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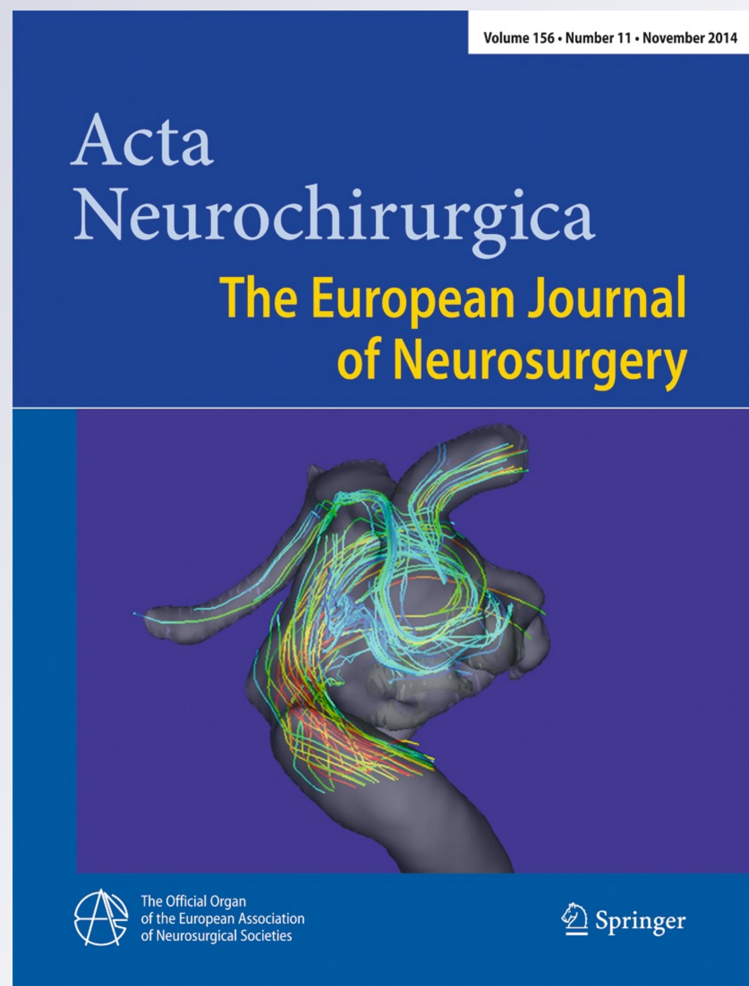

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EXPERIMENTAL RESEARCH - VASCULAR

Attenuation of cerebral vasospasm and secondary injury by testosterone following experimental subarachnoid hemorrhage in rabbit

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Abstract

Background The vasodilatator effects of testosterone have been widely studied and demonstrated. Based on previous studies of these vasodilatatory activities, we hypothesized that testosterone might have potential effects on subarachnoid hemorrhage-induced cerebral vasospasm.

Methods Thirty-two adult male New Zealand white rabbits were randomly divided into four groups of eight rabbits in each group: group 1 (control); group 2 (subarachnoid hemorrhage); group 3 (subarachnoid hemorrhage + vehicle); and group 4 (subarachnoid hemorrhage + testosterone). Testosterone (15 mg/kg, intraperitoneally) was administered 5 min after the intracisternal blood injection and continued for 72 h once per day in the same dose for group 4. Animals were killed 72 h after subarachnoid hemorrhage. Basilar artery cross-sectional areas, arterial wall thicknesses, and hippocampal degeneration scores were evaluated in all groups.

Results Intraperitoneal administration of testosterone was found to attenuate cerebral vasospasm and provide neuroprotection after subarachnoid hemorrhage in rabbits. Testosterone treatment was determined to be effective at increasing the luminal area and reducing the wall thickness of the basilar artery.

Conclusions Our findings show that testosterone has some preventive effects on SAH-induced vasospasm and secondary neuronal injury in rabbits. We propose that the vasodilatatory activity of testosterone is due to its effects on inhibiting calcium channels, activating potassium channels, augmenting nitric oxide synthesis, and inhibiting oxidant stress and inflammation.

