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MORPHOMETRIC ANALYSIS OF THE EFFECT OF ROYAL JELLY ON THE VASOSPASTIC FEMORAL ARTERY IN RATS: AN EXPERIMENTAL STUDY

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ABSTRACT

This study aimed to examine the efficacy of royal jelly, which has potent anti-inflammatory and antioxidant properties, on the vasospastic femoral artery in rats. In total, 24 Wistar- albino strain rats were divided into three groups: group 1, control (n = 8); group 2, vasospasm (n = 8) and group 3, vasospasm + royal jelly treatment (n = 8). The wall thickness (W) of the femoral arteries and the luminal diameters (L) were morphometrically measured. Data were analyzed using the SPSS software. Statistical significance was set at an alpha level of $p < 0.05$. The mean L values were significantly lower in group 2 than in groups 1 and 3 ($p = 0.001$, for all). In addition, the mean L values were significantly lower in group 3 than in group 1 ($p = 0.001$, for all). The mean W values were not significantly different between groups 1 and 3 ($p = 0.325$). The mean W values were statistically significantly higher in group 2 than in groups 1 and 3 (all $p < 0.001$). The statistical analysis of our results suggested that royal jelly can effectively prevent vasospasm because of its anti-inflammatory and antioxidant properties.

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INTRODUCTION

The exact aetiopathogenesis of developing cerebral vasospasm (CV) after subarachnoid haemorrhage (SAH) has not been completely elucidated, and to date, no effective therapeutic modalities have been developed for managing this condition (Egemen *et al*., 1993). Some of the mechanisms implicated in CV aetiopathogenesis include inflammation induced by blood products and free oxygen radicals. Consequently, the therapeutic role of various antioxidant and anti-inflammatory molecules has been investigated. The well-documented

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antioxidant and anti-inflammatory effects of royal jelly occur through numerous active enzymes and substances in its composition and through different mechanisms (Aslan and Aksoy 2015). A review of the English literature revealed that no studies have assessed the role of royal jelly in preventing CV after SAH. Thus, this study aimed to examine the effect of royal jelly on the development of vasospasm in an experimental rat femoral artery model of SAH and to investigate the in vivo applicability of royal jelly around viable vascular tissues.

MATERIALS AND METHODS

The protocol for this experimental study was approved by the ethics committee of the Experimental Research and Application Center for Medical Sciences, Cukurova University. The surgical phase of the study was conducted at the Experimental Animals Research Laboratory, Institution for Neurological Sciences, Marmara University, and the histopathological and morphometric analyses were performed at the pathology laboratory, Bakırkoy Dr. Sadi Konuk Training and Research Hospital., In total, 24 female Wistar-albino rats, weighing 220–250 g, were used developing the femoral artery vasospasm model, as described previously. The rats were divided into three groups: group 1 (n = 8), control; group 2 (n = 8), vasospasm and group 3 (n = 8), vasospasm + royal jelly treatment. Before the surgery to induce femoral arterial vasospasm, the rats were anaesthetised with intraperitoneal 50 mg/kg ketamine HCl (Ketalar flacon, 50 mg/ml; Pfizer) and laid supine on cork blocks. The inguinal area of the rats was shaved and disinfected with povidone–iodine (PVD) solution. Under surgical microscope guidance, a 2-cm-long longitudinal skin incision was made and the femoral neurovascular bundle was accessed. The femoral artery was gently dissected away from the femoral vein and nerve to avoid surgical trauma. A 1- to 1.5-cm-long segment of the femoral artery was wrapped with a silastic sheath, which was held in place by sutures. Autologous cardiac blood samples of the rats were collected as whole blood samples. Intra-cardiac arterial blood samples (0.1 cc), percutaneously obtained using insulin injectors, were injected into the silastic sheath to induce peripheral vasospasm in rats of groups 2 and 3. In group 3, 0.1 mL royal jelly was locally applied to the silastic sheath of the rats. In group 1, 0.1ml of 0.9% physiologic saline was locally applied. The rats were kept at normal room temperature and in separate cages and were fed standard pellet food.

