

## Original Article

# Calcitriol promotes survival of experimental random pattern flap via activation of autophagy

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**Abstract:** Calcitriol can promote random pattern flap survival and increase autophagy activity. However, effect of autophagy on flap survival after calcitriol treatment, along with the underlying mechanism, is undefined. In this study, the "McFarlane flap" model was established in 72 rats divided into control, calcitriol, and calcitriol+3-methyladenine (3MA) groups and injected with saline, calcitriol, and calcitriol plus 3MA, respectively. The percentage flap survival area and tissue water content were tested after 7 days. The extent of autophagy was evaluated by the expression of the autophagy markers LC3-II/I, Beclin1, and p62. Flap angiogenesis was assessed via hematoxylin and eosin (H&E) staining and immunohistochemistry (for CD34 levels). The level of vascular endothelial growth factor (VEGF) protein/mRNA was measured. Oxidative stress was assessed by measuring the activity of tissue superoxide dismutase (SOD) and the contents of glutathione (GSH) and malondialdehyde (MDA). Our results showed that 3MA decreased autophagy in random skin flap treated with calcitriol. Compared with the calcitriol group, the calcitriol+3MA group showed a smaller mean flap survival area and greater tissue edema, had a markedly decreased level of VEGF mRNA/protein and SOD activity, and a significantly higher level of MDA and GSH. H&E staining and immunohistochemistry showed that angiogenesis was inhibited in this group. In conclusion, calcitriol increased angiogenesis and reduced oxidative stress via activation of autophagy, contributing to a significant increase in random skin flap survival.

**Keywords:** Calcitriol, random pattern flap survival, autophagy, angiopoiesis, oxidative stress

## Introduction

Random pattern skin flap is widely used in plastic and reconstructive surgery to cover a variety of defects caused by traumatic skin injury or body surface tumor removal [1]. Random skin flaps lack specific vessels and their blood supply depends mainly on the connected new vasoganglions in the pedicle bed of the flap [2]. The blood supply of vasoganglions gradually decreases from the pedicle to the distal part; thus, the distal end of the random pattern skin flap usually suffers ischemia and necrosis. Although flap design and surgical techniques have improved, the ratio of length to width is unable to exceed 1.5 to 2:1, limiting clinical application [3]. An increasing number of studies have demonstrated that ischemia, and even

necrosis, is due to oxidative stress and an inadequate blood supply [4]. As a consequence, inhibition of oxidative stress and acceleration of angiogenesis are the main strategies for promoting the survival of random pattern skin flap.

Calcitriol (C<sub>27</sub>H<sub>44</sub>O<sub>3</sub>), known as 1,25-dihydroxyvitamin D<sub>3</sub>, has biological functions in bone and calcium metabolism and plays a key role in osteoporosis [5]. In addition, its functions of promoting angiopoiesis, reducing oxidative stress, and improving the activity of autophagy, etc., have recently been investigated [6-8]. Because of this, calcitriol was primarily used for the improvement of random pattern skin flap survival in our previous study. Our results suggested that calcitriol increased angiogenesis and reduced oxidative stress, contributing to a

significant increase in random flap survival. Furthermore, we found that autophagy was increased in flaps treated with calcitriol [9].

Autophagy, literally “self-eating”, is the process by which cells degrade cytosolic macromolecules and organelles in lysosomes, and is a survival tactic used to protect against metabolic stress [10]. On the other hand, autophagy is also generally considered to be a specific type of programmed cell death that shares many of the pathways and mechanisms of cell apoptosis [11]. The role of autophagy in the molecular mechanism underlying calcitriol treatment in random pattern flap, however, has yet to be defined. Therefore, the compound 3-methyladenine (3MA), a widely-used and powerful inhibitor of autophagy [12], was applied to down-regulate the level of autophagy in the current study. The effect of autophagy on random skin flap survival after calcitriol treatment, and the underlying mechanism of action of calcitriol with respect to its association with angiopoiesis or oxidative stress, was explored in this study.

### Materials and methods

#### *Animals*

Healthy Sprague-Dawley rats (Male, 250-300 g) were provided from Wenzhou Medical University (license no. SCXK [ZJ] 2005-0019). The animal operation and treatment conformed to the Guide for the Care and Use of Laboratory Animals of China National Institutes of Health. All procedures were approved by the Animal Care and Use Committee of Wenzhou Medical University (wydw 2012-0079). The best efforts were made to reduce the suffering of rats in the experiment. All rats were removed from the study and euthanized with an overdose of pentobarbital sodium. 72 rats were randomly separated into three groups (each group having 24 rats), including Control group, Calcitriol group and Calcitriol+3MA group.

#### *Animal model of random skin flap*

The administration of 2% (w/v) pentobarbital sodium (40 mg/kg, Solarbio Science & Technology, Beijing, China) was applied to anaesthetize rats via intraperitoneal injection. The modified “McFarlane” flap model [13] was employed

in the rat dorsum (in the same position in each rat). The caudal skin/panniculus carnosus flap in the size of 3 cm × 9 cm was outlined on the rat's back. After that, both sacral arteries in right and left were sectioned. After separation from the underlying fascia, the flap was immediately sutured to the donor bed using 4-0 silk and a wedged-on cutting needle. The area of random skin flap was separated into three equal zones: proximal (area I), intermediate (area II), and distal (area III).

#### *Experimental protocol*

The Calcitriol group ( $n = 24$ ) received intraperitoneal calcitriol (Cayman, Ann Arbor, MI, USA) injection at 2  $\mu\text{g}/\text{kg}/\text{day}$  for 7 consecutive days. The Calcitriol+3MA group ( $n = 24$ ) received the intraperitoneal administration of 2  $\mu\text{g}/\text{kg}/\text{day}$  calcitriol and 0.5 mg/kg/day 3MA (Sigma-Aldrich, St Louis, MO, USA) for 7 successive days. The saline group ( $n = 24$ ) received equal volumes of saline for 7 days. The first drug injection was given 2 h after the surgical procedure. All animals were housed individually in standard experimental cages in an environmentally controlled room and were provided with standard rat chow and water *ad libitum*. All rats were euthanized with an overdose of pentobarbital sodium at 7 days.

