bonferroni correction demonstrated that the SAH + Vehicle group evaluated on day 8 exhibited significantly lower neurological scores compared with both the control group and the thymoquinone-treated SAH group evaluated at the same time point (adjusted $p = 0.002165$ for both comparisons).

In contrast, no statistically significant difference was observed between thymoquinone-treated and untreated SAH groups during the early phase (day 3) (adjusted $p > 0.00238$). Descriptive statistics for neurological deficit scores are provided in Table III.

Cerebral Blood Flow (CBF)

Cerebral blood flow (CBF) values differed significantly among the experimental groups (Kruskal–Wallis test, $p < 0.001$), as illustrated in Figure 5. In the SAH + Vehicle group evaluated on day 8, relative CBF values were markedly reduced.

Post hoc analyses indicated that relative CBF in this group was significantly lower than that observed in both the control group (adjusted $p = 0.0013$) and the thymoquinone-treated SAH group evaluated on day 8 (adjusted $p = 0.0018$). By contrast, no statistically significant difference in relative CBF was detected between treated and untreated SAH groups during the early phase (day 3) ($p > 0.05$). Descriptive CBF data are presented in Table IV.

Basilar Artery Luminal Area

Basilar artery luminal area values differed significantly among the experimental groups (Kruskal–Wallis test, $H = 26.32$, $df = 6$, $p < 0.001$), with a very large effect size ($\eta^2 = 0.89$), as shown

Table II: Baseline Characteristics of Animals in the Experimental Groups

Group	n	Body weight (g), mean±SD	Age (months), mean±SD
Control	6	285±13	3.1±0.4
Sham + Vehicle (3 d)	6	282±11	3.0±0.3
SAH	6	287±12	3.1±0.3
SAH + Vehicle (3 d)	6	284±10	3.0±0.3
SAH + TQ (3 d)	6	286±14	3.1±0.2
SAH + Vehicle (8 d)	6	283±12	3.0±0.3
SAH + TQ (8 d)	6	285±11	3.1±0.4

Values are presented as mean±standard deviation (SD). Sham and control groups were evaluated only at day 3 to reduce animal use. SAH: Subarachnoid haemorrhage; TQ: Thymoquinone; d: Days.

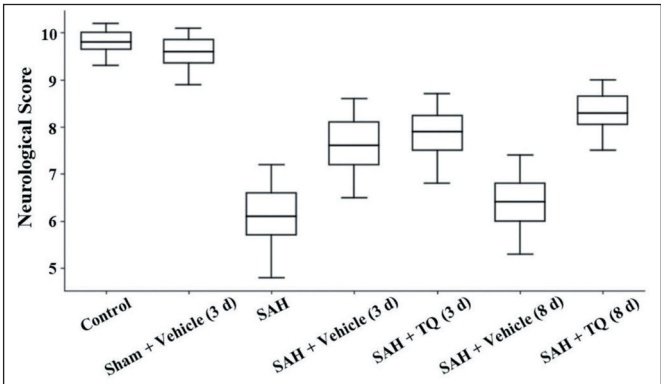


Figure 4: Neurological deficit scores across experimental groups. Box-and-whisker plots illustrating neurological deficit scores assessed on days 3 and 8 following subarachnoid haemorrhage. The central line represents the median, boxes indicate the interquartile range (25th–75th percentiles), and whiskers denote minimum and maximum values. Each group consisted of six animals ($n = 6$). Intergroup comparisons were performed using the Kruskal–Wallis test followed by Mann–Whitney U test with Bonferroni correction. Statistical significance was set at $p < 0.00238$. SAH: Subarachnoid haemorrhage.

