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Effects of induced pressure and clamping force by vascular clamps on the vascular endothelium of rat aorta

Sıçan aortu vasküler endotelinde vasküler klempler tarafından oluşturulan basınç ve sıkma kuvveti etkileri

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ABSTRACT

Background: This study aims to investigate the effects of vascular clamps on the vascular endothelium of rat aorta.

Methods: The study included 32 male Sprague-Dawley rats (weight 242±26 g; age 9 to 11 weeks). Rats were divided into four equal groups: control group: no clamp was applied on abdominal aorta; group 1: plastic bulldog clamp was applied on abdominal aorta for 10 minutes; group 2: metal bulldog clamp was applied on abdominal aorta for 10 minutes; group 3: microvascular clamp was applied on abdominal aorta for 10 minutes. At the end of 10 minutes, segments occluded with vascular clamp were excised in all groups and endothelial structures were evaluated histopathologically.

Results: Normal cellular sequencing and structure were determined in control group. Most severe injury of the endothelial surface was observed in group 3, moderate level endothelial injury was observed in group 2, while mildest endothelial injury was observed in group 1. Increased vascular endothelial growth factor expression levels were detected histopathologically in groups 1 and 2 (2.8±0.5 and 3.3±0.5, respectively) when compared with the control subjects (2.0±0.5).

Conclusion: Due to their induced pressure and clamping force, vascular clamps may cause endothelial injury.

Keywords: Endothelial injury; histopathology; vascular clamps; vascular endothelial growth factor.

ÖZ

Amaç: Bu çalışmada sıçan aortu vasküler endotelinde vasküler klemplerin etkileri araştırıldı.

Çalışma planı: Çalışmaya 32 erkek Sprague-Dawley sıçan (ağırlık 242±26 g; yaş 9-11 hafta) dahil edildi. Sıçanlar dört eşit gruba ayrıldı: Kontrol grubu: Abdominal aorta klemp uygulanmadı; grup 1: Abdominal aorta 10 dakika plastik bulldog klemp uygulandı; grup 2: Abdominal aorta 10 dakika metal bulldog klemp uygulandı; grup 3: Abdominal aorta 10 dakika mikrovasküler klemp uygulandı. On dakikanın sonunda tüm gruplarda vasküler klemple oklüde edilen segmentler çıkarıldı ve endotel yapılar histopatolojik olarak değerlendirildi.

Bulgular: Kontrol grubunda normal hücresel dizilim ve yapı tespit edildi. En ciddi endotel yüzey yaralanması grup 3'te, orta düzeyde endotel yaralanması grup 2'de, en hafif endotel yaralanması ise grup 1'de gözlendi. Histopatolojik olarak vasküler endotelyal büyüme faktörü ekspresyonunun grup 1 ve 2'de (sırasıyla 2.8±0.5 ve 3.3±0.5) kontrol örneklere göre (2.0±0.5) artmış olduğu saptandı.

Sonuç: Vasküler klempler, oluşturduğu basınç ve sıkma kuvvetine bağlı olarak endotel yaralanmasına neden olabilir.

Anahtar sözcükler: Endotel yaralanması; histopatoloji; vasküler klempler; vasküler endotelyal büyüme faktörü.

In cardiovascular surgery, vascular clamps are useful in controlling blood flow and avoiding bleeding.[1,2] However, vessel walls and vascular endothelium are sensitive to environmental factors, such as pressure, compression, or heat.[2-4] While controlling vascular blood flow with vascular clamps, the vascular endothelium is exposed to crushing by clamp compression (tissue compression between the tips of

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the clamp), and it is exposed to the local increase in intravascular pressure.[2,3] These factors lead to various degrees of endothelial injury that can threaten further blood flow in this vessel postoperatively. The degree of vessel damage can lead to different pathological situations, such as flap, dissection, and arteriovenous fistula.[2] Intravascular thrombosis and, later, stricture are other important complications that may potentially lead to the disruption of continuous blood flow. In particular, these complications most likely reduce the success rates of anastomotic interventions.[1,2]

Despite the adverse effects, vascular clamps are commonly used for bleeding control during cardiovascular procedures. Nevertheless, investigators have aimed to reduce the injury potential of these instruments. We attempted to reduce the clamping force as much as possible to provide minimal occlusion force to decrease the severity of endothelial injury.[2,5] Therefore, in this study, we aimed to investigate the effects of vascular clamps on the vascular endothelium of rat aorta.