#### *General observation and flap survival assessment*

The macroscopic changes of the random skin flap was noted for 7 days, which included appearance, colour, texture, and hair condition. 7 days after operation, the survival areas of flaps were measured by superimposition of photographs on graph paper. The percentage of viable area was calculated as: extent of viable area  $\times 100\%$ /total area (viable and ischemic).

#### *Tissue edema measurement*

Tissue edema of flap was reflected by water content. On postoperative day 7, flap tissues were weighed and then dehydrated in an autoclave at 50°C. All samples were weighed every day until the weight was stabilized for 2 days. The percentage of water content was determined as follows: Tissue % water content =  $([\text{wet weight} - \text{dry weight}]/\text{wet weight}) \times 100\%$ .

## Role of calcitriol and autophagy in experimental pattern flap

### *Hematoxylin and eosin (H&E) staining*

Three samples (1 cm × 1 cm) of central tissue from area II of each group were collected and biopsied after animal sacrifice. These samples were post-fixed in 4% (v/v) paraformaldehyde for one day and embedded in paraffin wax for transverse sectioning. The sections (10 μm in thickness) were mounted on poly-L-lysine-coated slides for hematoxylin and eosin staining. Under a light microscope (× 200 magnification), the thickness of granulation tissue, edema, and neutrophil infiltration were observed. Finally, we calculated the number of microvessels per unit area (/mm<sup>2</sup>), which is a common indicator of microvascular density.

### *Superoxide dismutase (SOD) activity, glutathione (GSH) level and malondialdehyde (MDA) content test*

SOD, GSH, and MDA testing kits were used to detect the oxidative stress status of ischemic flaps. At day 7 after the operation, tissue samples (0.5 cm × 0.5 cm) were separated from the middle area of Area II flaps in each group (n = 6), weighed, homogenized, and diluted to 10% (v/v) in an ice bath. Then homogenate was centrifuged at 3500 r.p.m (revolutions per min) for 15 min. After that, the supernatant solution of homogenate was collected for following indices testing. SOD activity is tested via a xanthine oxidase method. 0.1 ml homogenate was mixed with 1.4 ml xanthine oxidase and then maintained in a water bath at 37°C for 40 min. Afterwards, 2.0 ml developer was added for shade selection at 550 nm following 10 min of incubation at room temperature. GSH content was detected by using a modified DTNB method: 0.5 ml homogenate was mixed with 2 ml DTNB, and then centrifuged at 4000 r.p.m for 10 min. Then the supernatant (1 ml) was collected, and then reacted with 1.55 ml developer for 5 min incubation at room temperature. Finally, this reacted solution was carried out for shade selection at 420 nm. MDA content was tested via a modified TBA test: 0.1 ml homogenate was mixed with 0.1 ml dehydrated alcohol, 0.1 ml TBA and 4.0 ml developer, and maintained in a water bath at 95°C for 40 min. Then this mix solution cooled in running water and centrifuged at 4000 r.p.m for 10 min. The supernatant was collected and its absorbance was measured at 532 nm.