Table III: Neurological Deficit Scores Across Experimental Groups

Group	Neurological deficit score (mean±SD; median (IQR))
Control	9.8±0.4; 9.8 (9.65–10.00)
Sham + Vehicle (3 d)	9.6±0.5; 9.6 (9.35–9.85)
SAH	6.1±0.9; 6.1 (5.70–6.60)
SAH + Vehicle (3 d)	7.6±0.8; 7.6 (7.20–8.10)
SAH + TQ (3 d)	7.9±0.7; 7.9 (7.50–8.25)
SAH + Vehicle (8 d)	6.4±0.8; 6.4 (6.00–6.80)
SAH + TQ (8 d)	8.3±0.6; 8.3 (8.05–8.65)

Data are presented as mean±standard deviation (SD) and median (interquartile range, IQR). Each group consisted of six animals (n=6). **SAH:** Subarachnoid haemorrhage; **TQ:** Thymoquinone; **d:** Days.

Table IV: Relative Cerebral Blood Flow (CBF) Values Across Experimental Groups

Group	Measurement Day	Relative CBF (% of baseline), mean±SD; median (IQR)
Control	3	99.8±4.5; 99.9 (97.25–102.35)
Sham + Vehicle (3 d)	3	96.8±5.2; 96.8 (94.65–100.05)
SAH	3	58.4±7.8; 58.4 (54.75–63.05)
SAH + Vehicle (3 d)	3	60.1±8.0; 60.1 (56.65–65.00)
SAH + TQ (3 d)	3	62.3±7.5; 62.3 (58.15–66.75)
SAH + Vehicle (8 d)	8	59.6±7.9; 59.6 (56.05–63.75)
SAH + TQ (8 d)	8	69.5±6.8; 69.5 (65.80–72.90)

Data are presented as mean±standard deviation (SD) and median (interquartile range, IQR). Each group consisted of six animals (n=6). **CBF:** Cerebral blood flow, **SAH:** Subarachnoid haemorrhage, **TQ:** Thymoquinone, **d:** days.

in Figure 6. Descriptive statistics for each group (mean±standard deviation) are provided in Table V.

Pairwise comparisons performed using the Mann–Whitney U test with Bonferroni correction demonstrated that basilar artery luminal area was significantly reduced in the SAH + Vehicle group evaluated on day 8 compared with the control group (adjusted p=0.002165). In addition, basilar artery luminal area was significantly greater in the SAH + Thymoquinone group than in the SAH + Vehicle group evaluated on day 8 (adjusted p=0.002165).

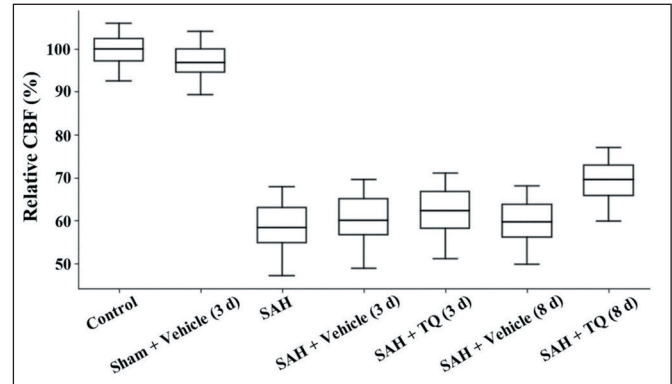


Figure 5: Relative cerebral blood flow (CBF) across experimental groups. Box-and-whisker plots showing relative cerebral blood flow values measured on days 3 and 8 and expressed as a percentage of baseline (pre-subarachnoid haemorrhage) values. The median is indicated by the central line, boxes represent the interquartile range, and whiskers denote minimum and maximum values. Each group consisted of six animals (n=6). Statistical analysis was performed using the Kruskal–Wallis test followed by Mann–Whitney U tests with Bonferroni correction.

SAH: Subarachnoid haemorrhage; **CBF:** Cerebral blood flow.

