The effect of atorvastatin on survival of rat ischemic flap

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Abstract  Management of skin avulsion with tissue exposure is a challenge for plastic surgeons. Clinical observations have suggested that longer survival of skin flap prevents further contamination and infection. Less well known is the role of atorvastatin in avulsion skin flap. Therefore, we attempted to determine whether atorvastatin could alleviate avulsion skin flap in a rat model. Twenty male Sprague–Dawley rats were randomized into two groups: the atorvastatin group and the control. Before operation, each rat received an initial blood perfusion scan as baseline data. Then, each rat received an operation of skin flap incision, elevation, and resuturing to the original position under general anesthesia. Another blood perfusion scan was performed on each rat 30 minutes, 4 days, and 7 days postoperatively. On the 7th postoperative day, the necrotic area of skin flap was measured as the skin flap viability. The skin flap tissues at 2.5 and 5 cm distal to the skin flap base were collected for histopathological analysis, as well as measurement of vascular endothelial growth factor (VEGF) mRNA expression, and vascular density. Compared with 30 minutes postoperation, there was a significant increase in the ratio of skin flap blood perfusion on the 4th and 7th days postoperation in both control and atorvastatin groups (p < 0.05). Compared with the control group, there was a significant decrease in necrotic area, significant increase in ratio of skin flap blood perfusion on postoperation days 4 and 7, and significant increase in vascular density under high field at 2.5 cm distal to the base of skin flap in the atorvastatin group (p < 0.05). The VEGF121 and VEGF165 mRNA expression at 2.5 cm distal to the base of skin flap differed significantly between the two groups (p < 0.05). Compared with the control group, atorvastatin treatment improved skin flap blood perfusion, vascular density, and necrotic area dependent on VEGF mRNA expression.

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Introduction

Skin abrasion wounds over a large area are common. Recovery of damaged skin over exposed tissue is beneficial not only for further tissue growth but also reduces the risk of infection. Various studies have addressed ischemic skin flap survival. Vascular endothelial growth factor (VEGF; by either pharmacological or gene facilitation) has shown great viability and neovascularization over ischemic skin flaps [1–3]. Other agents such as captopril (angiotensin-converting enzyme inhibitor) [4], sildenaf (phosphodiesterase inhibitors) [5], and aspirin have been demonstrated to be beneficial for skin flap viability, although the detailed mechanisms are still unclear.

Statins are drugs for treatment of hypercholesterolemia which inhibit 3-hydroxy-3-methylglutaryl coenzyme A. In addition to being a cholesterol-lowering class of drug, many studies have suggested that statins are of benefit in the prevention and treatment of various infections [6,7], as well as in wound strengthening and healing [8,9], angiogenesis [10–12], and apoptosis [13]. It was reported that statins stimulated expression of several angiogenic mediators [VEGF, interleukin-8, angiopoietin (Ang)-1, Ang-2, eNOS, and hemoxidase (HO)-1] in an ischemic hind limb model [14]. Reports on the role of statins in preventing the progression of ischemic skin flap are rare. Therefore, in this study, we aimed to determine whether statins alleviated the necrotic area and blood perfusion of the rat ischemic skin flap and to examine the underlying mechanisms.

Materials and methods

Animals

All procedures were performed using Sprague-Dawley rats obtained from the National Science Council, and they were approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University.

Experimental groups

In this study, 20 Sprague-Dawley (300–350 g) rats were divided into two groups. All experimental rats were fed and housed at Kaohsiung Medical University, following the approved guidelines. All rats were handled as follows:

Control group (n = 10): feed normal saline after surgery by lavage for 7 days.

Atorvastatin group (n = 10): feed atorvastatin after surgery (10 mg/kg/day) by lavage for 7 days.

Atorvastatin (Lipitor®; Abbott Co. Ltd., Chicago, IL, USA) was purchased from Kaohsiung Medical University Hospital.