At the completion of day 7, the rats were sedated with percutaneous intraperitoneal administration of 2 mg/kg ketamine and were fixed on cork blocks in the supine position. The silastic sheaths around the femoral artery were approached via the previous incision, and the sheaths were opened to reveal the femoral artery. In all rats, the presence of the femoral artery inside the silastic sheath was ascertained. In the meantime, the skin over the sternum was shaved, and after disinfecting with PVD solution, the sternum was dissected away from the costa following the line of the sternocostal junctions using a dissection scissors; the thorax was then explored. The pericardium was exposed, and a green injection needle was placed in the left ventricle. An infusion set was mounted to the tip of the catheter (the infusion set was placed 10 cm above the heart level to maintain the physiologic arterial blood pressure), and a mixture comprising 100 ml of 0.03 M phosphate buffer, 200 ml of 4% paraformaldehyde and 1% glutaraldehyde solution was delivered via the left ventricle. Infusion was continued until outflow of clear fluid from the previously dissected right atrium was observed; this indicated complete vascular circulation of the solution. A 1- to 1.5-cm-long segment of the right femoral artery was harvested from all the rats for histopathological morphometric analyses. This was followed by killing the rats via cervical dislocation. Femoral artery specimens immersed in buffered 10% formaldehyde were placed in cassettes, which were kept in a tissue maintenance device, and were treated with formaldehyde for fixation. After dehydration with graded alcohol and processing with xylene, the specimens were embedded in paraffin. Following completing this procedure, paraffinembedded tissue specimens were frozen as paraffin blocks. Sections of 5-µm thickness were cut using a microtome and were deparaffinised in an incubator at 60°C for 1h. The deparaffinisation procedure was continued using xylene, which was administered thrice. The specimens were then treated with graded alcohol for rehydration and were rinsed with water before staining with toluidine blue. The samples were examined under the microscope at ×100, ×200 and×400 magnification and were photographed for morphometric analysis. The vascular wall thickness (W) and luminal cross-sectional areas were measured using the ImageJ 1.34 software and were expressed as unit values. The measurements were performed using photos taken at ×40 magnification. The L and W values of the three groups were correlated, and the specimens were compared for morphometric analyses.

Statistical Analysis

For data analyses, the SPSS 15.0 software for Windows was used. Descriptive statistics were expressed as mean, standard deviation, minimum, maximum and mean for numerical variables. The results were assessed with Kruskal–Wallis test. For the subgroup analyses, Mann–Whitney U test was used and interpreted with Bonferroni correction. Statistical significance was expressed at an alphalevel of $p < 0.05$.