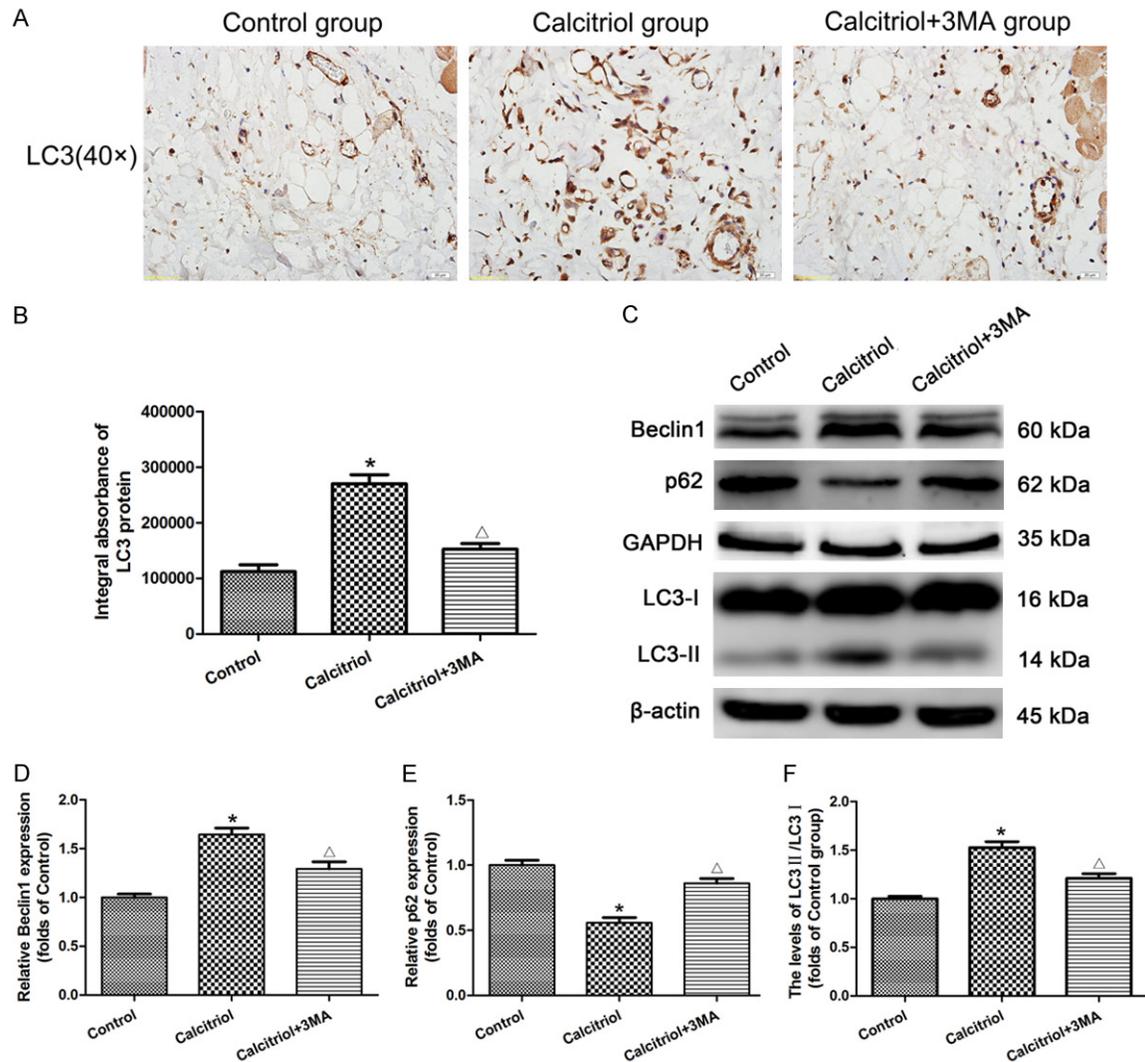
### *Immunohistochemistry*

Six sections specimens of area II of each group were deparaffinized in xylene and rehydrated through a graded set of ethanol baths. After washing, sections were blocked with 3% (v/v) H<sub>2</sub>O<sub>2</sub> and treated with 10.2 mM sodium citrate buffer (antigenretrieval) for 20 min at 95°C. After blocking with 5% (w/v) bovine serum albumin and 1% (v/v) Tween-20 (Solarbio Science and Technology, Beijing, China) in phosphate-buffered saline for 10 min, the sections were incubated with antibody against vascular endothelial growth factor (VEGF) (1:300; Bioworld, Nanjing, China), LC3 (1:400; Cell Signaling Technology; Danvers, MA, USA) and CD34 (1:100, Abcam, Cambridge, MA, USA) overnight at 4°C. Finally, the sections were incubated with an appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and counterstained with hematoxylin. Flap tissues were imaged at × 40 magnification using a DP2-TWAN image-acquisition system (Olympus Corp., Tokyo, Japan). Observation parameters (white balance, aperture, shutter speed, and time) were held constant. Images were saved using Image-Pro Plus software, version 6.0 (Media Cybernetics, Rockville, MD, USA) and the integral absorbance (IA) values were used as indicators of VEGF and LC3 expression levels. The numbers of CD34-positive blood vessels per unit area (mm<sup>2</sup>) were calculated.

### *In situ hybridization*

A VEGF mRNA in situ hybridization kit (Boster Inc., Wuhan, China) was used to detect the level of VEGF mRNA. The probe sequences were 5'-GCTCT ACCTC CACCA TGCCA AGTGG TCCCA-3', 5'-GACCC TGGTG GACAT CTTCC AGGAG TACCC-3', and 5'-GCAGC TTGAG TTAAG CGAAC GTACT TGCAG-3'. The procedure was carried out according to the kit instructions. After staining with DAB, the sections were dehydrated with graded ethanol, mounted with xylene, and sealed. Then the flap tissues were imaged at × 400 magnifications using a DP2-TWAN image-acquisition system (Olympus Corp). Observation parameters (white balance, aperture, shutter speed, and time) were held constant. Images were saved using the Image-Pro Plus software (ver. 6.0; Media Cybernetics) and the IA values were used as indicators of VEGF mRNA levels.

## Role of calcitriol and autophagy in experimental pattern flap



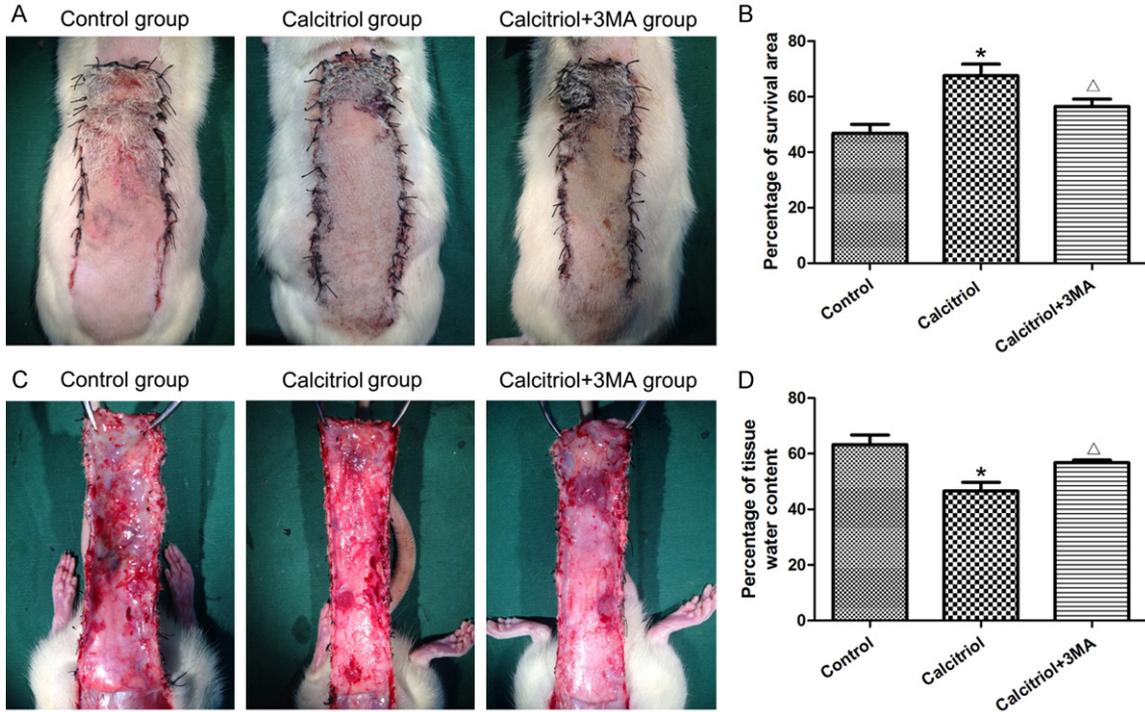
**Figure 1.** The treatment of 3MA suppressed the calcitriol-induced autophagy in random skin flap. A. LC3II punctuated dots were showed in all groups through Immunohistochemistry (original magnification × 400). B. Integral absorbance (IA) value of LC3 in control group was  $112260.80 \pm 12790.34$ , in the calcitriol group was  $270010.00 \pm 36742.35$  and  $152373.2 \pm 23553.52$  in Calcitriol+3MA group. C. Protein expression of LC3II/I, Beclin1 and p62 in each group through Western Blot Analysis. D-F. The optical density analysis of the ratio of LC3II/LC3I, Beclin1 and p62 proteins expression was presented in these groups. Values are expressed as the mean  $\pm$  SEM,  $n = 6$  per group. \* $P < 0.05$  versus the control group;  $^{\Delta}P < 0.05$  versus the calcitriol group.