MATERIALS AND METHODS

The study was conducted at Medical Faculty of Dicle University. All procedures were designed in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals prepared by the Ethics Committee. All protocols were presented to the Local Ethical Committee of the Animal Research Committee of the University after study designation and ethical approval was obtained. The study was conducted in accordance with the principles of the Declaration of Helsinki. Prior to the commencement of the study, animal subjects were maintained in standard humidity (50%±5%) and temperature-controlled (22 °C ±2 °C) cages with a 12-hour light/dark cycle controlled by the Laboratory of Animal Production Unit of the University.

The study included 32 male Sprague-Dawley rats (weight 242±26 g, age 9 to 11 weeks). Rats were divided into four equal groups as follows:

Control group was used for sampling the normal histology of the abdominal aorta of rat genus using standard median laparotomy. Abdominal aortas of these rats were harvested without any application, and normal vascular endothelium was evaluated histopathologically.

Group 1: Abdominal aortas of these rats were explored with standard median laparotomy. The explored abdominal aorta was clamped with a plastic bulldog for 10 minutes. Afterwards, the clamped segment of the abdominal aorta was harvested for histopathological evaluation of the vascular endothelium.

Group 2: Abdominal aortas of these rats were explored with standard median laparotomy. The explored abdominal aorta was clamped with a metal bulldog for 10 minutes. Afterwards, the clamped segment of the abdominal aorta was harvested for histopathological evaluation of the vascular endothelium.

Group 3: Abdominal aortas of these rats were explored with standard median laparotomy. The explored abdominal aorta was clamped with a microvascular clamp for 10 minutes. Afterwards, the clamped segment of the abdominal aorta was harvested for histopathological evaluation of the vascular endothelium.

Ketamine hydrochloride (Ketalar, Pfizer) at a dose of 130 mg/kg and xylazine (Rompun, Bayer) at a dose of 20 mg/kg were used for anesthetic management with intraperitoneal injection during the surgical procedure. Anesthetic maintenance was continued with ketamine hydrochloride at a dose of 50 mg/kg.

A maxi 45°-angled plastic bulldog clamp (Vascu-Statt II®; Scanlan, USA) (given a clamping pressure of 165 g-175 g, catalog no. 1001-505) [31 g clamping force and 8 mmHg systemic pressure increase] was used for clamping the aortas of rats in group 1. A DeBakey-Dietrich metal micro bulldog clamp (E type, Geister® Medizintechnik GmbH, Tuttlingen, Germany) was used for clamping the aortas of rats in group 2 (clamping length: 46/1 ½ mm, jaw: 14 mm, weight: 3.0 g, closing pressure: 180 g, catalog no. 20-0321) [47 g clamping force and 16 mmHg systemic pressure increase]. A DeBakey peripheral vascular clamp (M 22-0106 clamping length: 10 cm/4) [65 g clamping force and 27 mmHg systemic pressure increase] was used for clamping the aortas of rats in group 3. The case-specific clamping forces of clamps were calculated according to previously described methods with given general clamping force and producer parameters.[2,5] Vessel thickness (histopathologically determined mean aortic thickness was 108.7±2.7 100 μm), clamping area, and the given clamping pressures by producer were used as determinants for calculating the applied clamping pressures of the metal and plastic bulldog clamps on the rat aortas.[2] The applied clamping pressure of an atraumatic microvascular clamp, which has six levels of force, was generated by closing the clamp at three, four, five, and six notches of closure on the rat aorta and was calculated according to closed notch number (the closure of vessel was applied at the sixth notch for

this experiment to provide uniformity), clamped vessel thickness, applied force (given clamping pressures by producer), area of the clamped artery, and external diameter of the clamped artery.[2] All clamping forces were expressed as Newtons (N), and clamping pressures on the affected vessel area were expressed as N/mm^2.

The aortic biopsies were fixed in 10% neutral buffered formalin solution for 24 hours, dehydrated, cleared, and embedded in paraffin as usual. Serial tissue sections at a thickness of 4 µm-5 µm were cut using the microtome, stained with hematoxylin and eosin, and evaluated with a Nikon eclipse TSE 100 f model.