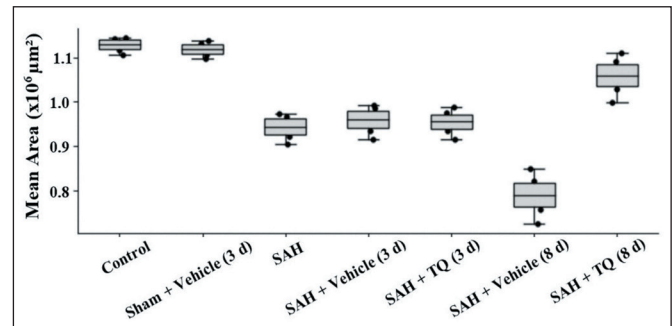


Figure 6: Basilar artery luminal area across experimental groups. Box-and-whisker plots illustrating basilar artery luminal area measurements obtained on days 3 and 8 following subarachnoid haemorrhage. Boxes represent the interquartile range with median values, whiskers indicate minimum and maximum values, and individual points represent single animals. Each group consisted of six animals (n=6). Intergroup comparisons were performed using the Kruskal–Wallis test followed by Mann–Whitney U tests with Bonferroni correction.

SAH: Subarachnoid haemorrhage.

No significant difference in luminal area was detected between the SAH + Vehicle and SAH + Thymoquinone groups evaluated during the early phase (day 3) (adjusted p=0.699134). Additionally, a significant increase in luminal area was observed between the SAH + Thymoquinone groups evaluated on days 3 and 8 (adjusted p=0.002165). Detailed results of post hoc pairwise comparisons are presented in Table VI.

Basilar Artery Tunica Media Area

No statistically significant differences were detected among the experimental groups with respect to basilar artery tunica

Table V: Basilar Artery Luminal Area Across Experimental Groups

Group	n	Basilar artery luminal area ($\times 10^6 \mu\text{m}^2$), mean $\pm$ SD
Control	6	1.1276 $\pm$ 0.0171
Sham + Vehicle (3 d)	6	1.1183 $\pm$ 0.0170
SAH	6	0.9427 $\pm$ 0.0284
SAH + Vehicle (3 d)	6	0.9584 $\pm$ 0.0328
SAH + TQ (3 d)	6	0.9538 $\pm$ 0.0300
SAH + Vehicle (8 d)	6	0.7897 $\pm$ 0.0463
SAH + TQ (8 d)	6	1.0577 $\pm$ 0.0446

Values are presented as mean $\pm$ standard deviation (SD). Luminal area values are expressed as $\times 10^6 \mu\text{m}^2$. Each group consisted of six animals (n=6). **SAH:** Subarachnoid haemorrhage, **TQ:** Thymoquinone, **d:** Days.

Table VI: Post Hoc Pairwise Comparisons of Basilar Artery Luminal Area Following Bonferroni Correction

Comparison	Adjusted p-value (Bonferroni)
Control vs SAH + Vehicle (8 d)	0.002165
SAH + Vehicle (3 d) vs SAH + Vehicle (8 d)	0.002165
SAH + Vehicle (3 d) vs SAH + TQ (3 d)	0.699134
SAH + Vehicle (8 d) vs SAH + TQ (8 d)	0.002165
SAH + TQ (3 d) vs SAH + TQ (8 d)	0.002165

Pairwise comparisons were performed using the Mann–Whitney U test with Bonferroni correction following a significant Kruskal–Wallis test. The adjusted significance threshold was set at $p < 0.00238$.

SAH: Subarachnoid haemorrhage, **TQ:** Thymoquinone, **d:** Days.

Table VII: Basilar Artery Tunica Media Area Across Experimental Groups

Group	n	Basilar artery tunica media area (μm^2), mean $\pm$ SD
Control	6	288.017 $\pm$ 13.364
Sham + Vehicle (3 d)	6	281.450 $\pm$ 9.787
SAH	6	277.583 $\pm$ 8.794
SAH + Vehicle (3 d)	6	275.650 $\pm$ 8.899
SAH + TQ (3 d)	6	280.667 $\pm$ 7.380
SAH + Vehicle (8 d)	6	275.833 $\pm$ 8.066
SAH + TQ (8 d)	6	277.833 $\pm$ 8.128

Values are presented as mean $\pm$ standard deviation (SD). Each group consisted of six animals (n=6). No statistically significant differences were detected among groups (one-way ANOVA, $p=0.294$).