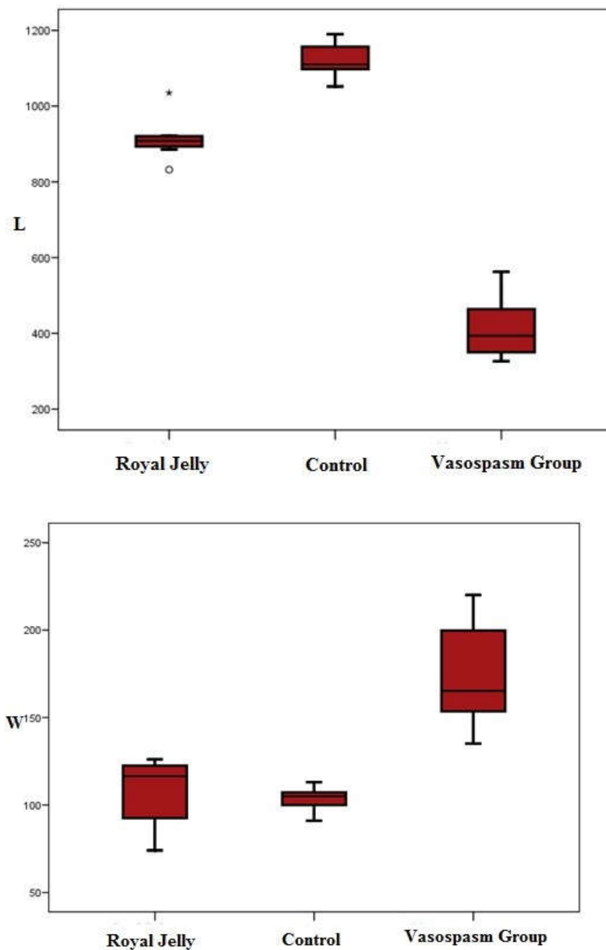
RESULTS

The resected samples of the femoral arteries were assessed under a surgical microscope, and various degrees of contracted femoral arteries, changing from mild to severe vasoconstriction, were observed in groups 2 and 3, whereas such changes were not observed in group 1 (Table I). Mean L and W values were statistically significantly different between groups 1 and 2 ($P< 0.001$ and $P = 0.001$, respectively) and between groups 2 and 3 ($P < 0.001$). The mean L value was statistically significantly lower in group 2 than in group 1, whereas the mean W value was significantly higher in group 2 than in group 1(both $p<0.001$). The mean W value was statistically significantly lower in group 3 than in group 2, whereas the mean L value was significantly higher in group 3 than in group 2 (for both $p < 0.001$). The mean W value in group 3 was similar to that in group 1, with no statistically significant difference ($p = 0.325$), whereas the mean L value in group 3 was statistically significantly lower than that in group 1 ($p < 0.001$; Graphics I and II).

Histopathological Changes: The femoral artery sections were examined under light microscopy in all groups. Arterial samples in group 1 had a thin and smooth endothelium in addition to a thin and uncoiled internal elastic lamina and concentrically arrayed smooth muscle cells, with no luminal stenosis and thickening of vascular walls (Figure 1).

Table I. The mean W and L values of each group and comparisons among the groups. Under microscopic examination, resected femoral arteries show arterial stenosis that increases in severity from group 2 (mild) to group 3 (severe), with no change observed in group 1

		Mean ± SD	Minimum–Maximum	Median
L	Group 1	1123.0 ± 47.3	1052–1190	1110
	Group 2	412.9 ± 82.0	326–562	393.5
	Group 3	913.9 ± 56.7	832–1035	908
	P		0.001	
W	Group 1	103.4 ± 7.2	91–113	105
	Group 2	174.0 ± 29.2	135–220	165.25
	Group 3	107.9 ± 19.4	74–126	116.5
	P		0.001	
	group analysis			
Sub				
			L	W
			P	*P*
	Groups 1 vs. 2	0.001		0.001
	Groups 1 vs. 3	0.001		0.325
	Groups 2 vs. 3	0.001		0.001



Graphics 1 and 2. Mean L and W values of the three groups were statistically significantly different (for both p = 0.001). The mean L value was statistically significantly lower in group 2 than in groups 1 and 3. However, the mean W value was significantly higher in group 2 than in groups 1 and 3 (for all p < 0.001). The mean L value was statistically significantly lower in group 3 than in group 1(p < 0.001), whereas the mean W value was not statistically significantly different in groups 1 and 3 (p = 0.325)

Group 2 exhibited marked narrowing of the arterial lumens and thickening of the vascular walls, disruption of the endothelial integrity, coiled internal elastic lamina and vacuolisation of the muscular layer (Figure 2). In group 3, similar to group 1, arteries were surrounded by a thin and smooth endothelial layer, having a thin internal elastic lamina with areas of slight coiling and concentrically arrayed smooth muscle cells (Figure 3).

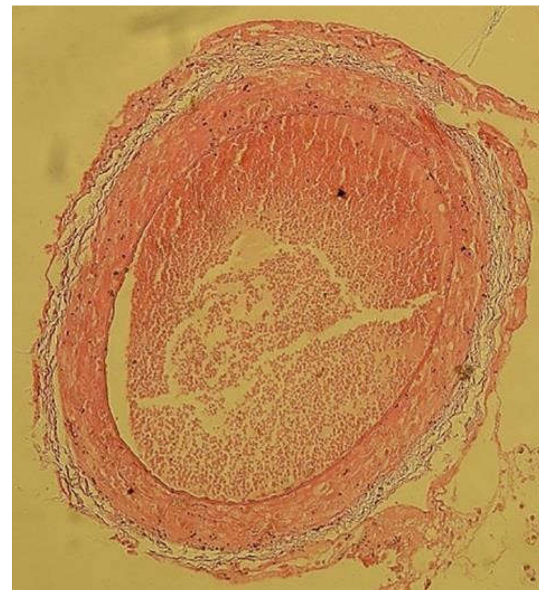


Figure 1. Group 1 (control group). Thin and smooth endothelium, thin and uncurled internal elastic lamina and concentrically arrayed smooth muscle cells of the arteries are observed, whereas stenotic arterial lumen and thickened arterial wall are not observed.