The antigen retrieval process was performed twice in a citrate buffer solution (pH: 6.0); the first time was during the first seven minutes and the second time was in the later five minutes boiled in a microwave oven at 700 W. They were allowed to cool to room temperature for 30 minutes and washed twice in distilled water for five minutes. Endogenous peroxidase activity was blocked in 0.1% hydrogen peroxide for 20 minutes. Ultra V block (Cat.No: 85-9043, Invitrogen, Carlsbad, CA, USA) was applied for 10 minutes prior to the application of the primary antibody vascular endothelial growth factor (VEGF) (vWF antibody rabbit-anti-vWF, 1/800, ab6994, Abcam), and then a secondary antibody was applied for 20 minutes. The slides were then exposed to streptavidin-peroxidase for 20 minutes. Diaminobenzidine (Invitrogen, Carlsbad, CA, USA) was used as a chromogen. The control slides were exposed to streptavidin-peroxidase for 20 minutes. The control slides were prepared as mentioned above, but the primary antibodies were omitted. After counterstaining with hematoxylin, washing in tap water for eight minutes and in distilled water for 10 minutes, the slides were mounted with Entellan. The VEGF expressions were classified as follows: 0: no expression, 1: low expression; 2: moderate expression; 3: high expression; and 4: extremely high expression.

Endothelium injury was classified histomorphologically according to the literature 1. The injury classification is as follows:

Grade 0 (unscathed endothelium): Regular interaction of endothelial cells and normal cellular morphology with or without adhesion of platelets and other blood cells to the endothelium.

Grade 1: Maintained cellular integrity and interactions but with change in cellular content and diameter (flattening) with the adhesion of platelets and other blood cells to the endothelium.

Grade 2: Disrupted cellular interactions, decomposition of cellular adherence, and isolated cellular loss.

Grade 3: Naked subendothelial tissue due to the peeling of the endothelium.

Statistical analysis

The SPSS version 15.0 statistical analysis program (SPSS Inc., Chicago, IL, USA) was used for statistical evaluation of the obtained data. Mann-Whitney U test was used for comparing groups according to endothelial injury. The one-way analysis of variance was used to determine the differences between groups in terms of injury grade and VEGF expression levels. Tukey's honest significant difference was used as a post hoc test. Statistical significance was considered as $p<0.05$.

RESULTS

The clamping forces generated by the plastic bulldog clamp, metal bulldog clamp, and microvascular clamp were 5.0±0.2 N, 5.1±0.3 N, and 8.5±0.4 N, respectively. The clamping pressures on the rat aorta (in terms of the clamped surface area) were 83.8±0.3 N/mm^2 in group 1, 134.2±5.6 N/mm^2 in group 2, and 298.0±11.6 N/mm^2 in group 3.

The normal endothelial morphology detected with the periodic acid-Schiff staining in the control group is shown in Figure 1a. Mild-moderate (grade 1-2) endothelial injury (Figure 1b, c) was observed in groups 1 and 2 (mean injury degrees: 2.0±0.5 and 2.8±0.0, respectively). Moderate-severe (grade 2-3) endothelial injury (Figure 1d) was observed in group 3 (mean injury degree 3.6±0.8). The injury grade distribution for each rat in each group is presented in Table 1.

Increased VEGF expression levels were detected in groups 1 and 2 (2.8±0.5 and 3.3±0.5, respectively) when compared with the control subjects (2.0±0.5). However, markedly lower VEGF expression levels were observed in group 3 (0.6±0.5). The VEGF expression levels for each rat in each group are presented in Table 2. The VEGF expressions in groups are illustrated histopathologically in Figure 2a-d.

DISCUSSION

Increased clamp pressures lead to advanced injury on the vascular endothelium, according to the results of this study. Both clamping forces of clamps and clamping pressures on endothelium seem to affect the injury grade. In particular, extremely

