

Effect of Notoginsenoside R1 on autologous adipose graft in rats

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Abstract. Autologous fat particle transplantation has been widely used by surgeons. The present study evaluated the effect of Notoginsenoside R1 (NR1) treatment on rat autologous fat graft, along with the quality and retention rates. Male Sprague-Dawley rats (n=60) received fat particle auto-transplantation from the left abdominal cavity into lateral dorsum. A total of 14 days after surgery, NR1 in different doses (50, 100 and 200 mg/kg/day) was injected into rats, following which blood and fat graft samples were harvested at days 7, 14 and 28. Assessments were carried out by hematoxylin and eosin staining, western blotting, ELISA and immunohistochemistry (IHC). The survival rate of fat grafts was increased in three experimental groups, as detected by weight measurement. Histological scoring demonstrated that there were significant differences in tissue integrity between the 100 mg/kg/day group and the other 3 groups. hepatocyte growth factor, vascular endothelial growth factor, fibroblast growth factor, angiotensin and S100 levels in the 100 mg/kg/day NR1 group was increased compared with the other 2 treatment groups; however, all 3 treatment groups demonstrated increased expression of these proteins compared with the control group. Additionally, cluster of differentiation (CD)68 exhibited negative expression and CD31 showed weakly positive expression in all three experiments, as assessed by IHC. In conclusion, 100 mg/kg/day NR1 may potentially promote the retention rate and enhance the quality of autologous fat grafts via increasing vascularity in the recipient site. These results implicate NR1 as a therapeutic strategy for the improvement of outcome following fat graft surgery.

Introduction

Soft tissue defects caused by a variety of reasons not only lead to appearance disfigurement, but also seriously affect the

quality of life, which is one of the most significant challenges in reconstructive and plastic surgery (1). The use of fat to improve adherent scars goes back to the end of the 19th Century when Gustav Neuber described the first transplantation of parcels of arm adipose tissue to the lower margin of the orbit (2). To date, autologous fat grafting has been widely used in repair of soft tissue defects because of its rich material resources, convenience in obtaining, and no immunity reaction, which has rapidly become a popular approach to treat contour deformities in both cosmetic and reconstructive plastic surgery (3). However, the limitations of fat transplantation are well known. Unfortunately, one of the greatest and unclear issues is associated with graft resorption. Its resorption and retention rates are unpredictable in the long term and highly varies from 20 to 90% which leads to either overcorrection or additional fat grafting negatively impacting the procedure, as the survival rate depends on the fat harvesting method, processing, transplanting and recipient site condition, according to previous studies (4,5). A high percentage of fat graft resorption reduces the clinical efficacy and often results in further engrafting (6,7).

Notoginsenoside R1 (NR1), formula $C_{47}H_{80}O_{18}$, is a major ingredient isolated from *Panax notoginseng* (Sanqi) roots. It is a well-known traditional Chinese herbal medicine as known to possess anti-inflammatory, anti-oxidative and anti-ischemia-reperfusion injury properties, and facilitates the treatment of cardiovascular disease (8,9). Previous studies have demonstrated that *Panax notoginseng* saponins improve ischemia/reperfusion-induced hepatic microcirculation disturbance (10), inhibit platelet aggregation (11,12) and adhesion molecule expression (13-15), and improve vascular endothelium function (16). The expression of lipopolysaccharide-induced vascular endothelial tumor necrosis factor- α is inhibited by NR1 by inhibiting degradation of inhibitor- κ B (17). To date, the most effective method of autologous fat tissue transplantation remains to be fully investigated. The present study aimed to investigate the effect of NR1 on the quality of autologous fat engrafting in rats, which may be an effective solution for soft tissue defects and further clinical applications.

Materials and methods

Chemicals. NR1 was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany); other chemicals were commercially available and purchased at reagent grade from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

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Animal experiments. Male Sprague-Dawley rats (age, 12 weeks; weight, 230-250 g; n=60) were obtained from Southern Medical University (Guangzhou, China). All animal experiments were approved by the Southern Medical University Committee. All animals were maintained at $22\pm 1^{\circ}\text{C}$ in $55\pm 5\%$ humidity in a 12-h light/dark cycle and had free access to sterilized water and food, in a specific pathogen-free animal room of Southern Medical University. After a 3-day acclimation period, all animals were randomly divided into groups. There were <6 rats in every cage.