### Western blot analysis

On day 7 after operation, samples ( $1 \times 1$  cm) from area II were dissected and stored at  $-80^{\circ}\text{C}$  prior to Western blot analysis. The concentration of protein was determined using the BCA assay (Thermo, Rockford, IL, USA). Seventy microgram amounts of protein were separated on a 12% (w/v) gel and transferred onto PVDF membranes (Roche Applied Science, Indianapolis, IN). After blocking with 5% (w/v) non-fat milk for 2 h, the membranes were incubated

with antibodies against VEGF (1:300; Bioworld, Nanjing, China), GAPDH (1:1000; Abcam, Cambridge, MA, USA) and Beclin1, p62, LC3 (1:1000; Cell Signaling Technology, Beverly, MA, USA), and  $\beta$ -actin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After that, the membranes were incubated with a goat-anti-rabbit secondary antibody for 2 h at room temperature and bands were detected using the ECL-plus reagent kit (PerkinElmer, Waltham, MA, USA). Band intensity was quantified using the Image Lab 3.0 software (Bio-Rad).

## Role of calcitriol and autophagy in experimental pattern flap



**Figure 2.** Down-regulation of autophagy reduced the effect of calcitriol on flap survival area and tissue oedema. A. Digital photographs showed the characterization of postoperative flaps of the control group, the calcitriol group and the calcitriol+3MA group on Day 7. B. The histogram of percentages of survival area in the control group was  $46.80 \pm 7.19\%$ ,  $67.60 \pm 9.24\%$  in the calcitriol group and  $56.40 \pm 6.07\%$  in the Calcitriol+3MA group, respectively. C. Digital photographs exhibited the tissue oedema of postoperative flaps of all groups on Day 7. D. The histogram of percentages of tissue water content in the control group, the calcitriol group and the calcitriol+3MA group was  $63.20 \pm 7.89\%$ ,  $46.60 \pm 6.95\%$  and  $56.80 \pm 2.05\%$  respectively. Values are expressed as the mean  $\pm$  SEM,  $n = 6$  per group. \* $P < 0.05$  versus the control group;  $^{\Delta}P < 0.05$  versus the calcitriol group.

### Statistical analysis

Statistical analyses were carried out using the SPSS.20 statistical software. All data were presented as mean  $\pm$  S.E.M. Statistical evaluation of the data was performed by one-way analysis of variance followed by a post hoc comparison test using the least significant difference (equal variances assumed) or Dunnett's T3 (equal variances not assumed) method.  $P < 0.05$  was considered to indicate statistical significance.

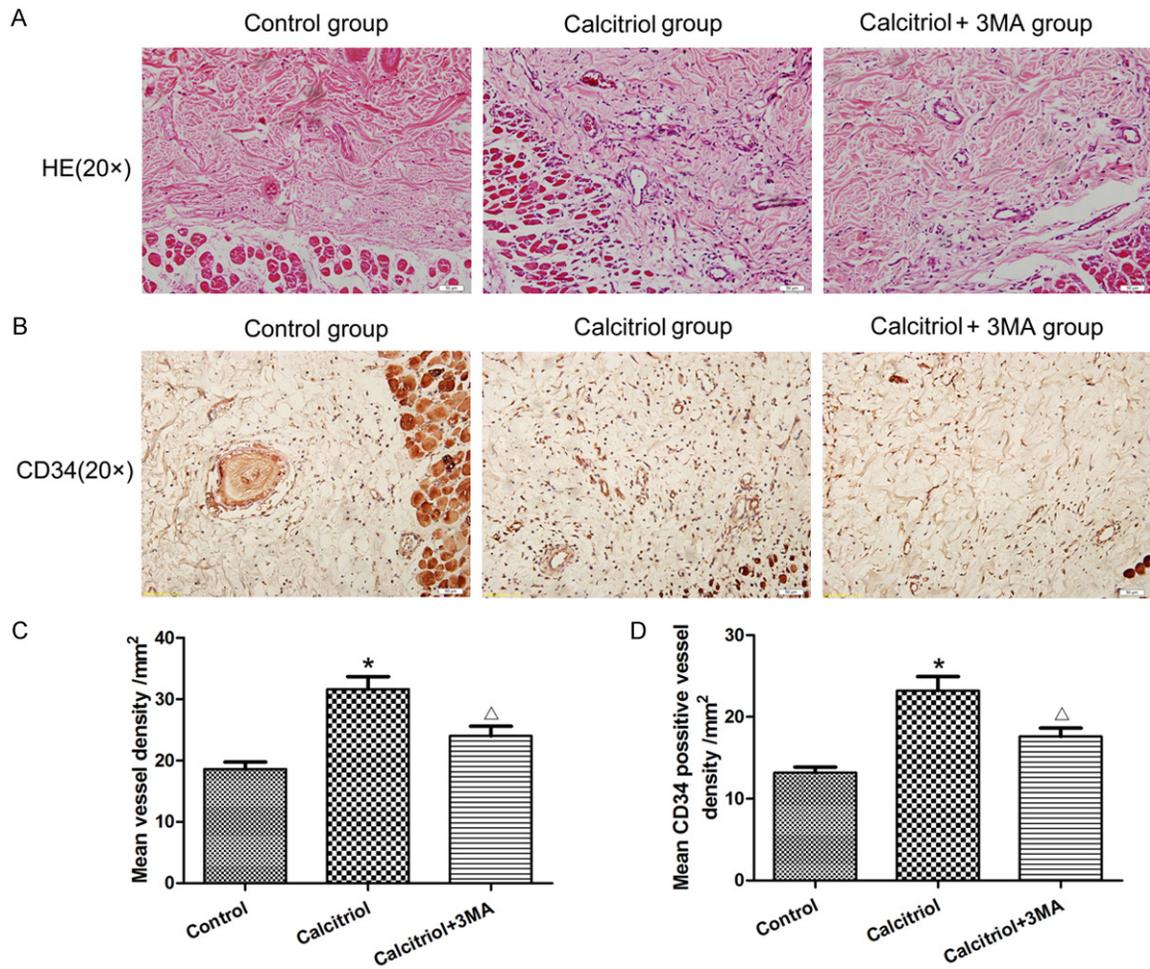
### Results

#### *Treatment with 3MA suppressed calcitriol-induced autophagy in random pattern flap*

To assess autophagy activity in random skin flap, Immunohistochemistry staining for LC3-II punctate dots was performed in each group 7 days after the operation (**Figure 1A**). As shown in **Figure 1B**, calcitriol significantly increased