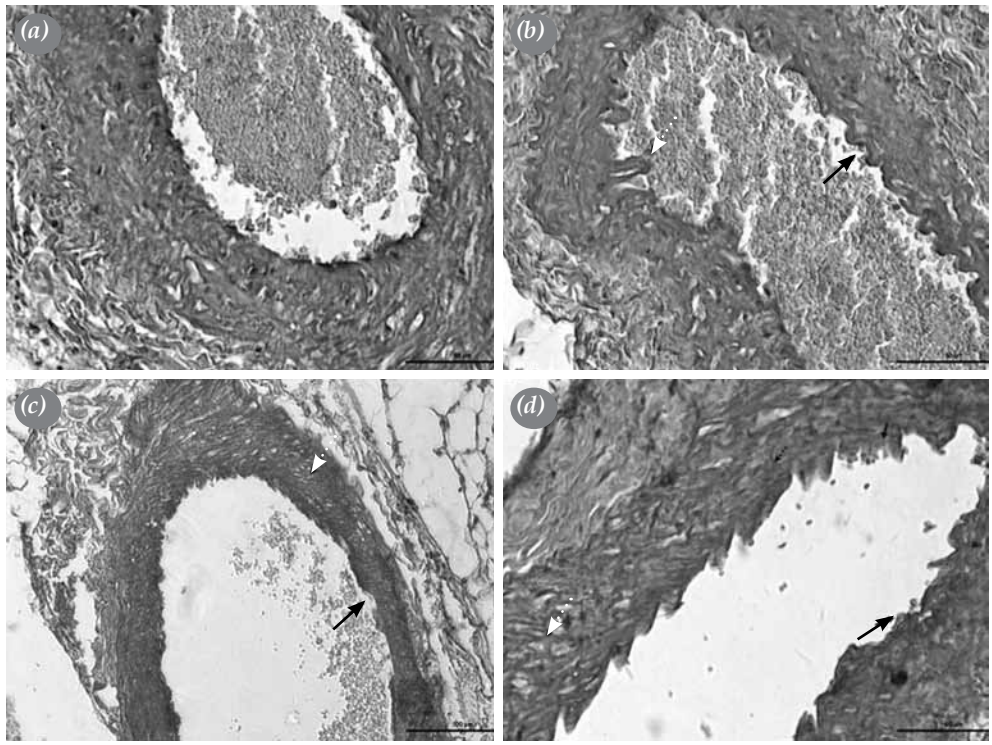

Figure 1. (a) Normal aortic endothelium of rat aorta [periodic acid-Schiff (PAS) staining, Bar=100 μm]; **(b)** Group 1: oval and centric placement of muscle cells, regular elastic fibers, invagination of elastic membrane to the vessel lumen (dashed arrow), regular endothelial cells (straight arrow) [PAS staining, Bar=100 μm]; **(c)** Group 2: local endothelial loss (straight arrow) and impairment of membrane structure windows with vacuolar formation (dashed arrow) [PAS staining, Bar=100 μm]; **(d)** Group 3: cellular loss of endothelium, thinning of basal membrane and desquamation of vessel wall (straight arrow), structural impairment of elastic membrane, degeneration and hyalinization of muscle cells and local vacuolar formations (dashed arrow) [PAS staining, Bar=100 μm].

high forces lead to cellular loss of endothelium and decreased VEGF expression. Conversely, low clamping forces lead to reversible cellular damage, which can activate VEGF expression and result in proliferation. To the best of our knowledge, this study is the first to demonstrate the levels of VEGF expressions from the vascular endothelium after vascular clamping.

Table 1. Injury grading in each group and comparison of grades between groups

Experimental subject	Control group	Group 1	Group 2	Group 3
1	+	++	++++	++++
2	+	+++	+++	++++
3	+	++	++++	++++
4	+	+	++	+++
5	+	++	+++	+++
6	+	++	++	++++
7	+	++	+++	++++
8	+	++	++	+++
Mean±SD	1.0±0.0	2.0±0.5	2.8±0.8	3.6±0.8
p (ANOVA)			0.000	

	Groups	*p*
Control	Group 1	0.007
	Group 2	0.000
	Group 3	0.000
Group 1	Group 2	0.020
	Group 3	0.000
Group 2	Group 3	0.055

Tukey HSD

+: Grade 0 injury; ++: Grade 1 injury; +++: Grade 2 injury; ++++: Grade 3 injury; SD: Standard deviation; p<0.05 is considered significant; ANOVA: One-way analysis of variance; HSD: Honest significant difference.

Table 2. Vascular endothelial growth factor expression levels in each group and comparison of vascular endothelial growth factor expression between groups

Experimental subject	Control group	Group 1	Group 2	Group 3
1	1	3	3	0
2	2	3	3	0
3	2	3	4	1
4	3	2	3	1
5	2	3	4	0
6	2	3	3	1
7	2	2	4	1
8	2	3	3	1
Mean±SD	2.0±0.5	2.7±0.5	3.3±0.5	0.6±0.5
p (ANOVA)			0.000	

		Groups	*p*
Tukey HSD	Control	Group 1	0.031
		Group 2	0.000
		Group 3	0.000
	Group 1	Group 2	0.089
		Group 3	0.000
	Group 2	Group 3	0.000

0: No expression; 1: Low expression; 2: Moderate expression; 3: High expression; ANOVA: One-way analysis of variance; HSD: Honest significant difference; SD: Standard deviation.