Rats were divided into 4 groups (n=15/group) as follows: (A) Group I: Positive control group (receiving fat particle auto-transplantation only); (B) group II: NR1 (50 mg/kg/day) group [receiving fat particle auto-transplantation and 50 mg/kg/day NR1 via intraperitoneal (i.p.) injection for 2 weeks after surgery]; (C) group III: NR1 (100 mg/kg/day) group (receiving fat particle auto-transplantation and 100 mg/kg/day NR1 via i.p. injection for 2 weeks after surgery); (D) group IV: NR1 (200 mg/kg/day) group (receiving fat particle auto-transplantation and 200 mg/kg/day NR1 via i.p. injection for 2 weeks after surgery). Serum and adipose tissues were collected and stored at -80°C for less than a week before they were tested.

Fat removal and transplantation surgeries. Surgeries were performed under isoflurane anesthesia in a sterile operating theater fitted with high efficiency particulate air filters. (Induction at 3-4% and maintained at 1-2% with a flow rate of 0.2-0.3 l/min. Isoflurane was purchased from Yuyan Instruments, Shanghai, China.) Fig. 1 represents from where adipose tissue was removed and where it was relocated.

Following anesthetization with isoflurane, surgeries were performed through a midventral abdominal incision. In the current study, a 2-cm incision was made under loupe magnification (magnification, x2.5). Rats with adipose tissue relocation (excision of fat from one side with subsequent relocation within the lateral dorsum of the same rat) were termed to have undergone auto transplantation. For all groups, ~400 mg white adipose tissue was removed from the left abdominal cavity. The excised subcutaneous adipose tissue was immediately transplanted to the lateral dorsum. The transplants were attached via VETBOND tissue adhesive. Samples of fat were obtained from all groups at days 7, 14 and 28 after surgery. After muscle and skin were closed, absorbable suture meloxicam analgesic (0.025 mg/10 g body weight) was injected subcutaneously.

Survival rate of the fat graft. The transplanted fat tissues were extirpated. Skin incision was performed in a square to surround on the transplanted region. The half samples of three transplanted groups were taken out only integrally without envelope, and their volumes were measured. The weight of grafted fat was examined for animals sacrificed at 7, 14 and 28 days after NR1 injection. Chloral hydrate (10%) was used to sacrifice the animals through intraperitoneal injection with the dose of 0.3 ml/100 g weight. The grafted fat was carefully dissected from the nearby tissues in order to measure its weight. The fat graft survival percentage was expressed as the weight/weight ratio of the initial weight to the final weight, as follows: Fat graft survival (%)=[final weight (g)/initial weight (g)] x100. After measurement of the weight of grafted fat, fat was used



Figure 1. Schematic of autologous fat grafting in Sprague-Dawley rats. The oval dash in the center is the fat tissue in visceral cavity. The black oval represents the engrafting adipose depots in lateral dorsum. The black arrow indicates where adipose tissue was removed and to where it was relocated.

for western blotting, histopathology and immunohistochemistry assays.

Histopathology assays. At days 7, 14 and 28, fat grafts were harvested, immediately fixed in 10% formalin and embedded in paraffin for sectioning (5 μm) and hematoxylin and eosin (H&E) staining which was performed according to the method of Foster *et al* (18) and Bartness *et al* (19). A total of 10 sections were selected from each group for histological analysis and scoring. Bright-field images were obtained with a x40 objective. Four blinded, independent investigators assessed the fat grafts according to a previously published protocol (20). Each investigator evaluated integrity (assessed by presence of intact, nucleated fat cells), cyst/vacuoles, inflammation and fibrosis.