SAH: Subarachnoid haemorrhage, **TQ:** Thymoquinone, **d:** Days.

media area (one-way ANOVA, $p=0.294$), indicating comparable medial thickness across groups. Corresponding descriptive data are provided in Table VII.

DISCUSSION

In the present study, we quantitatively demonstrate that thymoquinone exerts a time-dependent neurovascular effect on basilar artery morphology following experimental subarachnoid haemorrhage (SAH). Using unbiased stereological analysis, we show that prolonged thymoquinone administration attenuates delayed basilar artery luminal narrowing and is associated with partial preservation of cerebral blood flow, whereas short-term treatment does not produce a measurable morphometric effect. These findings indicate that treatment duration and temporal alignment with the evolution of vasospasm, rather than drug exposure alone, are critical determinants of the vascular response to thymoquinone. Importantly, by applying unbiased stereology, our study provides one of the first quantitative demonstrations that the vascular effect of TQ after SAH is duration-dependent, and that short-term administration is insufficient to induce a detectable morphometric benefit.

Basilar artery luminal area differed significantly among experimental groups, with pronounced luminal narrowing observed in the SAH + Vehicle group evaluated during the delayed phase (day 8). In contrast, animals receiving thymoquinone throughout this period exhibited a significant attenuation of luminal narrowing. The observation that control animals consistently displayed the largest basilar artery luminal areas supports the notion that basilar artery morphology remains stable under physiological conditions. Prior experimental studies similarly suggest that basilar artery morphology remains stable over comparable timeframes in the absence of SAH; however, baseline measurements at day 8 were not directly obtained in the present study. Therefore, the luminal narrowing observed following SAH most plausibly reflects progressive neurovascular dysfunction. Experimental and translational studies have implicated inflammatory signaling, oxidative stress, endothelial dysfunction, and altered vasoactive mediator balance as key contributors to delayed cerebral vasospasm (3,7).

Within this pathophysiological framework, the absence of significant changes in basilar artery tunica media area across experimental groups suggests that luminal narrowing in the present model is mediated predominantly by functional vascular mechanisms, rather than by permanent structural remodeling of the vessel wall. This interpretation is consistent with previous experimental observations indicating that delayed vasospasm is largely driven by dynamic alterations in vascular tone and endothelial function (5,23,26,31,32).

Previous experimental studies investigating thymoquinone in SAH models have primarily employed oral administration at doses of approximately 10 mg/kg/day, with outcome measures focused mainly on biochemical markers, histopathological injury, or neurological function (6,9,20). While these studies have consistently highlighted the antioxidant and anti-inflammatory properties of thymoquinone, the relationship between

treatment duration and vascular morphology has received limited attention. In this context, the present study extends existing knowledge by demonstrating that prolonged administration encompassing the delayed phase of vasospasm is required to elicit a measurable morphometric benefit at the level of the basilar artery.

Cerebral vasospasm following subarachnoid haemorrhage typically emerges around the third day and reaches peak severity between days 7 and 10 (22,24,30). Within this established temporal profile, the absence of a significant early-phase effect observed in the present study appears biologically plausible. This pattern suggests that thymoquinone preferentially modulates vascular processes developing during the delayed phase of vasospasm, rather than mechanisms associated with the immediate post-haemorrhagic period or acute early brain injury (24,30).