Endothelial cells remain stable for years until any traumatic reason leads to their damage and initiates endothelial dependent reactions.[6] Vascular endothelial cells are sensitive to external trauma. After mechanical or heat-induced trauma, mediators are induced from endothelial cells to trigger cellular and systemic response immediately.[7] These reactions lead to the initiation of a series of events that cause the chemoattraction of inflammatory modulator cells to the injury site.[7] Although endothelial cells regenerate rapidly, a partial or complete function loss occurs in the new cellular formation.[6] Therefore, recent reports have

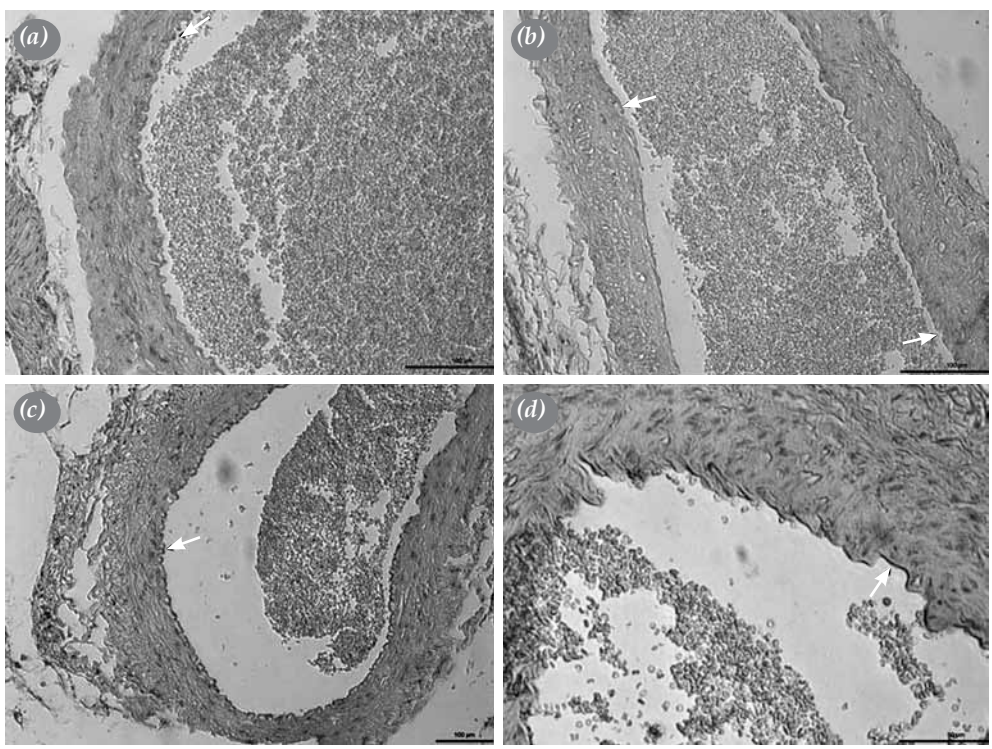

Figure 2. (a) Normal aortic endothelium of rat aorta, positive vascular endothelial growth factor (VEGF) expression (white arrow) (VEGF Immune Staining, Bar=100 µm); **(b)** Group 1: impairment of elastic membrane and increased VEGF expression (white arrows) (VEGF Immune Staining, Bar=100 µm); **(c)** Group 2: partially regular elastic membrane and increased VEGF expression (white arrow) (VEGF Immune Staining, Bar=100 µm); **(d)** Group 3: endothelial loss and locally VEGF expression loss on discontinuous elastic membranes (white arrow) (VEGF Immune Staining, Bar=100 µm).