Protein isolation and western blotting. To evaluate if NR1 affected the protein expression levels of hepatocyte growth factor (HGF) in fat grafts, western blotting was performed to detect the expression of HGF. Protein from adipose tissue was extracted by Minute™ Total Protein Extraction kit for Adipose Tissues/Cultured Adipocytes (AT-022; Invent Biotechnologies, Inc., Plymouth, MN, USA). The concentration of protein extracts from rat adipose graft were determined using a Bicinchoninic Acid kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Proteins (20 μg) were separated by 10% SDS-PAGE followed by transfer to nitrocellulose membranes. Membranes were blocked by QuickBlock™ western blotting blocking reagent (P0252; Beyotime Institute of Biotechnology, Beijing, China) for 1 h at room temperature and washed by 1XTBST for three times (10 min per wash). Following washing, the membranes were incubated with mouse anti-rat HGF (sc-374422; 1:4,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse anti-GAPDH (AF0006; 1:1,000; Beyotime Institute of Biotechnology) antibodies at 4°C overnight. Following washing three times with 1XTBST (10 min per wash), membranes were incubated with horseradish peroxidase-conjugated secondary antibody (A4416; 1:10,000; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. Proteins were visualized an Enhanced Chemiluminescence system (EMD Millipore, Billerica, MA, USA).

Enzyme linked immunosorbent assay (ELISA). Vascular endothelial growth factor (VEGF), fibroblast growth factor (bFGF) and angiotensin (ANG) concentrations in serum were quantified using a commercial rat VEGF/Basic Fibroblast Growth Factor/Angiogenin heterodimer ELISA kit (69-50063; Wuhan

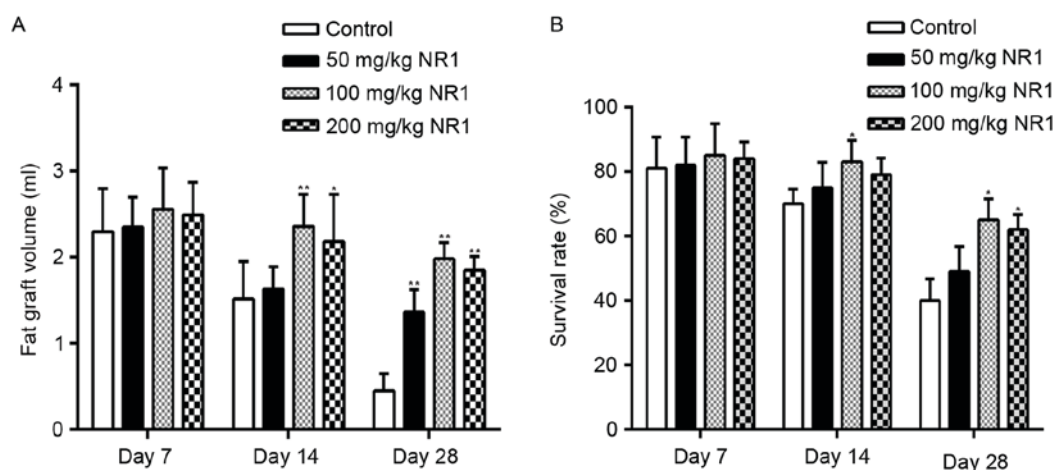


Figure 2. Effect of NR1 on fat grafts in all groups. (A) Measurement of fat graft volume and (B) percentage of survival rate of fat grafts in all groups. Data are presented as the mean \pm standard deviation. * $P<0.05$, ** $P<0.01$ vs. control group at same time point. NR1, Notoginsenoside R1.

Merck Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's protocol. All samples were assayed in duplicate. The mean concentration was determined for each sample. The sensitivity of the assay was 0.13 ± 0.01 ng/ml. The color reaction was halted with $50\ \mu\text{l}$ 1 M HCl per well, and the optical density measured after 1 h at a wavelength of 492 nm on an Infinite M200 microtiter plate reader (Tecan Group, Ltd., Männedorf, Switzerland). Results were normalized using β -actin (included in the ELISA kit) as control and presented as percentage of expression.