The association between prolonged thymoquinone administration and partial preservation of cerebral blood flow during the delayed phase further supports the haemodynamic relevance of the morphometric findings. The concordance between basilar artery luminal area and cerebral perfusion suggests that attenuation of delayed luminal narrowing is accompanied by functionally meaningful alterations in cerebral haemodynamics. The observed preservation of cortical CBF should therefore be interpreted as a haemodynamic correlate consistent with improved vascular conditions rather than as a direct measure of basilar artery blood flow.

By contrast, the limited improvement observed in neurological deficit scores is consistent with the multifactorial nature of neurological dysfunction following subarachnoid haemorrhage, in which vascular alterations represent only one of several contributing mechanisms. In addition to large-artery vasospasm, early brain injury, microcirculatory impairment, blood-brain barrier disruption, neuroinflammation, and cortical spreading depolarizations may contribute to delayed neurological deficits and poor functional recovery. Consequently, attenuation of basilar artery luminal narrowing and partial preservation of CBF may not translate proportionally into neurological score improvement, particularly in models where secondary injury pathways remain active (22,36).

Although previous studies have demonstrated that thymoquinone modulates nitric oxide bioavailability, endothelin-1 expression, and inflammatory cytokine release, these molecular pathways were not directly assessed in the present study (6,16,28). A parsimonious working hypothesis is that TQ exerts cumulative endothelial and redox-modulating effects that require sustained exposure to influence the delayed vasospasm milieu. Given the time-dependent evolution of oxidative stress, inflammatory signaling, and vasoactive mediator imbalance after SAH, prolonged administration may be necessary to restore nitric oxide bioavailability, dampen endothelin-driven vasoconstrictive tone, and reduce oxidative endothelial dysfunction sufficiently to yield a detectable morphometric benefit. This framework is consistent with the observed lack of early-phase effect and the preferential benefit during the delayed phase. Accordingly, the findings should be interpreted as structural, haemodynamic, and functional ev-

idence supporting a time-dependent neurovascular effect of thymoquinone, rather than as a comprehensive mechanistic characterization of its molecular actions.

Several limitations of the present study should be acknowledged. The analysis relied primarily on morphometric outcomes, and biochemical markers of oxidative stress or inflammation were not assessed. In addition, direct angiographic confirmation of vasospasm was not performed. Control and sham groups were evaluated only at day 3 to reduce animal use, representing a methodological limitation. Although control and sham animals were not evaluated at day 8 to reduce animal use, prior experimental literature indicates that basilar artery morphology remains stable over comparable timeframes in the absence of SAH; therefore, the delayed luminal narrowing observed here is most plausibly attributable to SAH-related pathophysiology rather than temporal baseline drift (33,35). Nevertheless, the application of unbiased stereological methods represents an important methodological strength, enhancing the objectivity and reproducibility of the morphometric measurements.

Overall, the present findings demonstrate that prolonged thymoquinone administration preserves basilar artery luminal area and is associated with partial maintenance of cerebral perfusion during the delayed phase of experimental vasospasm (6,9). These results support a time-dependent neurovascular effect of thymoquinone in experimental subarachnoid haemorrhage. Further studies incorporating dose-response analyses, alternative routes of administration, and molecular assessments are warranted to clarify the mechanisms underlying these observations and to further define the therapeutic potential of thymoquinone (27,29).

■ CONCLUSION

This study demonstrates that intraperitoneal thymoquinone administration exerts duration-dependent neurovascular effects in an experimental subarachnoid haemorrhage model. Prolonged treatment for eight days, encompassing the delayed phase of vasospasm, attenuated delayed basilar artery luminal narrowing and was associated with partial preservation of cerebral blood flow, whereas short-term administration did not produce a measurable morphometric effect. These findings indicate that temporal alignment of treatment with vasospasm development is a critical determinant of the vascular response to thymoquinone. Further experimental studies incorporating dose-response analyses, alternative routes of administration, and molecular assessments are warranted to clarify the underlying mechanisms and better define the neurovascular actions of thymoquinone.

Declarations

Funding: This study was supported by an institutional scientific research funding program.