Keywords Rabbit · Subarachnoid hemorrhage · Testosterone · Vasospasm

Introduction

Cerebral vasospasm is the leading cause of poor outcome and death, adversely affecting more than one in five of all patients suffering from subarachnoid hemorrhage (SAH). About 70 % of patients may develop arterial narrowing but only 20–30 % will manifest neurological deficit [1, 2]. Developments in the treatment regimens of vasospasm during the last three decades have resulted in a definite reduction of morbidity and mortality; from 25–30 % in the 1970s to 15–20 % in the 1980s, and to the 5–10 % currently observed [2, 3]. Nonetheless, there remains an absence of consistently effective preventive and therapeutic treatments for SAH-induced cerebral vasospasm.

In addition, although experimental data suggests that free calcium (Ca^{+2})-dependent vasoconstriction plays an important role [2, 4, 5], the exact pathogenesis of this unique entity remains unclear.

Testosterone (TES), the gonadal sex steroid hormone, has a variety of physiological actions on a variety of tissues including bone, cardiovascular, reproductive, skeletal muscle, and the central nervous system (CNS) [6]. In the last two decades, vasodilatator effects of TES has been widely studied and demonstrated in various vessels, such as coronary arteries

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[7-10], thoracic aorta [10-12], mesenteric arteries [13], pulmonary artery [14, 15], radial artery [16], and basilar artery [17]. The mechanisms responsible for TES-induced vasorelaxation remain under debate [16, 18, 19]. Briefly, the vasodilatator effect of TES is mainly associated with the cell membrane ion channel function, including inactivation of Ca^{+2} channels and the activation of potassium (K^{+}) channels [8, 9, 11-18]. In addition, the roles of endothelium-derived nitric oxide (NO) and neuronal nitric oxide synthetase (nNOS) were also assumed [13, 18]. Based on these vasodilatatory activities, we hypothesized that TES might have effects on SAH-induced cerebral vasospasm.

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. Thirty-two adult male New Zealand white rabbits, weighing 2.900–3.450 g were randomly divided into the following four groups of eight rabbits:

- Group 1: Control group (n=8); 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=8); the SAH protocol was used to induce vasospasm as described below.
- Group 3: Vehicle group (n=8); cerebral vasospasm was induced by SAH protocol as described below, and the animals received a single daily intraperitoneal dose of 10 % benzoate in 1 ml olive oil starting 5 min after induction of SAH.
- Group 4: TES group (n=8); as for group 3, but rabbits received a single daily intraperitoneal dose of 15 mg/kg TES (Sustanon 250, Schering-Plough, Turkey; containing testosterone propionate 30 mg, testosterone phenylpropionate 60 mg, testosterone isocaproate 60 mg, and testosterone decanoate 100 mg). The treatment was started 5 min after the intracisternal blood injection and continued for 72 h once per day in the same dosage. This dosage of TES was selected based on past studies [20, 21].

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 by intramuscular administration of 70 mg/kg ketamine (Ketalar, Parke Davis Eczacıbaşı, Turkey) and 5 mg/kg xylazine (Rompun, Bayer, Turkey) combination. 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, where only those animals with PO_2 >70 mmHg and PCO_2 <40 mmHg were included to the study. Heart rate and arterial blood pressure were measured with the use of an ear arterial catheter. Physiological parameters of the experimental groups were 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 as well as an equal volume of fresh, non-heparinized autologous arterial blood, which was obtained from the ear artery injected into cisterna magna within 2 min. The animals were then placed in a head-down position at 30 °C 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 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. The brains were then removed and stored in formaldehyde solution at 4 °C overnight.

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Table 1 Physiological parameters of the experimental groups

Variables	Control	SAH	Vehicle	Testosterone	Statistics
pH	7.46 (0.02)	7.44 (0.02)	7.46 (0.02)	7.45 (0.02)	χ^2=3.895, p=0.273[a]
PCO_2 (mmHg)	35.7±0.82	36.1±0.85	36.5±0.74	36.3±0.76	F=1.315, p=0.289[b]
PO_2 (mmHg)	95.5±1.47	94.5±0.76	95.1±1.28	95.3±1.21	F=1.159, p=0.343[b]
MABP (mmHg)	105.5 (6.00)	105.5 (5.50)	104.5 (6.25)	106.0 (3.25)	χ^2=1.346, p=0.718[a]
HR (bpm)	167.9±4.76	164.9±3.68	163.7±5.97	165.1±4.01	F=1.115, p=0.360[b]