the IA value of the LC3 expression in skin flap ( $P < 0.05$ ). However, the calcitriol+3MA group showed a significantly decreased IA value of LC3 expression relative to the calcitriol-only group ( $P < 0.05$ ). Western blotting was carried out to detect LC3-II/LC3-I, Beclin1, and p62 expressions in Area II of random skin flap (**Figure 1C**). The LC3-II/LC3-I ratio and Beclin1 expression were significantly increased in flap following treatment with calcitriol, but the effect of calcitriol was suppressed after treatment with 3MA (**Figure 1D** and **1E**;  $P < 0.05$ ). The expression level of p62 in calcitriol group was also detected by Western blotting (**Figure 1C**), and was found to be much lower than that in the control group ( $P < 0.05$ ). However, 3MA significantly down-regulated p62 level in the flap after administration of calcitriol (**Figure 1F**,  $P < 0.05$ ). Taken together, these results provide evidence that the treatment of 3MA suppressed calcitriol-induced autophagy in random skin flap.

## Role of calcitriol and autophagy in experimental pattern flap



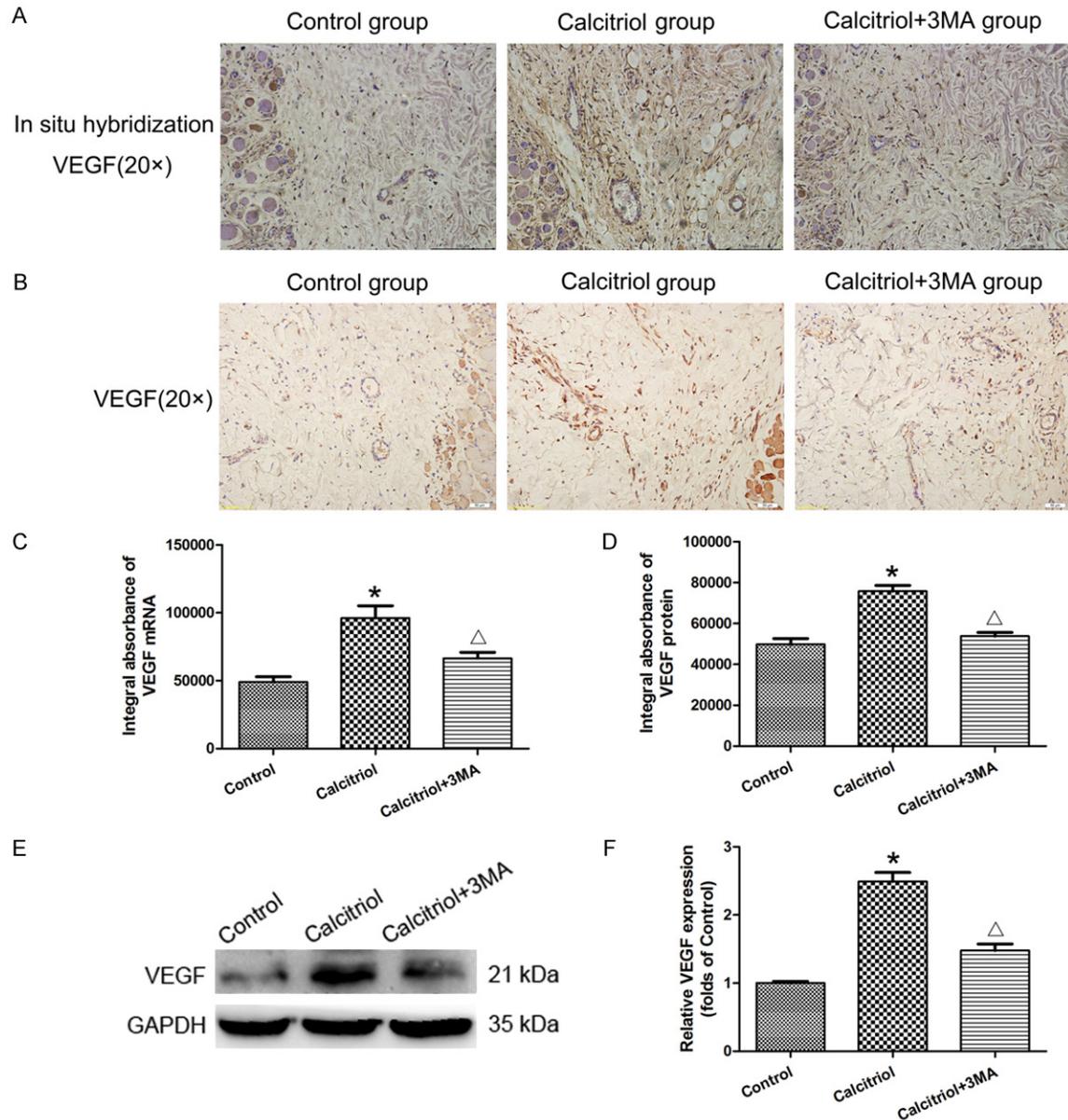
**Figure 3.** Inhibition of autophagy reduced vascularisation in calcitriol-treated skin flap. A. Neovascularization in the control, the calcitriol and the calcitriol+3MA groups by H&E staining. (Original magnification × 200). C. The histogram of percentages of MVDs in the control group and the calcitriol group were  $18.60 \pm 2.61/\text{mm}^2$  and  $31.60 \pm 4.67/\text{mm}^2$ , and  $24.01 \pm 3.54/\text{mm}^2$  in the calcitriol+3MA group respectively. B. Immunohistochemistry for CD34 positive vessels in these three groups. D. The number of CD34 positive vessels/ $\text{mm}^2$  in the control group was  $13.20 \pm 3.89/\text{mm}^2$ , in the calcitriol group was  $24.67 \pm 3.89/\text{mm}^2$  and  $17.60 \pm 2.30/\text{mm}^2$  in the calcitriol+3MA group, respectively. Values are expressed as the mean  $\pm$  SEM, n = 6 per group. \* $P < 0.05$  versus the control group;  $\Delta P < 0.05$  versus the calcitriol group.