Availability of data and materials: The data supporting the findings of this study are available from the corresponding author upon reasonable request. Due to ethical considerations related to animal experimentation, raw data are not publicly available.

Disclosure: The authors declare that there are no conflicts of interest regarding this study.

AUTHORSHIP CONTRIBUTION

Study conception and design: CA, MEO, AHM
Data collection: CA, AHM
Analysis and interpretation of results: CC, MEO
Draft manuscript preparation: CA, MAE
Critical revision of the article: MAE, CC, AHM
Other (study supervision, fundings, materials, etc.): AHM
All authors (CA, MAE, CC, MEO, AHM) reviewed the results and approved the final version of the manuscript.

REFERENCES

- Akkaya E, Evran Ş, Çalış F, Çevik S, Katar S, Karataş E, Koçyiğit A, Sağlam MY, Hatiboğlu MA, Hanımoğlu H: Thymoquinone ameliorates delayed cerebral injury and cerebral vasospasm secondary to experimental subarachnoid haemorrhage. *Neurol Neurochir Pol* 54:576-584, 2020. <https://doi.org/10.5603/PJNNS.a2020.0090>
- Altunkaynak BZ, Altunkaynak E, Unal D, Unal B: A novel application for the cavalieri principle: a stereological and methodological study. *Eurasian J Med* 41:99, 2009.
- Cahill WJ, Calvert JH, Zhang JH: Mechanisms of early brain injury after subarachnoid hemorrhage. *J Cereb Blood Flow Metab* 26:1341-1353, 2006. <https://doi.org/10.1038/sj.jcbfm.9600283>
- Chan AY, Choi EH, Yuki I, Suzuki S, Golshani K, Chen JW, Hsu FP: Cerebral vasospasm after subarachnoid hemorrhage: developing treatments. *Brain Hemorrhages* 2:15-23, 2021. <https://doi.org/10.1016/j.heest.2020.08.003>
- Crowley RW, Medel R, Dumont AS, Ilodigwe D, Kassell NF, Mayer SA, Ruefenacht D, Schmiedek P, Weidauer S, Pasqualin A: Angiographic vasospasm is strongly correlated with cerebral infarction after subarachnoid hemorrhage. *Stroke* 42:919-923, 2011. <https://doi.org/10.1161/STROKEAHA.110.597005>
- Darakhshan S, Pour AB, Colagar AH, Sisakhtnezhad S: Thymoquinone and its therapeutic potentials. *Pharmacol Res* 95:138-158, 2015. <https://doi.org/10.1016/j.phrs.2015.03.011>
- Dodd WS, Laurent D, Dumont AS, Hasan DM, Jabbour PM, Starke RM, Hosaka K, Polifka AJ, Hoh BL, Chalouhi N: Pathophysiology of delayed cerebral ischemia after subarachnoid hemorrhage: a review. *J Am Heart Assoc* 10:e021845, 2021. <https://doi.org/10.1161/jaha.121.021845>
- Etminan N, Chang H-S, Hackenberg K, De Rooij NK, Vergouwen MD, Rinkel GJ, Algra A: Worldwide incidence of aneurysmal subarachnoid hemorrhage according to region, time period, blood pressure, and smoking prevalence in the population: a systematic review and meta-analysis. *JAMA Neurol* 76:588-597, 2019. <https://doi.org/10.1001/jamaneurol.2019.0006>
- Farkhondeh T, Samarghandian S, Shahri AMP, Samini F: The neuroprotective effects of thymoquinone: a review. *Dose-response* 16:1559325818761455, 2018. <https://doi.org/10.1177/1559325818761455>
- Garcia JH, Wagner S, Liu K-F, Hu Xj: Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats: statistical validation. *Stroke* 26:627-635, 1995. <https://doi.org/10.1161/01.str.26.4.627>