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

SAH subarachnoid hemorrhage, *MABP* mean arterial blood pressure, *HR* heart rate, *bpm* beats per minute

[a] Kruskal–Wallis test

[b] One-way ANOVA

Histological morphometric analysis of basilar artery

Each brainstem specimen was embedded in paraffin. The entire basilar artery was sectioned into five segments at 2 mm in length (Fig. 1), and stained with hematoxylin-eosin (H&E). The morphometric measurements on all five segments of the basilar were performed using 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 the 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 lumen and external border of muscle layer was measured at four quadrants of each segment of basilar artery. If an undulating luminal border was encountered, an extra measurement was performed 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- 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 light microscope, morphological signs of neuronal degeneration such as; neuronal shrinkage, hyperchromasia, and nuclear pyknosis

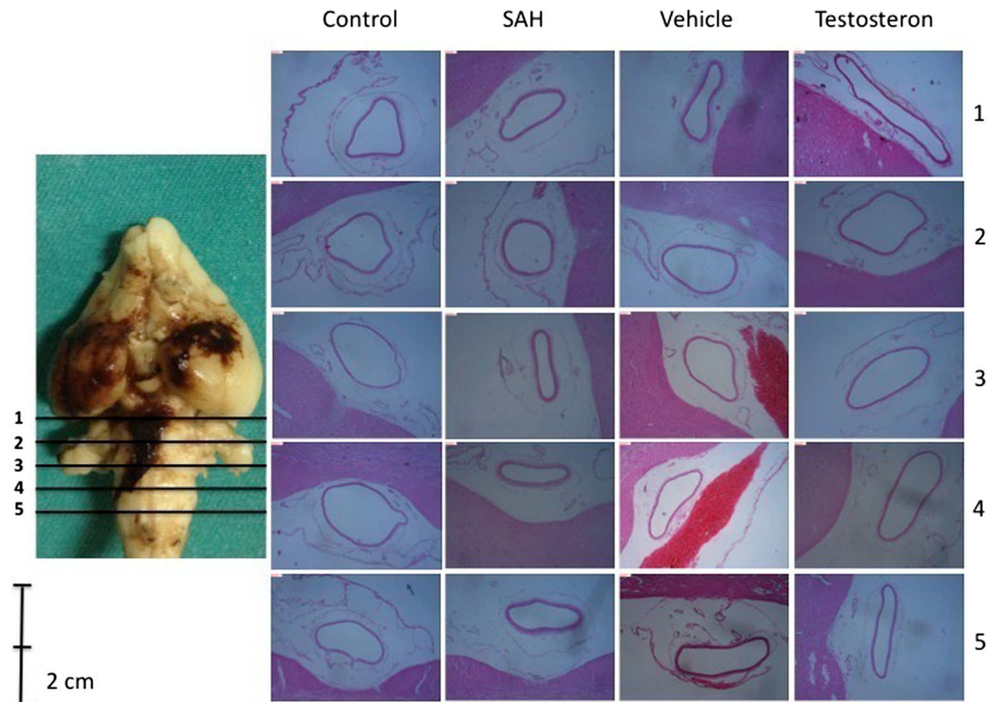

Fig. 1 Macroscopic view of a basis of the rabbit brain from the SAH group (*left*) and representative histological cross-sections of the basilar artery corresponding to all study groups (*right*, H&E, 40X obj.). *SAH* subarachnoid hemorrhage

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Table 2 Intra- 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	0.983	0.923–0.996	0.859	0.490–0.970
2nd observer	0.997	0.985–0.999	0.963	0.843–0.992
Inter-observer				
1st measurement	0.827	0.358–0.963	0.660	<0–0.921
2nd measurement	0.838	0.390–0.965	0.703	0.069–0.932

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

were evaluated. The presence and extent of neuronal degeneration were scored semi-quantitatively in the CA1, CA3, and dentate gyrus 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 [22]. 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.

Statistical analysis

Data analyses were performed using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, USA). Whether the distributions of continuous variables were normally distributed was determined by using Shapiro–Wilk test. Levene test was used for the evaluation of homogeneity of variances. The data were shown as mean ± SD, median (IQR) or median (min-max), where applicable.

The mean differences among groups were compared by one-way ANOVA, whereas the Kruskal–Wallis test was applied for comparisons of the median values. When the p values from Kruskal–Wallis test statistics were statistically significant the Conover's non-parametric multiple comparison test was used to identify which group(s) differed.