### *Down-regulation of autophagy reduced the effect of calcitriol on the flap survival area and tissue edema*

The random skin flaps in all groups become pale and swollen on the first day after the operation, and were gray or purple in color (without obvious necrosis) in Area III of these groups. However, 3 days postoperatively, the flaps were darker, and showed some evidence of necrosis associated with a brown nidus at Areas II and III in all groups. On the 7<sup>th</sup> day after the operation, Area I of each group had survived, whereas Area III had become darker with necrosis spreading to Area II, along with scabbing and hardening. The boundaries between the necro-

sis and areas of survival were clear (**Figure 2A**). As shown in **Figure 2A**, the survival area in the calcitriol group was larger than that in the control group. However, the calcitriol+3MA group showed a significantly decreased survival area relative to the calcitriol-only group. The percentage of skin flap surviving 7 days after the operation was  $46.80 \pm 7.19\%$  and  $67.60 \pm 9.24\%$ , in the control group and calcitriol group (**Figure 2B**,  $P < 0.05$ ), respectively. In the calcitriol+3MA group, the percentage ( $56.40 \pm 6.07\%$ ) was significantly lower than that in the calcitriol group. Moreover, as shown in **Figure 2C** and **2D**, the percentage of tissue water content in the control and calcitriol groups was  $63.20 \pm 7.89\%$  and  $46.60 \pm 6.95\%$ , respective-

## Role of calcitriol and autophagy in experimental pattern flap



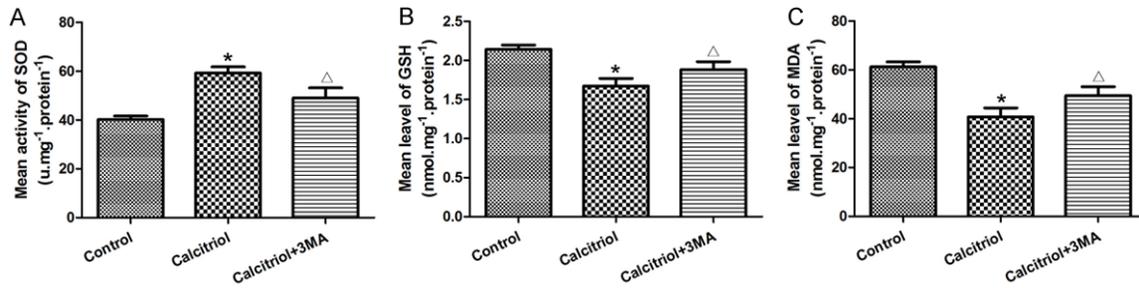
**Figure 4.** The suppression of autophagy down-regulated the levels of VEGF mRNA/protein in calcitriol-treated skin flap. A. In situ hybridization for VEGF mRNA in all groups (original magnification × 200). B. VEGF expression in all groups through Immunohistochemistry (original magnification × 200). C. The integral absorbance (IA) value VEGF mRNA in the control, the calcitriol and the calcitriol+3MA groups was  $49056.80 \pm 8673.32$ ,  $96172.20 \pm 20242.82$  and  $62606.40 \pm 5883.64$ , respectively. D. The integral absorbance (IA) value of VEGF protein in the control group was  $49833.06 \pm 6258.63$ , in the calcitriol group was  $75796.20 \pm 6333.97$ , and  $53823.80 \pm 4076.39$  in the calcitriol+3MA group. E. Protein expression of VEGF in these three groups by western blot analysis. F. Densitometry results of VEGF protein expression in all groups. Values are expressed as the mean ± SEM, n = 6 per group. \* $P < 0.05$  versus the control group; <sup>Δ</sup> $P < 0.05$  versus the calcitriol group.

ly ( $P < 0.05$ ). In the calcitriol+3MA group, the value of  $56.80 \pm 2.05\%$  was significantly higher than that in the calcitriol group ( $P < 0.05$ ). This indicated that calcitriol decreased tissue edema in the skin flap and that this function was restrained after the addition of 3MA.

### *Inhibition of autophagy reduced vascularization in calcitriol-treated flap*

On day 7 after the operation, the histology of Area II of all flaps in the control, calcitriol, and calcitriol+3MA groups were observed via H&E

## Role of calcitriol and autophagy in experimental pattern flap



**Figure 5.** Down-regulation of autophagy abolished the effect of calcitriol on oxidative stress status in flap. A. The level of SOD activity was  $40.20 \pm 3.19 \text{ u mg}^{-1} \text{ protein}^{-1}$  in the control group,  $59.20 \pm 5.72 \text{ u mg}^{-1} \text{ protein}^{-1}$  in the calcitriol group and  $49.00 \pm 9.35 \text{ u mg}^{-1} \text{ protein}^{-1}$  in the calcitriol+3MA group. B. The level of GSH content was  $2.14 \pm 0.59 \text{ nmol mg}^{-1} \text{ protein}^{-1}$  in the control group,  $1.71 \pm 0.08 \text{ nmol mg}^{-1} \text{ protein}^{-1}$  in the calcitriol group and  $1.90 \pm 0.12 \text{ nmol mg}^{-1} \text{ protein}^{-1}$  in the calcitriol+3MA group. C. Treatment with calcitriol demonstrated level of MAD was  $49.40 \pm 7.12 \text{ nmol mg}^{-1} \text{ protein}^{-1}$ ; the content in the control group and the calcitriol+3MA group was  $61.60 \pm 5.94 \text{ nmol mg}^{-1} \text{ protein}^{-1}$  and  $41.01 \pm 3.87 \text{ nmol mg}^{-1} \text{ protein}^{-1}$ , respectively. Values are expressed as the mean  $\pm$  SEM,  $n = 6$  per group. \* $P < 0.05$  versus Control group;  $^{\Delta}P < 0.05$  versus Calcitriol group.