- Gökçe EC, Kahveci R, Gökçe A, Cemil B, Aksoy N, Sargon MF, Kısa Ü, Erdoğan B, Güvenç Y, Alagöz F: Neuroprotective effects of thymoquinone against spinal cord ischemia-reperfusion injury by attenuation of inflammation, oxidative stress, and apoptosis. *J Neurosurg Spine* 24:949-959, 2016. <https://doi.org/10.3171/2015.10.spine15612>
- Guan D, Li Y, Peng X, Zhao H, Mao Y, Cui Y: Thymoquinone protects against cerebral small vessel disease: Role of antioxidant and anti-inflammatory activities. *J Biol Regul Homeost Agents* 32:225-231, 2018.
- Gundersen H, Bendtsen TF, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard J, Pakkenberg B, Sørensen FB, Vesterby A: Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS* 96:379-94, 1988. <https://doi.org/10.1111/j.1699-0463.1988.tb05320.x>
- Hosseinzadeh H, Parvardeh S, Asl MN, Sadeghnia HR, Ziaee T: Effect of thymoquinone and Nigella sativa seeds oil on lipid peroxidation level during global cerebral ischemia-reperfusion injury in rat hippocampus. *Phytomedicine* 14:621-627, 2007. <https://doi.org/10.1016/j.phymed.2006.12.005>
- Idris-Khodja N, Schini-Kerth V: Thymoquinone improves aging-related endothelial dysfunction in the rat mesenteric artery. *Naunyn Schmiedeberg's Arch Pharmacol* 385:749-758, 2012. <https://doi.org/10.1007/s00210-012-0749-8>
- Isaev NK, Genrikhs EE, Stelmashook EV: Antioxidant thymoquinone and its potential in the treatment of neurological diseases. *Antioxidants* 12:433, 2023. <https://doi.org/10.3390/antiox12020433>
- Karadağ MK, Kadioğlu HH: Nigella sativa ekstresinin CAS:490-91-5 deneysel subaraknoid kanamaya bağlı vasküler değişiklikler üzerine olan etkisinin ışı mikroskopik ve stereolojik incelenmesi. *Atatürk Univ Fac Med J Surg Med Sci* 1:29-39, 2018.
- Kassell N, Sasaki T, Colohan A, Nazar G: Cerebral vasospasm following aneurysmal subarachnoid hemorrhage. *Stroke* 16:562-572, 1985. <https://doi.org/10.1161/01.str.16.4.562>
- Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG: The ARRIVE guidelines animal research: reporting in vivo experiments. *PLoS Biol* 8:e1000412, 2010. <https://doi.org/10.1371/journal.pbio.1000412>
- Lu Y, Feng Y, Liu D, Zhang Z, Gao K, Zhang W, Tang H: Thymoquinone attenuates myocardial ischemia/reperfusion injury through activation of SIRT1 signaling. *Cell Physiol Biochem* 47:1193-1206, 2018. <https://doi.org/10.1159/000490216>
- Lucke-Wold BP, Logsdon AF, Manoranjan B, Turner RC, McConnell E, Vates GE, Huber JD, Rosen CL, Simard JM: Aneurysmal subarachnoid hemorrhage and neuroinflammation: a comprehensive review. *Int J Mol Sci* 17:497, 2016. <https://doi.org/10.3390/ijms17040497>
- Macdonald RL: Delayed neurological deterioration after subarachnoid haemorrhage. *Nat Rev Neurol* 10:44-58, 2014. <https://doi.org/10.1038/nrneurol.2013.246>
- Macdonald RL, Weir B: A review of hemoglobin and the pathogenesis of cerebral vasospasm. *Stroke* 22:971-982, 1991. <https://doi.org/10.1161/01.str.22.8.971>