Immunohistochemistry. Immunohistochemistry was performed to detect the expression of cluster of differentiation (CD)31, CD68 and S100. Fat grafts were carefully dissected and then fixed overnight in 4% paraformaldehyde with a 0.1 M phosphate buffer solution (pH 7.4). Following this, they were embedded in paraffin and sliced into 5- μm -thick serial sections using a Microtome (Leica Microsystems Inc., Buffalo Grove, IL, USA). The sections were deparaffinized and fixed after hydration. Sections were incubated with anti-CD31 (ab119339; 1:20), anti-CD68 (ab955; 1:40) and anti-S100 (ab14849; 1:50) antibodies (all from Abcam, Cambridge, UK) at 4°C overnight. Following washing three times with PBS, sections were incubated with Biotin-labeled goat anti rabbit (A0277; 1:100; Beyotime Institute of Biotechnology) or mouse IgG (H+L) secondary antibody (A0286; 1:100; Beyotime Institute of Biotechnology) for 1 h at room temperature. A total of three identified adjacent areas per section were imaged (magnification, x400).

Statistical analysis. The statistical analyses were performed by SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean \pm standard deviation. Statistical significance was assessed by Student's t-test and one-way analysis of variance followed by Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Fat graft measurement. The volume of fat graft harvested from the lateral dorsum in the 4 groups was measured. As presented

in Fig. 2A, at days 7, 14 and 28 after auto transplantation surgery, the volume of fat graft in group III was increased compared with groups II and VI. Groups II and VI had increased fat graft volume compared with group I; however, did not have significant differences between them.

NR1 promotes the survival rate of the fat graft. As presented in Fig. 2B, 7 days after fat grafting, the fat graft survival rates were $82.4\pm8.8\%$ (group II), $85.2\pm9.9\%$ (group III), $84.1\pm5.2\%$ (group IV) and $81.1\pm9.8\%$ in group I; 14 days after fat grafting, the fat graft survival rates were $75.0\pm7.9\%$ (group II), $83.1\pm6.8\%$ (group III), $78.9\pm5.3\%$ (group IV) and $70.1\pm4.6\%$ in group I; 28 days after fat grafting, the fat graft survival rates were $49.1\pm7.8\%$ (group II), $64.9\pm6.6\%$ (group III), $62.2\pm4.8\%$ (group IV) and $39.9\pm6.8\%$ in group I. No significant differences were observed at 7 days; however, at 14 days, group III demonstrated significantly improved survival rates compared with the control group. This was also true of group IV at 28 days. Transplants were viable and revascularized; however, those with apparent fibrosis were excluded from the study ($n=1$).

NR1 promotes the quality of the fat graft. Assessment of H&E stained sections of fat sections from rats at 7, 14 and 28 days post-fat grafting resulted in significant difference in all fat characteristics. Fat grafts in the NR1 groups exhibited greater integrity compared with the control group. Proliferation of fibrous tissue around the graft, extensive necrosis and fusion, and formation of large vacuoles were observed in the fat grafts in all 4 groups in a time-dependent manner. However, the 100 mg/kg/day NR1-treated group displayed fewer cyst/vacuoles, inflammation and fibrosis at days 7, 14 and 28 after surgery, compared with the control group (Fig. 3). There were no significant differences in adipocyte damage between groups II and IV.

HGF expression. The expression of HGF in fat grafts after NR1 treatment was examined *in vivo*. NR1-treated rats induced the expression of HGF at different doses (Fig. 4). Among these doses, protein expression levels of HGF were markedly increased in the 100 mg/kg/day NR1-treated rats compared with all other groups.

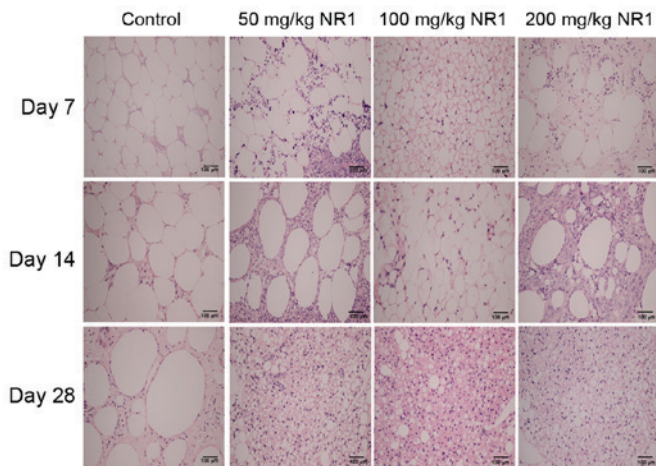


Figure 3. Effect of Notoginsenoside R1 on fat grafts in all groups, as assessed by hematoxylin and eosin staining. Representative histochemical images. Scale bar, 100 μ m. Notoginsenoside R1, NR1.