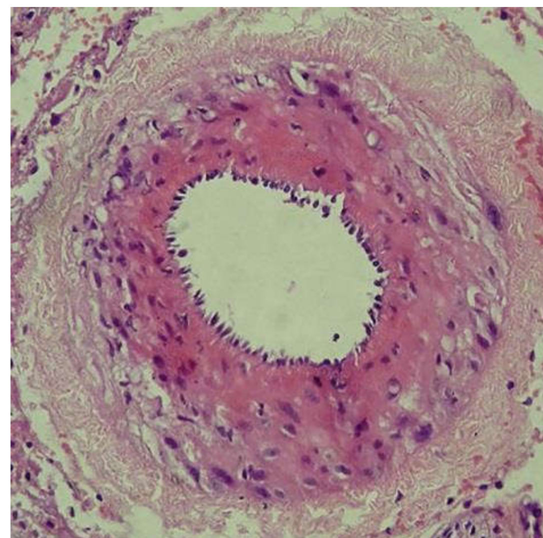


Figure 2. Group 2 (vasospasm group). Marked narrowing of the arterial lumen, impairment of endothelial integrity, curling of internal elastic lamina and vacuolisation of the muscular layer are observed

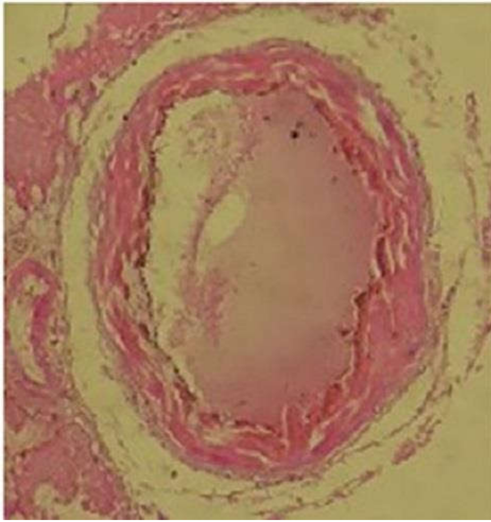


Figure 3. Group 3 (vasospasm + royal jelly treatment group). Arterial morphology is surrounded with thin and smooth outer endothelial layer of the artery, similar to that observed in the control group. There are patchy areas of slightly curved internal elastic lamina and concentric smooth muscle cells

DISCUSSION

CV is associated with reduced perfusion in the distal part of the involved artery and is caused by pathological arterial vasoconstriction that results because of the accumulation of blood and blood elements in the cerebral cisternae after an SAH incident (Anderson *et al*., 2000, Chehrazi *et al*., 1989). CV physiopathology of CV is complex and multifactorial and includes the potential role of the following factors in vasospasm development: the presence of blood clots in the subarachnoid space, excessive arterial injury, intracranial hypertension, cerebral ischaemia, inflammation and oxidative species (Gunaldi *et al*., 2015, Joshita *et al*., 1992, Kobayashi *et al*., 1992, Kongable *et al*., 1996, Smith *et al*., 1983). Initial studies of experimental SAH, which assessed the causes of CV, date back to the 1960s, and studies have investigated many different agents for preventing or treating vasospasm (Gumus *et al*., 2014, Gunaldi *et al*., 2010). From a physiopathological perspective, the role of oxyhaemoglobin in directly or indirectly inducing smooth muscle contraction in CV remains unclear. The cerebral vascular tonus is largely regulated by the vascular endothelium via the balance between the endothelium-derived relaxation factor (nitric oxide) and the endothelium derived constriction factor (endothelin). Vasospasm that develops after SAH is associated with increased endothelin concentrations in the plasma and cerebrospinal fluid.