Intra-class correlation coefficient for area and wall thickness was calculated for determining both inter- and also intra-observer reliability levels. A p value <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 307,162.6±44,418.3 μm^2. In the SAH and vehicle groups, the mean basilar artery cross-sectional area decreased to 146,189.4±13,462.9 and 141,346.9±8,920.8 μm^2, respectively. These decreases were statistically significant ($p<0.001$ for both). There was no statistically significant difference between the SAH and the vehicle groups ($p=0.474$). In the TES group, the mean basilar artery cross-sectional area was 262,727.4±29,659.7 μm^2. Treatment with TES increased the cross-sectional area of the basilar artery when compared to the SAH and the vehicle groups ($p<0.001$ for both) (Fig. 2).

The mean value of the basilar artery wall thickness was 25.3±2.1 μm in the control group, 47.8±2.4 μm in the SAH group, and 48.7±1.2 μm in the vehicle group. The TES group had a mean value of 30.9±1.6 μm for the basilar artery wall thickness. When the SAH and the vehicle groups were compared to the control group, following the induction of SAH, basilar artery wall thicknesses were increased ($p<0.001$ for both). Basilar artery wall thicknesses in the TES group was smaller than in the SAH and vehicle groups ($p=0.001$ and $p<0.001$, respectively). There was no statistically significant difference between the SAH and the vehicle groups ($p=0.185$) (Fig. 3). Mean basilar artery cross-sectional areas and arterial wall thickness 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. 4a). In the SAH and vehicle groups, almost complete degeneration of the neurons was observed (Fig. 4b and c). The

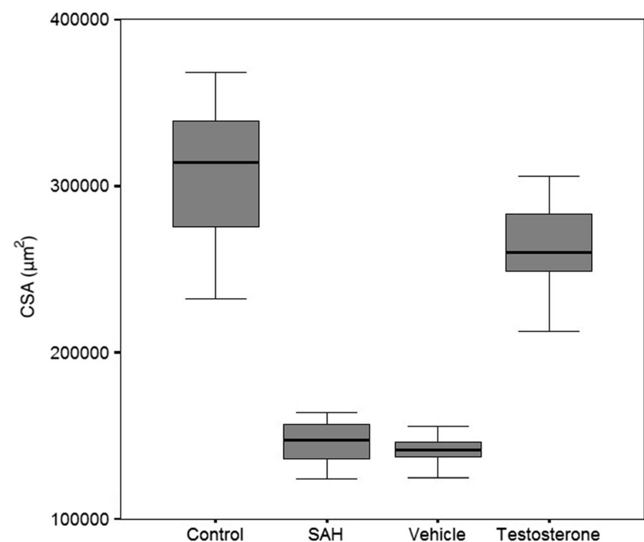

Fig. 2 Mean basilar artery cross-sectional areas of the study groups. The *horizontal lines* in the middle of each box indicate the median, while the *top and bottom borders* of the box mark the 25th and 75th percentiles, respectively. The *whiskers* above and below the box mark indicate the maximum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

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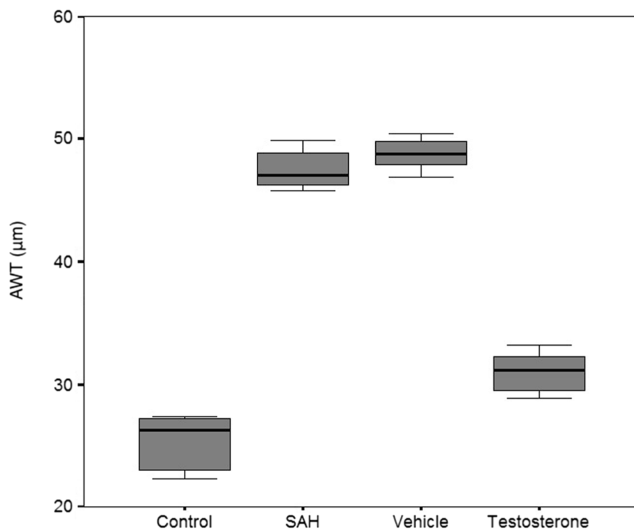

Fig. 3 Mean basilar artery wall thicknesses of the study groups. The *horizontal lines* in the middle of each box indicate the median, while the *top and bottom borders* of the box mark the 25th and 75th percentiles, respectively. The *whiskers* above and below the box mark indicate the maximum and minimum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

TES group revealed better pathological appearance of the CA1, CA3, and the dentate gyrus, where few degenerated neurons with hyperchromasia and nuclear pyknosis were noticed (Fig. 4d).