Surgical procedure

All surgical procedures were performed under Zoletil 50 anesthesia (Tiletamine and Zolazepam; Virbac Co. Ltd., Carros Cedex, France; 5 mg/kg, intraperitoneal injection). Dorsal hair was shaved completely before operation. All rats had caudally based McFarlane-type dorsal skin flaps (3 × 10 cm) [15,16]. Along the designated area, the skin including the panniculus carnosus muscle was incised and elevated vertically with the base over the bilateral iliac crest. To leave the skin flap with blood supply only from the pedicle, an elastic sheet of the same area was placed below the flap, and the flap was sutured with a 4–0 nylon suture back to its original location.

Measurement of microcirculation in skin flap

To assess the changes in blood flow in the flap, the laser Doppler imager (MoorLDI2-2®; Moor Instruments, Millwey, UK) was used. Serial measurements of skin vascularity were taken at four time points: before operation, and 30 minutes, 4 days, and 7 days after operation. The blood perfusion change rate was expressed as percentage of value obtained at 30 minutes, 4 days, or 7 days after operation/value obtained before operation.

Evaluation of skin flap survival rate

The flap survival was evaluated on postoperative day 7. A millimetric paper template was used to record the necrotic area of each flap, and the necrotic area was calculated as the percentage of necrotic area to the whole flap.

Tissue harvest

All rats were sacrificed on postoperative day 7. Skin samples 2.5 and 5.0 cm distal to the base of the pedicle were harvested and stored in liquid nitrogen and formalin for further reverse transcription polymerase chain reaction (RT-PCR) and histological assessment, respectively.

Measurement of VEGF mRNA expression

Total RNA was extracted from frozen skin sample with TRIzol (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed with SuperScriptIII (Invitrogen) according to the manufacturer’s instructions. Primers used for amplification of VEGF were as follows: forward 5’-TGC ACC CAC GAC AGG GGA-3’, reverse 5’-TCA CCG CCT TGG CTT GTA ACA T-3’, GAPDH: forward 5’-GTA TGA TCA CAC TCA CGG CAA AT-3’, reverse 5’-GAC TCC ACG ACA TAC TCA GCA C-3’ [17]. The PCR reaction for amplified VEGF and GAPDH was as follows: 95°C for 5 minutes, and than 30 cycles of 95°C for 15 seconds, 58°C for 1 minute and 72°C for 1 minute. The PCR product was analyzed by electrophoresis using 1.5% agarose gel; and the predicted lengths of the PCR products were 360 bp for VEGF [12], 492 bp for VEGF, 564 bp for VEGF, 360 bp for VEGF, 152 bp for GAPDH, respectively.

Histopathological analysis

In each group, the harvested tissue was fixed in 10% buffered formalin and 5-mm section was prepared from paraffin-embedded tissues. Paraffin sections (5 μm thick) were cut and stained with hematoxylin–eosin (H&E) under standard histological methods. Histopathological analysis
was carried out by a pathologist blinded to the research groups.

**Vascular density**

Quantitative assessment of neovascularization was executed under ×400 magnification of the light microscope. The number of vessels within the dermis level under five random high power fields was calculated. An average number of vessels of all fields represented vascular density.

**Statistical analysis**

The data were expressed as mean ± SD. Comparisons of ratio of skin flap blood perfusion at different time points in both control and Atorvastatin groups were determined by one-way analysis of variance, followed by Tukey’s test for post hoc comparisons. Comparisons of other parameters between control and Atorvastatin groups were performed by Mann–Whitney test. A p value of less than 0.05 was considered statistically significant. GraphPad Prism 5.0 was used for all statistics.

**Results**

**Effect of atorvastatin on viability of skin flap**

The effect of atorvastatin on viability of skin flap is illustrated in Fig. 1. The percentages of necrotic area of control and atorvastatin groups were 66.08 ± 4.36% and 43.83 ± 13.12%, respectively. There was a significant difference between the groups (p < 0.05). Fig. 1A and B show the representative skin flap viability on postoperative day 7 in control and atorvastatin-treated rats, respectively.