24. Marques IP, de Albuquerque CRC, de Oliveira Souza NV, de Andrade JBC, Silva GS, Kurtz P: Delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage: a narrative review. *Arq Neuropsiquiatr* 83:1-14, 2025. <https://doi.org/10.1055/s-0045-1809885>
25. Mehri S, Shahi M, Razavi BM, Hassani FV, Hosseinzadeh H: Neuroprotective effect of thymoquinone in acrylamide-induced neurotoxicity in Wistar rats. *Iran J Basic Med Sci* 17:1007, 2014.
26. Pluta RM, Hansen-Schwartz J, Dreier J, Vajkoczy P, Macdonald RL, Nishizawa S, Kasuya H, Wellman G, Keller E, Zauner A: Cerebral vasospasm following subarachnoid hemorrhage: time for a new world of thought. *Neurol Res* 31:151-158, 2009. <https://doi.org/10.1179/174313209x393564>
27. Pottot FH, Ibrahim AM, Alammari A, Alsinan R, Aleid M, Alshehri A, Alshehri M, Mishra S, Alhajri N: Thymoquinone: review of its potential in the treatment of neurological diseases. *Pharmaceuticals* 15:408, 2022. <https://doi.org/10.3390/ph15040408>
28. Saadat M, Dahmardeh N, Sheikhabaei F, Mokhtari T: Therapeutic potential of thymoquinone and its nanoformulations in neuropsychological disorders: a comprehensive review on molecular mechanisms in preclinical studies. *Naunyn-Schmiedeberg's Arch Pharmacol* 397:3541-3564, 2024. <https://doi.org/10.1007/s00210-023-02832-8>
29. Samarghandian S, Farkhondeh T, Samini F: A review on possible therapeutic effect of *Nigella sativa* and thymoquinone in neurodegenerative diseases. *CNS Neurol Disord Drug Targets* 17:412-420, 2018. <https://doi.org/10.2174/1871527317666180702101455>
30. Schmidt TP, Weiss M, Hoellig A, Nikoubashman O, Schulze-Steinen H, Albanna W, Clusmann H, Schubert GA, Velde-man M: Revisiting the timeline of delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage: toward a temporal risk profile. *Neurocrit Care* 37:735-743, 2022. <https://doi.org/10.1007/s12028-022-01545-9>
31. Sehba FA, Bederson JB: Mechanisms of acute brain injury after subarachnoid hemorrhage. *Neurol Res* 28:381-398, 2006. <https://doi.org/10.1179/016164106x114991>
32. Sehba FA, Friedrich V: Early events after aneurysmal subarachnoid hemorrhage. *Neurovascular Events After Subarachnoid Hemorrhage: Towards Experimental and Clinical Standardisation*:23-28, 2014. https://doi.org/10.1007/978-3-319-04981-6_4
33. Sobey CG, Heistad DD, Faraci FM: Effect of subarachnoid hemorrhage on dilatation of rat basilar artery in vivo. *Am J Physiol Heart Circ Physiol* 271:H126-H132, 1996. <https://doi.org/10.1152/ajpheart.1996.271.1.H126>
34. Suarez JI, Tarr RW, Selman WR: Aneurysmal subarachnoid hemorrhage. *N Engl J Med* 354:387-396, 2006. <https://doi.org/10.1056/nejmra052732>
35. Vatter H, Weidauer S, Konczalla J, Dettmann E, Zimmermann M, Raabe A, Preibisch C, Zanella FE, Seifert V: Time course in the development of cerebral vasospasm after experimental subarachnoid hemorrhage: clinical and neuroradiological assessment of the rat double hemorrhage model. *Neurosurgery* 58:1190-1197, 2006. <https://doi.org/10.1227/01.NEU.0000199346.74649.66>
36. Weir B: The pathophysiology of cerebral vasospasm. *Br J Neurosurg* 9:375-390, 1995. <https://doi.org/10.1080/02688699550041386>
37. Woo CC, Kumar AP, Sethi G, Tan KHB: Thymoquinone: potential cure for inflammatory disorders and cancer. *Biochem Pharmacol* 83:443-451, 2012. <https://doi.org/10.1016/j.bcp.2011.09.029>