The mean degeneration score for the control group was 3.7±0.8; the mean degeneration scores for the SAH and the vehicle groups were 11.2±0.8 and 10.8±0.8, respectively; and the mean degeneration score for the TES group was 7.6±0.7. The difference between the control, and the SAH and the vehicle groups were statistically significant ($p<0.001$ for both). The mean degeneration scores were significantly lower in the TES group when compared with the SAH and the vehicle groups ($p<0.001$ for both). Pathological examinations of the hippocampus are summarized in Table 4.

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

Group	CSA (μm^2)	AWT (μm)
Control	314,317.2 (70,374.2)^{a,b}	26.2 (4.2)^{a,b,c}
SAH	147,626.7 (22,913.8)^{a,d}	47.0 (3.3)^{a,d}
Vehicle	141,675.9 (10,127.8)^{b,e}	48.8 (2.1)^{b,e}
Testosterone	259,834.2 (45,004.3)^{d,e}	31.1 (3.2)^{d,e,f}
Statistics	χ^2=24.480, $p<0.001$†	χ^2=26.480, $p<0.001$†

Data are shown as the medians (IQR)

†Kruskal–Wallis test

CSA cross-sectional area, *AWT* arterial wall thickness, *SAH* subarachnoid hemorrhage

[a] Control vs. SAH ($p<0.001$)

[b] Control vs. vehicle ($p<0.001$)

[c] Control vs. testosterone ($p<0.001$)

[d] SAH vs. testosterone ($p<0.001$)

[e] Vehicle vs. testosterone ($p<0.001$)

[f] Control vs. testosterone ($p=0.007$)

Discussion

Aneurysmal SAH is associated with high rates of morbidity and mortality. Delayed and sustained vasospasm of the large-diameter cerebral arteries is a major contributor to SAH-induced disability and death [23]. The pathogenesis of SAH-induced cerebral vasospasm is complex, multifactorial, and still not fully understood. The catastrophic problem arising from cerebral vasospasm is ischemic neurological deficit. Both the treatment strategies and research are focused on these parameters [24].

Testosterone classically regulates cellular function in a variety of tissues via interaction with its nuclear androgen receptor. Testosterone can pass the blood–brain barrier due to its lipophilic structure, thus influencing the CNS [25]. Although TES is recognized to have important effects on metabolism and secondary sexual characteristics, many authors have also reported vasodilatatory effects on coronary arteries [7-10], thoracic aorta [10-12], mesenteric arteries [13], pulmonary artery [14, 15], radial artery [16], and the basilar artery [17]. Furthermore, some studies demonstrated that both acute administration of intravenous TES [26] and chronic administration of oral TES enhance brachial artery responsiveness to flow and nitrate-mediated dilatation in men with coronary artery disease [27].

Intracellular Ca^{+2} is a ubiquitous second messenger playing critical roles in a wide range of physiological processes including smooth muscle contraction [28]. The contractile force of arterial smooth muscle depends on Ca^{+2}, and Ca^{+2} influx through Ca^{+2} channels is one of the major regulators of vascular smooth muscle constriction [29]. Also, in the cerebral vasculature, intracellular Ca^{+2} concentration dictates smooth muscle contraction and arterial diameter [30]. Thus, an increase in intracellular Ca^{+2} leads to enhanced vasoconstriction and potentially a decrease in cerebral blood flow [31]. Following SAH, membrane potential depolarization and enhanced L-type voltage-dependent Ca^{+2} channel (VDCC) activity causes an increase in intracellular Ca^{+2} concentration leading to vasospasm [4, 32, 33].