In vivo studies have demonstrated that intra-cisternally injecting endothelin results in prolonged constriction of the cerebral arteries (Juvela 1992). Oxyhaemoglobin also causes a shift in the vascular tonus toward vasoconstriction, either by directly binding to nitric oxide or by abolishing nitric oxide via its role in superoxide anion radical formation. Moreover, oxyhaemoglobin stimulates vasospasm through both mechanisms by triggering the release of endothelin from endothelial cells (Shinego *et al*., 1982). In their histopathological study, Peterson *et al*., observed severe inflammation in the vasospastic vessel wall after SAH. They also reported that this inflammatory process was associated with vasospasm induction, which could be averted by preventing inflammation (Peterson *et al*., 1990). Fassbender *et al*. revealed that pro-inflammatory cytokines such as IL-β, IL-6 and TNF-α triggered the inflammatory step, leading to tissue damage. They also found that the inflammatory response was the main reason why patients with SAH developed vasospasm and that anti-inflammatory and anti-cytokine treatments might prevent ischaemic complications after SAH (Fassbender *et al*., 2001). Free radicals have also been implicated in CV development. Organisms are equipped with protective mechanisms that negate the effects of free radicals. Some of these mechanisms prevent the formation of free radicals, whereas others prevent the noxious effects of already formed free radicals. Collectively, these free radicals are referred to as antioxidants (Frei 1994). On the basis of the ability of antioxidants to protect tissues or cells against oxidative damage, these agents have also been proposed to have anti-ageing properties and have protective effects against toxic agents that lead to tissue damages (Verma *et al*., 2015, Ito and Hirose 1989). Royal jelly is secreted from the mandibular and hypopharyngeal gland that is located in the scalp of a young worker bee (Kubo *et al*., 1996, Zaluski *et al*., 2017). Worker bees use their royal jelly for supplying the food demands of larvae (Chen and Chen 1995). In addition, the queen bee is fed royal jelly for a lifetime and their differentiated from the worker bee. Constitutional proteins of the royal jelly possess antioxidant and anti-inflammatory properties and are used for treating conditions that results from an imbalance among reactive oxygen species. Studies have demonstrated the effect of coronary vasodilatation and bronchodilation of royal jelly (Krylov *et al*., 2004, Krylov *et al*., 2006). Furthermore, royal jelly is used as an anti-ageing agent in the cosmetic industry (Karacal *et al*., 2006). Royal jelly comprises water (60%–70%), raw protein (12%–15%), fats (3%–6%), various free fatty acids, various types of sugar (10%–16%), free amino acids, collagen, lecithin and vitamins A, B5, C, D and E. The pH of royal jelly ranges between 3.6 and 4.2 (Chen and Chen 1995, Jia *et al*., 1995, Kamakura *et al*., 2001).

On an average, each gram of royal jelly has 7.3 mg of free amino acids, with proline, lysine, glutamate, β-alanine, phenylalanine, aspartate and serine being the most abundant (Boselli *et al*., 2003). In the studies by Guo *et al*., 29 antioxidative peptides were identified, and shorter peptides with 2–4 amino acids had a more potent antioxidant activity (Guo *et al*., 2005, Guo *et al*., 2009). This study was based on the hypotheses that the anti-inflammatory effects of royal jelly occur via the inhibition of lipid peroxidation and the release of inflammatory cytokines such as TNF-α, IL-1β and IL-18 and that anti-inflammatory treatment may help prevent ischaemic complications after subarachnoid haemorrhage. The peripheral chronic vasospasm model and the autologous arterial whole blood sampling method used in our study were originally developed by Okada *et al*., (Okada *et al*., 1990). Our results showed a significant difference between groups 1 and 2 with respect to W and L values, indicating that the vasospasm model could be successfully reproduced. In groups 1 and 3, the W and L values were significantly higher and the W value was significantly lower than that in group 1. These results suggest that the vasospasm-preventing effects of royal jelly observed in group 3 were almost similar to those observed in group 1.

Conclusion

In this experimental rat femoral artery vasospasm model, the ability of royal jelly to prevent vasospasm was statistically demonstrated. Royal jelly has numerous active metabolites and

enzymes that act together, precluding the identification of individual molecule(s) that are responsible for preventing vasospasm after SAH. Therefore, one or more of the constitutional active metabolites of royal jelly may be more effective in preventing vasospasm. This was a study of a femoral artery vasospasm model, and further studies that involve CV models are warranted to better delineate its effects on cerebral arteries and potential noxious effects on cerebral tissues. In conclusion, we believe that further studies are required to assess the role of royal jelly in more advanced vasospasm cases because of its extremely rich constitutional mixture of active metabolites and enzymes.

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