**Effect of atorvastatin on blood perfusion of skin flap**

The effect of atorvastatin on blood perfusion of skin flap is illustrated in Fig. 2. In the control group, the blood perfusion ratios at 30 minutes, 4 days, and 7 days postoperation were 0.273 ± 0.067, 0.548 ± 0.148, and 0.493 ± 0.193, respectively (Fig. 2B). There was a significant increase in blood perfusion ratio on postoperative day 4 and day 7 compared with 30 minutes postoperation in the control group (p < 0.05). Fig. 2A shows the representative blood perfusion of skin flap in the control group before operation, and 30 minutes, 4 days, and 7 days postoperation.

In the atorvastatin group, the blood perfusion ratios at 30 minutes, 4 days, and 7 days after operation were 0.477 ± 0.208, 0.881 ± 0.279, and 0.905 ± 0.261, respectively (Fig. 2D). There was a significant increase in blood perfusion ratio on postoperative day 4 and day 7 compared with 30 minutes after operation in the control group (p < 0.05). Fig. 2A shows the representative blood perfusion of skin flap in the atorvastatin group before operation, and 30 minutes, 4 days, and 7 days after operation.

Compared with the control group, there was a significant increase in blood perfusion ratio in the atorvastatin group on postoperative day 4 and day 7 (p < 0.05; Fig. 3).

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**Figure 1.** Effect of atorvastatin on survival of skin flap on postoperative day 7. (A and B) Representative pictures of skin flap in the control and atorvastatin groups, respectively. Ratio of necrotic/original area in both groups is illustrated in (C). Each bar represents the mean ratio of necrotic area ± SD of the data pooled from each group (n = 10). *p < 0.05, significant difference in the ratio of necrotic area between control and atorvastatin groups.
Effect of atorvastatin on VEGF mRNA expression

The effect of atorvastatin on VEGF mRNA expression is illustrated in Fig. 4. Fig. 4A and B show VEGF mRNA expression at 2.5 and 5.0 cm distal to the base of the skin flap, respectively. The ratio of VEGF$_{121}$/GAPDH mRNA expression at 2.5 cm distal to the base of the skin flap was $0.223 \pm 0.042$ and $0.431 \pm 0.079$ in control and atorvastatin groups, respectively. The ratio of VEGF$_{165}$/GAPDH mRNA expression at 2.5 cm distal to the base of the skin flap was $0.151 \pm 0.042$ and $0.271 \pm 0.036$ in control and atorvastatin groups, respectively. The ratio of VEGF$_{121}$/GAPDH mRNA and VEGF$_{165}$/GAPDH mRNA expression at 2.5 cm distal to the base of the skin flap showed no significant difference between control and atorvastatin groups.

The ratio of VEGF$_{121}$/GAPDH mRNA expression at 5.0 cm distal to the base of the skin flap was $0.105 \pm 0.091$ and $0.232 \pm 0.093$ in control and atorvastatin groups, respectively. The ratio of VEGF$_{165}$/GAPDH mRNA expression at 5.0 cm distal to the base of the skin flap was $0.080 \pm 0.060$ and $0.093 \pm 0.040$ in control and atorvastatin groups, respectively. The ratio of VEGF$_{121}$/GAPDH mRNA and VEGF$_{165}$/GAPDH mRNA expression at 5.0 cm distal to the base of the skin flap showed no significant difference between control and atorvastatin groups.
Effect of atorvastatin on vascular density of skin flap

The effect of atorvastatin on vascular density of skin flap is illustrated in Fig. 5. Fig. 5A and B show representative histopathological pictures at 2.5 cm distal to the base of the skin flap in control and atorvastatin-treated rats, respectively. Fig. 5C and D show representative histopathological pictures at 5.0 cm distal to the base of the skin flap in control and atorvastatin-treated rats, respectively. The average vascular density under high field at 2.5 cm distal to the base of the skin flap was 1.5 ± 1.12 and 3.86 ± 0.82 in control and atorvastatin groups, respectively (Fig. 5E). There was a significant difference between groups at 2.5 cm distal to the base of the skin flap (p < 0.05). The average vascular density under high field at 5.0 cm distal to the base of the skin flap was 0.45 ± 0.88 and 0.32 ± 0.36 in control and atorvastatin groups, respectively (Fig. 5F). There was no significant difference between groups at 5.0 cm distal to the base of the skin flap (p > 0.05).