One of the most important mechanisms underlying the vasodilatatory effect of TES is the inhibition of Ca^{+2} channels [9, 14, 15, 17]. Previous studies had demonstrated that the key mechanism underlying the vasodilatator action of TES is associated with the modulation of vascular smooth muscle cell membrane ion channel function via inactivation of L-type VDCC [9, 14, 15, 34-39], and by a lower affinity inhibition of T-type VDCC [39]. It was observed that TES

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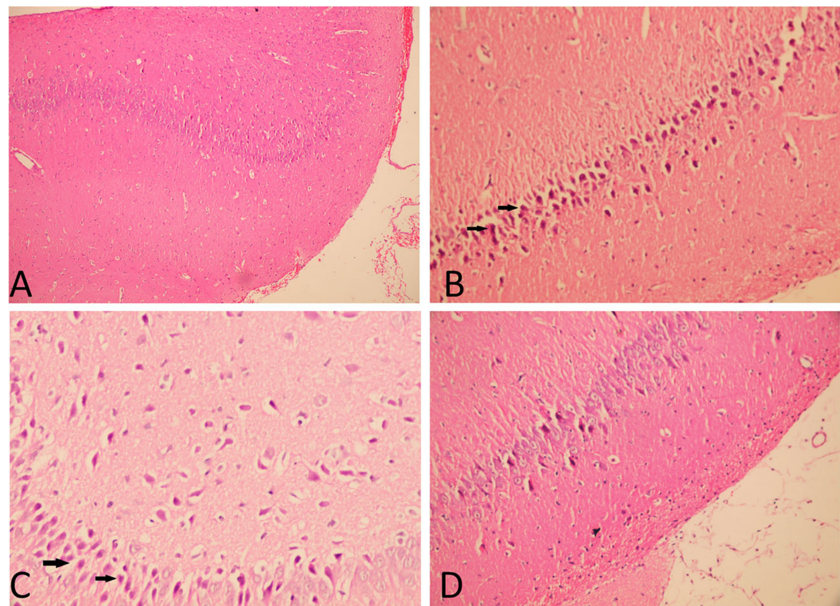

Fig. 4 Representative photomicrographs showing the normal appearing slices from the CA1 of the hippocampus of the control group (**a**) (H&E, 20X obj.). Slide from the SAH group showing the completely degenerated neurons (*arrows*) in the CA3 (**b**), and slide from the vehicle group showing the completely degenerated neurons (*arrows*) in the dentate gyrus (**c**) of the hippocampus (H&E, 40X obj.). Photomicrographs from the TES group show only mild degeneration of the neurons in the CA3 (**d**) of the hippocampus (H&E, 20X obj.)

inhibited L-type VDCC at nanomolar [40], physiological [41], and supraphysiological [42] concentrations. Moreover, Scragg et al. [40] observed that the L-type VDCC mutation at the nifedipine-binding site results in the loss of the vasorelaxant effects of TES. It was suggested that the vasodilatatory affect of TES on the basilar artery is also mediated by the blockage of Ca^{+2} influx through the inhibition of VDCC [17].

Harder et al. [43] reported that the membrane potential of cerebral artery myocytes is depolarized following SAH and several studies provided further evidence for a decreased voltage-dependent K^+ activity in pial arteries following SAH [4, 44, 45]. Inactivation of voltage-dependent K^+ channels may be one of the possible mechanisms causing cerebral vasospasm following SAH. A variety of studies have proposed that TES acts via opening voltage-dependent K^+ channel activation for its vasodilatatory action [11-13, 15, 46, 47].

As widely studied, TES has vasodilatatory activity via inhibiting VDCC and activating K^+ channels (Fig. 5a). Both these mechanisms provide evidence to explain the possible theuropathic activity of TES for SAH-induced vasospasm. Moreover, TES stimulates NO production via activating nNOS, which in turn evokes the formation of cyclic guanosine monophosphate to induce vasorelaxation [19, 35]. Previous studies reported that decreased NO availability and/or decreased NO synthesis from nNOS loss appear to contribute to the development of SAH-induced vasospasm [48] (Fig. 5b).

Moreover, it has been shown that inflammation and the formation of reactive oxygen species play an important role in

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

Variable	Control	SAH	Vehicle	Testosterone	p value [a]
CA 1	1 (1–2)[b,c,d]	4 (3–4)[b,e]	4 (3–4)[c,f]	3 (1–4)[d,e,f]	χ^2=27.338, p<0.001
CA 3	1 (1–2)[b,c,d]	4 (3–4)[b,e]	4 (3–4)[c,f]	2 (2–3)[d,e,f]	χ^2=22.090, p<0.001
Dentate gyrus	1 (1–2)[b,c,d]	4 (3–4)[b,e]	3.5 (2–4)[c,f]	3 (2–3)[d,e,f]	χ^2=21.920, p<0.001
Mean deg. score	3.5 (3–5)[b,c,d]	11.5 (10–12)[b,e]	11 (10–12)[c,f]	7.5 (7–9)[d,e,f]	χ^2=26.861, p<0.001