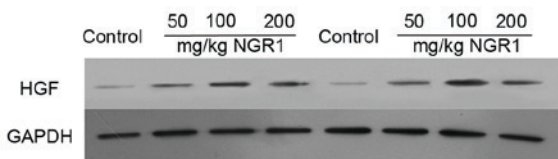


Figure 4. Expression of HGF in fat grafts. Representative western blot images of protein expression levels of HGF in the 4 groups. GAPDH served as an internal control. NR1, Notoginsenoside R1; HGF, hepatocyte growth factor.

Rat serum VEGF, bFGF and ANG levels, as detected by ELISA. The results of the ELISA assay are presented in Fig. 5. VEGF, bFGF and ANG levels were increased after NR1 treatment compared with the control group. Among the 3 experimental groups, serum VEGF (Fig. 5A), bFGF (Fig. 5B) and ANG (Fig. 5C) levels in rats treated with 100 mg/kg/day NR1 were increased compared with the other 2 groups.

CD31, CD68 and S100 expression, as assessed by immunohistochemistry assay. S100 staining in the cell membrane and nucleus increased time-dependently. Cell membrane and nuclear S100 protein expression of mature and new born adipocytes was observed in the nucleus of nascent adipocytes at various differentiation states, which was low during the early stages (7 days) but increased through their maturation from up to 28 days after transplantation. Among the 3 experimental groups, S100 distribution in rats treated with 100 mg/kg/day NR1 was increased in nascent adipocytes compared with the other 2 experimental groups (Fig. 6). In the groups, CD68 was expressed very weakly (Fig. 7A). CD31 was distributed clearly around new generated blood vessels, and the signal increased in a time-dependent manner. CD31 distribution was observed from days 14 to 28 after transplantation (Fig. 7B).

Discussion

Autologous fat tissue transplantation has been popular for >100 years, and currently is used in reconstructive and other forms of surgery to improve surgical appearance and benefit metabolic effects on certain diseases (21). Unfortunately, the