staining. As shown in **Figure 3A**, treatment with calcitriol significantly increased the rate of vascularization and reduced necrosis in Area II of the random skin flap, whereas these were inhibited by the administration of 3MA. The respective microvascular density (MVD) value for Area II in the control and calcitriol groups was  $18.60 \pm 2.61/\text{mm}^2$  and  $31.60 \pm 4.67/\text{mm}^2$  ( $P < 0.05$ ), whereas the value ( $24.01 \pm 3.54/\text{mm}^2$ ) in the calcitriol+3MA group was decreased compared with that of the calcitriol group (**Figure 3C**;  $P < 0.05$ ). CD34 is a common marker for endothelial cells. Therefore, the MVDs of Area II in the three groups were also directly reflected in the number of CD34-positive vessels/ $\text{mm}^2$  (**Figure 3B**). The administration of calcitriol significantly increased the number of CD34-positive vessels ( $24.67 \pm 3.89/\text{mm}^2$ ) in the calcitriol group compared with the number ( $13.20 \pm 3.89/\text{mm}^2$ ) in the control group ( $P < 0.05$ ). However, the number of vessels in the calcitriol+3MA group ( $17.60 \pm 2.30/\text{mm}^2$ ) was decreased compared with that in the calcitriol group (**Figure 3D**;  $P < 0.05$ ).

### *Suppression of autophagy down-regulated the level of vascular endothelial growth factor (VEGF) mRNA/protein in calcitriol-treated flap*

In situ hybridization was performed to assess the VEGF mRNA level in Area II of all groups. As shown in **Figure 4A**, a greater amount of VEGF mRNA was synthesized by vessels and stromal cells in the dermis of flap in the calcitriol group compared with the control group. However, the level of VEGF mRNA in the calcitriol+3MA group

was lower than that in the calcitriol group. Based on calculations of the IA (**Figure 4C**), the levels of VEGF mRNA in the control and calcitriol groups were  $49,056.80 \pm 8,673.32$  and  $96,172.20 \pm 20,242.82$ , respectively ( $P < 0.05$ ), whereas the value in the calcitriol+3MA group ( $62606.40 \pm 5883.64$ ) was significantly lower than that of the calcitriol group ( $P < 0.05$ ). Immunohistochemistry was performed to distinguish the VEGF protein-expressing cells (**Figure 4B**). This protein was expressed in vessels and stromal cells in the dermis of the random skin flaps of these groups. Greater expression of VEGF was observed in the calcitriol group compared with the control group. However, 3MA down-regulated the level of VEGF protein in calcitriol-treated skin flap. The IA values of the VEGF protein in the control and calcitriol groups were  $49,833.06 \pm 6,258.63$  and  $75,796.20 \pm 6,333.97$ , respectively ( $P < 0.05$ ), whereas the value in the calcitriol+3MA group ( $53,823.80 \pm 4,076.39$ ) was significantly lower than that of the calcitriol group (**Figure 4D**;  $P < 0.05$ ). Western blot analysis also indicated that the calcitriol group expressed more VEGF than the control group, but 3MA down-regulated the level of VEGF protein in the calcitriol-treated group (**Figure 4E and 4F**;  $P < 0.05$ ).

### *Down-regulation of autophagy abolished the effect of calcitriol on the oxidative stress status in flap*

Mean SOD activity and mean contents of MDA and GSH in each group ( $n = 6$  per group) were compared. As shown in **Figure 5A**, the level of

SOD activity was  $40.20 \pm 3.19$  U  $\text{mg}^{-1}$  protein $^{-1}$  in the control group and  $59.20 \pm 5.72$  U  $\text{mg}^{-1}$  protein $^{-1}$  in the calcitriol group ( $P < 0.05$ ). Moreover, the value ( $49.00 \pm 9.35$  U  $\text{mg}^{-1}$  protein $^{-1}$ ) in calcitriol+3MA group was significantly higher than that in calcitriol group ( $P < 0.05$ ). In **Figure 5B**, the content of GSH was  $2.14 \pm 0.59$  nmol  $\text{mg}^{-1}$  protein $^{-1}$  in control group and  $1.71 \pm 0.08$  nmol  $\text{mg}^{-1}$  protein $^{-1}$  in calcitriol group ( $P < 0.05$ ); in calcitriol+3MA group, the value ( $1.90 \pm 0.12$  nmol  $\text{mg}^{-1}$  protein $^{-1}$ ) was significantly higher than that in calcitriol group ( $P < 0.05$ ). As shown in **Figure 5C**, treatment with calcitriol was associated with a lower MDA level ( $49.40 \pm 7.12$  nmol  $\text{mg}^{-1}$  protein $^{-1}$ ) compared with that of the control group ( $P < 0.05$ ), whereas the value in the calcitriol+3MA group ( $41.01 \pm 3.87$  nmol  $\text{mg}^{-1}$  protein $^{-1}$ ) was significantly lower than that of the calcitriol group ( $P < 0.05$ ).

### Discussion

It is well known that calcitriol ( $\text{C}_{44}\text{H}_{80}\text{O}_4\text{Si}_2$ , also known as 1,25-dihydroxyvitamin  $\text{D}_3$ ), has biological functions in bone and calcium metabolism and plays an important role in osteoporosis [14]. Recently, however, an increasing number of studies have focused on additional bioactivities, including promotion of angiogenesis [6], reduction of oxidative stress [15], and up-regulation of autophagy [16]. In our previous study, calcitriol was primarily used to increase the area of survival in the distal section of flap. It was found that calcitriol could enhance random skin flap viability by promoting vascularization and reducing oxidative stress. Furthermore, autophagy was increased in skin flap treated with calcitriol. To our knowledge, the role of calcitriol-mediated activation of autophagy in random skin flap has not been defined. Moreover, any role played by autophagy in the skin flap model was unclear. Therefore, it was necessary to define the function of autophagy in random pattern flap after the administration of calcitriol.