Discussion

Ischemic flap treated with VEGF gene therapy showed improved ischemic flap survival and neoangiogenesis [1,2]. Agents such as sildenafil, celecoxib, and angiotensin-converting enzyme inhibitor were proven to improve ischemic flaps by induction of VEGF [4,5,18]. Some studies have suggested that statins in different doses seem to produce biphasic effects in angiogenesis. In their review study, Hindler et al. [19] suggested that low doses of statins facilitate tumor angiogenesis by stimulation of protein kinase B and/or activation of endothelial nitric oxide synthase, and high doses of statins inhibit angiogenesis by inhibition of capillary tube formation and/or decreased endothelial vascular growth factor release. In another inflammation-related study, the angiogenesis was enhanced with low-dose statin therapy (0.5 mg/kg/day) but was significantly inhibited with high concentrations of cerivastatin or atorvastatin (2.5 mg/kg/day) [20]. The result of our study suggested that atorvastatin (10 mg/kg/day) improved flap necrotic area in rat skin flap models when compared with the control group, but the dose in this study could be considered high-dose statin therapy compared with previous studies. However, one study suggested that atorvastatin (10 mg/kg/day) strongly induced angiogenesis in ischemic hind limbs [14]. Therefore, the definite borderline of high/low dose of statins seems to have no standard. More related studies are necessary.

The result of this study showed better blood perfusion of ischemic skin flap on postoperative days 4 and 7 than 30 minutes after operation in both control and atorvastatin groups. In a similar study presented by Lindenblat et al. [21], capillary widening in the wound bed appeared at day 1 after grafting and increased until day 4. The maximum expression of hypoxic-inducible factor-1α (HIF-1α) appeared within the 1st 48 hours, but the peak of VEGF expression occurred at 72 hours [21]. Multiple HIF-1 target genes have been shown to modulate angiogenesis by promoting the mitogenic and migratory activities of endothelial cells [22]. Lindenblat et al.’s finding may explain how the ischemic skin flap restored blood perfusion in the animal model.

In Matsumura et al.’s [14] study, atorvastatin strongly induced angiogenesis with increases in expressions of VEGF, interleukin-8, Ang-1, Ang-2, eNOS, and HO-1 proteins in ischemic hind limbs. Similar results were also proven in an in vitro study, which suggested that atorvastatin stimulated the expression of Ang-2, eNOS in human umbilical endothelial cells [23]. The findings in our study showed that atorvastatin significantly increased VEGF mRNA expression on postoperative day 7 as compared with those without treatments, which matched the finding of improved blood perfusion and vascular density.

In Uygur et al.’s [24] study, tissue was harvested from 1, 3, and 5 cm distal to the base of the flap. We considered that there should be no differences between control and...
The flap necrotic area and blood perfusion in this study were designed for gross observation of this flap. We found that atorvastatin does decrease the necrotic area (avascular zone) compared with the control group, as demonstrated by the blood perfusion scan. However, the perfusion scan showed non-homogenous distribution of vessel over all flap. Therefore, we harvested tissue 2.5 and 5 cm distal to the base of the flap to observe the vascular density and the expression of VEGF mRNA. We found that there is no difference between atorvastatin and control groups at 5 cm distal to the base of the flap, but that there is significant difference at 2.5 cm distal to the base of the flap. These results suggested that the increased vascular density and VEGF mRNA expression in proximal site of atorvastatin group could supply more reliable flap area although no differences were noted in vascular density and VEGF mRNA expression in the control portion.

In conclusion, compared with the control group, atorvastatin improved skin flap blood perfusion, vascular density, and necrotic area via VEGF-dependent pathway.

References