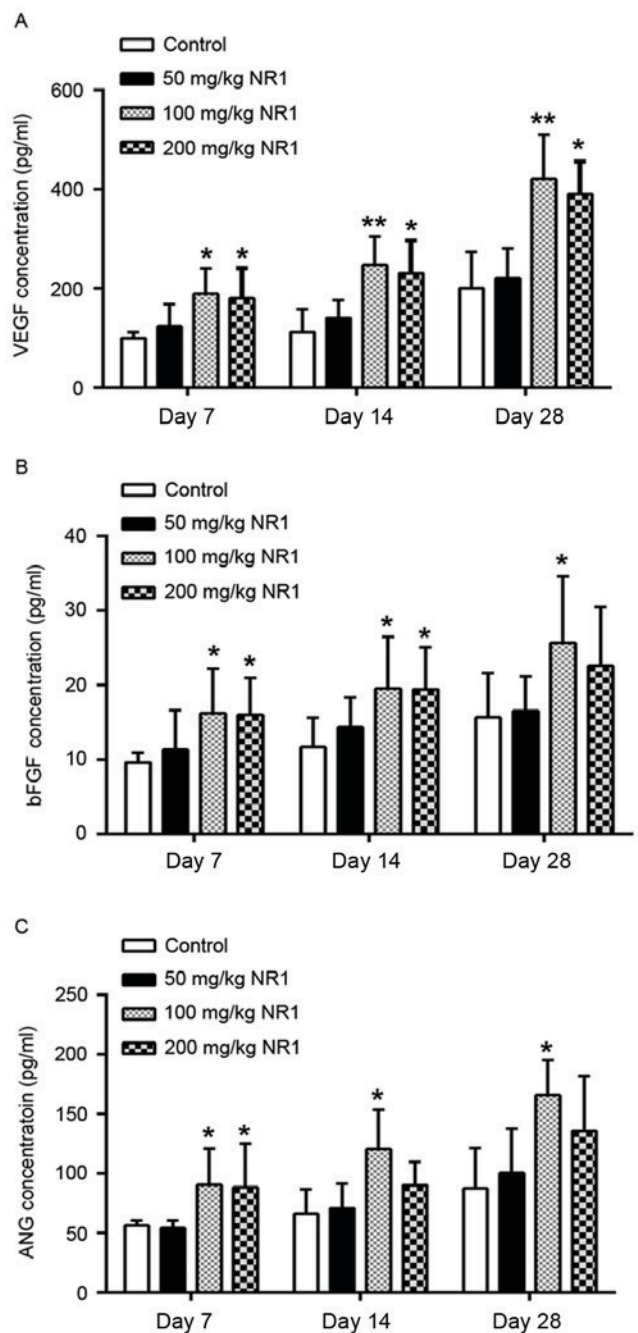


Figure 5. VEGF, bFGF and ANG levels in rat serum samples, as detected by ELISA. Serum (A) VEGF, (B) bFGF and (C) ANG levels at days 7, 14 and 28 after surgery. Data are presented as the mean \pm standard deviation. * P <0.05, ** P <0.01 vs. control group at same time point. NR1, Notoginsenoside R1; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; ANG, angiogenin.

main technical problem of fat graft usage for transplantation surgery has been maintenance of graft volume and survival rate while minimizing the inflammatory response. To investigate this, the present study investigated the effect of the herbal extraction NR1 on autologous fat transplantation. It was demonstrated that treatment of 100 mg/kg/day NR1 may improve the survival rate and quality of autologous fat graft, through increasing the recipient site vascularity and promoting angiogenesis. The primary finding of the present study was that the decrease in the weight and volume of 100 mg/kg/day NR1-treated rat fat grafts was smaller than

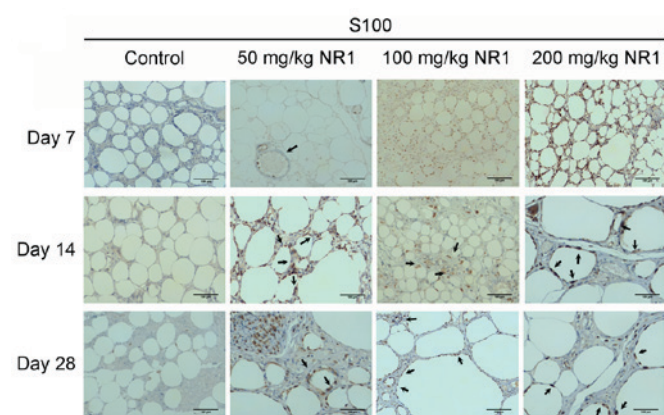


Figure 6. Expression of S100, as detected by immunohistochemistry. Representative micrograph images of S100 expression at days 7, 14 and 28 after surgery. Black arrows indicate positive staining. Scale bar, 100 μ m. CD, cluster of differentiation; NR1, Notoginsenoside R1.

the decrease in other groups. Treatment of the fat grafts with NR1 induced the expression of various angiogenic factors, including VEGF, bFGF and ANG. The histological assessments of the harvested fat grafts indicated that NR1 treatment led to improved fat tissue integration with fewer cysts, and reduced fibrosis and inflammatory cell infiltration, compared with the control group. From these results, it was concluded that NR1 treatment improves integration of the fat graft into the surrounding tissues and its long-term survival following fat transplantation. HGF is one of multi-potent growth factors, which exhibits a morphogenic and mitogenic response on cells (22). It has been suggested that functional expression of HGF and HGF receptor/c-met may serve a protective role of cell mobilization, tissue repair and wound healing in adult human mesenchymal stem cells (23). Previous studies have reported that adipose-derived stem cells induce high expression of HGF, which may promote new blood vessel generation, strengthen the collateral circulation after transplantation and finally increase fat graft survival rate, which was confirmed in the results of the present study (24-26).