Data are shown as the medians (min-max)

SAH subarachnoid hemorrhage, *deg* degeneration

[a] Kruskal–Wallis test

[b] Control vs. SAH (p<0.001)

[c] Control vs. vehicle (p<0.001)

[d] Control vs. testosterone (p<0.05)

[e] SAH vs. testosterone (p<0.01)

[f] Vehicle vs. testosterone (p<0.05)

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Fig. 5 **a** Figure summarizing the proposed sites of action of TES via voltage-dependent calcium channels and potassium channels, which underlie its vasodilatatory action. $[Ca^{+2}]$: intracellular calcium; DAG: diacyl glycerol; IP_3: inositol triphosphate; IP_3R: inositol triphosphate receptor; K_{ATP}: ATP-sensitive potassium channel; K_{Ca}: calcium-sensitive potassium channel; K_V: voltage-sensitive potassium channel; PIP_2: phosphatidylinositol bisphosphate; PLC: phospholipase C; R: receptor; RyrR: ryanodine receptor. **b** Summary model of testosterone action via NO and nNOS. AR: androgen receptor; Ca: calcium; CAPC: calcium activated potassium channel; cGMP: cyclic guanosine monophosphate; NO: nitric oxide; nNOS: neuronal nitric oxide synthetase; sGC: soluble guanylyl cyclase; PI_3: phosphoinositide 3-kinase; PKB: protein kinase B; PKG: cGMP dependent protein kinase

the development of SAH-induced vasospasm [2]. Anti-inflammatory [49-51] and antioxidant [49, 52] potency of TES has been demonstrated.

Treatment with TES provides a wide array of neuroprotective and neurotheuropathic effects [53]. Recent data suggest that TES might exert neurotrophic actions, increase the expression of nerve growth factor [54], and mediate promotion of neurite growth [55]. Furthermore, TES shows neuroprotective effects via antioxidant properties [56] and via inhibiting apoptotic pathways [57].

Here we investigated the effects of TES on SAH-induced vasospasm. Our data revealed that TES has some preventive

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effects on SAH-induced vasospasm and secondary neuronal injury in rabbits. As described in detail, TES achieves this vasodilatatory action via multiple mechanisms (VDCC blockage, K^+ channel activation, NO synthesis, anti-oxidation, and anti-inflammation), where both affect the pathogenesis of the SAH-induced vasospasm.

Despite that the normal blood TES levels of men are higher than women [58, 59], the incidence of vasospasm is not any lower in male patients than in female patients [2, 23]. The circulating free TES levels in human are measured in ng/dl [59], which is far lower than the theuropathic levels administered in this study. So we thought that the observed vasodilatatory effects of TES are due to the administration of higher doses of TES than the normal circulating levels. Therefore, for better understanding of this conflict, further studies with dose- and sex-dependent results of TES on SAH induced cerebral vasospasm are needed.

Non-castrated intact male animals can have variable endogenous androgen levels on time of day and environmental stress as evidenced by the wide range of androgen values reported. Therefore, further study models with castrated groups are needed to better assess the effects of TES on SAH-induced vasospasm. Delayed histopathological assessment of the vasospasm after SAH could also increase the potential value of TES in the treatment. Furthermore, comparative effects of TES with a nimodipine group may provide stronger results, which is another limitation of the current study. Many patients are admitted to hospital several days after SAH with signs of already established vasospasm. Whether TES could reverse already established vasospasm in this group of patients remains unclear. Thus, to address the effect of TES for both prevention and reversion of vasospasm would require additional studies.

Conclusions

Our findings show that TES has some preventive effects on SAH-induced vasospasm and secondary neuronal injury in rabbits. The possible mechanism of the vasodilatatory activity of TES is inhibiting Ca^{+2} channels, activating K^+ channels, augmenting NO synthesis, and producing antioxidative and anti-inflammatory effects. We suggest that TES might be worthy of further investigation.

Conflicts of interest None.

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Comment

This remains an observational rather than mechanistic study, but is well conceived and conducted. It provides hypothesis-generating data, and as such a contribution to the literature.

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