In our present study, to exploit the defined bio-function of stimulated autophagy in flap treated with calcitriol, the compound 3MA was used to inhibit the level of autophagy. 3MA has a widely-known biochemical mechanism that blocks Class III PI3K, inhibits the formation of autophagosomes, and reduces autophagic flux [17]. After intervention with 3MA in our research, autophagic responses were monitored by the

exploitation of biochemical reactions, which included the expressions of LC3, Beclin1, and p62 [18]. The autophagy formation process requires many autophagy-related genes. LC3, known as microtubule-associated protein 1 light chain 3, is essential for autophagy [19]. LC3 is lipidated upon the activation of autophagy (LC3-I, unlipidated; LC3-II, lipidated) [20]. LC3-II is involved in the expansion stage of autophagosome formation and is considered the most promising autophagosomal marker in mammals [21]. In addition, one of the other autophagic vacuoles markers is Beclin1 protein. The Beclin1-Vps34-Vps15 core complex is required in the pre-autophagosomal structure, and hence the expression of Beclin1 correlates closely to autophagosome activity [22]. Since autophagy is a dynamic mechanism which degrades damaged cellular organelles and unwanted proteins, it is useful to develop and test degradation markers. For example, p62 (also known as SQSTM1/sequestome1) is incorporated into autophagosomes by binding to LC3 and subsequently degraded through autophagy, hence p62 protein levels can be used to assess autophagic degradation [23]. In our present study, immunohistochemistry showed that LC3-II punctate dots generated in the cytoplasm of flap cells in calcitriol group were inhibited after treatment with 3MA. Furthermore, Western blotting showed that LC3-II/LC3-I and Beclin1 decreased, indicating that autophagy vesicles were reduced in calcitriol+3MA group compared with the calcitriol group. The level of p62 was also detected by Western blotting. Compared to the calcitriol group, p62 was significantly increased, indicating that autophagy flux was inhibited in the calcitriol+3MA group. Thus, 3MA appears to down-regulate the level of autophagy in random skin flap after treatment with calcitriol.

In our current research, the survival area in the calcitriol group was larger than that in the control group. However, the calcitriol+3MA group showed a significantly decreased survival area relative to the calcitriol-only group. Meanwhile, it was found that calcitriol decreased tissue edema in skin flap and that this function was restrained after treatment with 3MA. In other words, the inhibition of autophagy significantly decreases the therapeutic effect of calcitriol on random skin flap. In our previous study, the mechanism of calcitriol in the promotion of ran-

dom pattern skin flap survival was involved in promoting vascularization and attenuating oxidative stress [9]. However, it remained to be determined whether the up-regulation of autophagy by calcitriol promotes the survival of experimental random pattern flap by increasing angiogenesis and reducing oxidative stress. To answer this question, angiogenesis and oxidative stress were evaluated in calcitriol-treated skin flap after autophagy inhibition.

Previous studies have shown that calcitriol exerts a proangiogenic effect by stimulating VEGF expression. For an example, calcitriol was shown to promote the angiogenic process by up-regulating VEGF expression in breast cancer and skeletal muscle cells [24, 25]. In addition, the therapeutic treatment of anti-VEGF can reduce angiopoiesis in lung carcinoma cells [26]. Autophagy also plays an important role in angiogenesis, with studies reporting that its induction promotes angiogenesis, and its inhibition suppresses angiogenesis, including VEGF-induced angiogenesis [27]. Therefore, we hypothesized that calcitriol would increase angiogenesis in random skin flap by stimulating autophagy. In our research, the levels of VEGF mRNA/protein, in vessels and stromal cells in the dermis of the calcitriol group, were both down-regulated after autophagy inhibition. Western blotting also revealed lower levels of the expression in the calcitriol+3MA group compared to the calcitriol group. Furthermore, the mean microvascular density results from the H&E staining and CD34 staining both showed reduced neovascularization in the calcitriol+3MA group compared to the calcitriol group. Thus, we conclude that up-regulation of autophagy by calcitriol promotes the survival of experimental random pattern flap by increasing angiogenesis.

In our previous study, calcitriol significantly promoted the survival of distal random skin flap by attenuating oxidative stress. Moreover, the drug stimulated autophagy activity. These imply the potential relationship between autophagy and oxidative stress in the treatment of calcitriol for ischemic flaps. Recently, there has been growing evidence of a close connection between autophagy and oxidative stress. It is widely accepted that autophagy plays a crucial role in maintenance of healthy mitochondria and subsequent reduction of oxidative stress

[28, 29]. Under conditions of oxidative stress, the damaged mitochondria generate reactive oxygen species (ROS), where excess ROS oxidatively damage other cellular components and result in cell death [30]. Damaged mitochondria can be sequestered and degraded through the process of autophagy, subsequently helping cells escape oxidative stress induced death [31].

In our previous study, SOD activity and the levels of GSH and MDA were used to evaluate oxidative stress status. MDA is the end product of lipid peroxidation, and the content of MDA is an indicator of free radical levels [32]. GSH is a major intracellular antioxidant that evades free radicals and catalyzes the dis-mutation of superoxide anions [33], and can reflect the cell's ability to scavenge toxic free radicals [34]. SOD is an extreme defense against oxygen free radicals, and is an indicator of antioxidant activity with the function of clearing  $O_2^-$  and preventing tissue injury by toxic oxygen free radicals [35]. In our current research, we found that calcitriol markedly increased SOD activity and GSH, and decreased MDA level. Furthermore, 3MA, an autophagy inhibitor, inhibited these effects. This indicates that the down-regulation of autophagy abolishes the effect of calcitriol on oxidative stress in flap. Thus, we conclude that calcitriol increases the survival area in the distal part of flap and increases its angiogenesis by stimulation of autophagy.

In conclusion, calcitriol increased angiogenesis, reduced oxidative stress, and up-regulated autophagy, contributing to a significant increase in random skin flap survival. Additionally, the suppression of autophagy could reduce VEGF-induced vascularization and increase oxidative stress. Finally, we suggest that calcitriol increased angiogenesis and reduced oxidative stress via activation of autophagy, contributing to a significant increase in random skin flap survival. However, whether calcitriol also has therapeutic functions for the ischemia and necrosis of clinical random skin flap is unknown. Further experimental and clinical studies on calcitriol are needed to fully understand its effects.

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### Disclosure of conflict of interest

None.

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