VEGF and bFGF are known as potent angiogenic factors, which may induce angiogenesis of fat graft after surgery and influence endothelial proliferation and fat cell survival, viability and migration (27). A recent study revealed that modified adipose-derived stem cells with VEGF overexpression enhance the survival and quality of fat grafts through an angiogenesis-dependent mechanism (28). Other studies showed that bFGF together with adipose-derived stem cells can enhance the efficacy of autologous fat transplantation and increase blood vessel generation involved in the benefits from bFGF (29). Consistent with this, the present study demonstrated that NR1 treatment of rat fat grafts induced high levels of VEGF and bFGF. To date, there have been no studies about the direct effect of ANG on autologous fat graft; however, according to recent research, ANG can positively regulate adipose tissue and adipose-derived cells, which results in initiation of the renin-angiotensin system activation and affects pre-adipocyte differentiation (30-32). The results of the present study also strongly supported the hypothesis about NR1 treatment improving survival and quality of fat grafts via increasing ANG levels.

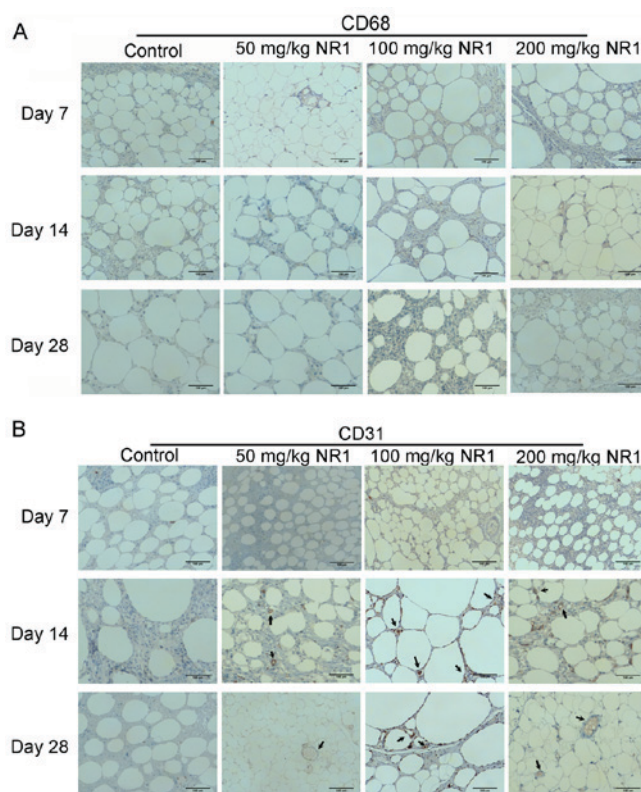


Figure 7. Expression of CD31 and CD68, as detected by immunohistochemistry. Representative micrograph images of (A) CD68, (B) CD31 expression at days 7, 14 and 28 after surgery. Black arrows indicate positive staining. Scale bar, 100 μ m. CD, cluster of differentiation; NR1, Notoginsenoside R1.

To identify if foam cells during late phase of transplantation come from nascent adipocytes, and to further validate the phenotype of obtained cells from fat grafts, several antibodies were applied. The differential expression of surface markers, including CD31, CD68 and S100, were detected by immunohistochemistry. CD31 and CD68 are well-known macrophage markers which indicate the functional activation of microvessels in the tissue (33). Therefore, various CD31/CD68-negative microvessels observed in culture could be interpreted as quiescent microvessels. S100 is reported as a glial marker, which is also expressed in adipocyte lineage cells (27). Usually most S100-positive cells are CD68 negative. In the present study, NR1 treatment was observed to induce an increase in the number of CD68-negative and S100-positive microvessels, while CD31 was expressed weakly.

In conclusion, the present study further optimized autologous fat grafts using a Chinese traditional medicine. The results demonstrated that 100 mg/kg/day NR1 may potentially promote retention rate and enhance the quality of autologous fat graft via increasing vascularity in the recipient site. These results implicate NR1 as a therapeutic strategy for the improvement of outcome following fat graft surgery.

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