recommended reducing the manipulation of vascular structures to avoid endothelial trauma.[8] Moreover, "no-touch" vascular harvesting techniques have been developed for preventing these undesirable endothelial effects.[8,9] Tsui et al.[9] reported better patency rates in this technique while preserving endothelial functions such as nitric oxide synthesis. However, accomplishing surgical protocols with free graft or bloodless elective procedures cannot be performed every time in vascular surgery. The surgeon's visual field must be clear to provide a good view of the vascular anastomoses. Surrounding tissues should be sufficiently retracted with sufficient exposure, and bleeding should be minimized.[10,11]

The traumatic effects of surgical procedures and devices that provide bloodless intervention have been known for decades.[12] Most vascular injury studies have focused on vascular clamps because of the common use of such devices.[1] Even clamp-free anastomosis techniques have been investigated in recent reports.[11] Despite the advanced techniques, vascular clamps are still the most highly available devices to stop bleeding and provide good operation conditions.[10,11] Manship et al.[13] demonstrated vascular endothelial and presumed medial injury in clamped vessels using all types of vascular clamps, and they claimed that vascular loops could protect the vascular endothelium better. Thereafter, Margovsky et al.[2] described a measurement of an endothelial injury method by staining an endothelial surface area, and reported that vascular clamps lead to increased endothelial injury. Recently, Babin-Ebell et al.[14] investigated the effects of intravascular and external pressure and the duration of vascular clamping on pig aorta in an experimental model, and reported that low force clamping is important to avoid notable endothelial injury. Moderate endothelial injury was even reported with microvascular clamps, which have minimal clamping forces, in another experimental study.[1] Vural et al.[15] and Hangler et al.[16] studied the endothelial effects of intravascular shunts rather than vascular clamps on coronary arteries (to avoid external pressure). However, endothelial denudation was reported in both studies.[15,16] We found significant endothelial injury with all types of sensitive vascular clamps similar to previous reports. Based on these studies and according to our findings, the general consensus is that all types of external and internal effects on the vascular endothelium can easily lead to endothelial injury.

Other conclusions have been asserted according to the results of studies that focused on vascular clamping on endothelial structures. Gücü et al.[1] claimed that endothelial injury from vascular clamping could lead to early thrombosis or late stenosis, which could result in malperfusion on the distal site of the vessels. After the mechanical disruption of endothelium, endothelial cells are produced and release proinflammatory and proangiogenic factors for providing neo-endothelialization and accelerating vascular healing.[7] The VEGF is an important example for these factors. Rapid elevation of VEGF levels is demonstrated after endothelial injury.[7] Furthermore, this factor is a key regulator of physiologic and pathologic angiogenesis that is sensitive for stress factors. dela Paz et al.[17] reported that VEGF expression could be affected by shear stress and that arterial shear stress could decrease apoptosis and increased VEGF expression. Briefly, optimum stress can be important for the vascular endothelial cycle. Endothelial cell homeostasis also depend on VEGF expression.[18] The VEGF has been reported to be responsible for neointimal hyperplasia in injured vascular endothelium.[19] We found a marked elevation of endothelial VEGF expressions after 10 minutes of clamping with bulldog clamps (plastic and metal bulldog) but not with the microvascular clamp, which has the highest clamping pressure. On the contrary, decreased VEGF expressions were demonstrated in the microvascular clamp group.

Endothelial impairment with vascular clamps was previously described. However, to the best of our knowledge, this is the unique study that reports the interaction between VEGF expression and clamping force with vascular clamps. Additionally, this experimental study determines the relationship between injury grades and VEGF expression. We believe that these results may provide a viewpoint for further studies to investigate the effects of external forces on vascular endothelial structures.

This study has two major limitations. First, the clamp effects were investigated on rat aorta. Therefore, the findings reflect the animal genus, and further studies should be conducted on human subjects for confirmation. Second, a threshold force for clamping pressure, which leads to definitive injury, was not set. This study could not define a threshold force to induce vascular injury.

In conclusion, endothelial injury seems to occur with all types of vascular clamps, according to the results of the current study. Additionally, partially lower clamping pressures lead to increased vascular endothelial growth factor expression with cellular damage, which can cause late stenosis with neointimal hyperplasia. However, higher clamping pressures

seem to lead to cellular loss and decreased vascular endothelial growth factor expression, which could result in early vascular occlusion with thrombosis. Therefore, no-touch techniques are convenient for endothelial protection strategies. Despite the knowledge about the damage clamps can cause, they are still widely used vascular devices in the field of vascular surgery, and further studies are required for determining safe and available strategies.

Declaration of conflicting interests

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