Therapeutic Angiogenesis by Ultrasound-Mediated MicroRNA-126-3p Delivery

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Objective—MicroRNAs are involved in many critical functions, including angiogenesis. Ultrasound-targeted microbubble destruction (UTMD) is a noninvasive technique for targeted vascular transfection of plasmid DNA and may be well suited for proangiogenic microRNA delivery. We aimed to investigate UTMD of miR-126-3p for therapeutic angiogenesis in chronic ischemia.

Approach and Results—The angiogenic potential of miR-126-3p was tested in human umbilical vein endothelial cells in vitro. UTMD of miR-126-3p was tested in vivo in Fischer-344 rats before and after chronic left femoral artery ligation, evaluating target knockdown, miR-126-3p and miR-126-5p expression, phosphorylated Tie2 levels, microvascular perfusion, and vessel density. In vitro, miR-126-3p–transfected human umbilical vein endothelial cells showed repression of sprouty-related protein-1 and phosphatidylinositol-3-kinase regulatory subunit 2, negative regulators of vascular endothelial growth factor and angiopoietin-1 signaling, increased phosphorylated Tie2 mediated by knockdown of phosphatidylinositol-3-kinase regulatory subunit 2 and greater angiogenic potential mediated by both vascular endothelial growth factor/vascular endothelial growth factor R2 and angiopoietin-1/Tie2 effects. UTMD of miR-126-3p resulted in targeted vascular transfection, peaking early after delivery and lasting for >3 days, and resulting in inhibition of sprouty-related protein-1 and phosphatidylinositol-3-kinase regulatory subunit 2, with minimal uptake in remote organs. Finally, UTMD of miR-126-3p to chronic ischemic hindlimb muscle resulted in improved perfusion, vessel density, enhanced arteriolar formation, pericyte coverage, and phosphorylated Tie2 levels, without affecting miR-126-5p or delta-like 1 homolog levels.

Conclusions—UTMD of miR-126 results in improved tissue perfusion and vascular density in the setting of chronic ischemia by repressing sprouty-related protein-1 and phosphatidylinositol-3-kinase regulatory subunit 2 and enhancing vascular endothelial growth factor and angiopoietin-1 signaling, with no effect on miR-126-5p. UTMD is a promising platform for microRNA delivery, with applications for therapeutic angiogenesis. (Arterioscler Thromb Vasc Biol. 2015;35:2401-2411. DOI: 10.1161/ATVBAHA.115.306506.)

Key Words: angiogenesis  ■  gene therapy  ■  microRNA  ■  ischemia  ■  ultrasound

Abnormalities of various transcriptional and posttranscriptional regulators have been shown to play important roles in human vascular disease. Manipulation of these target genes remains a major focus of ongoing research, with the goal of developing novel therapeutic strategies to combat ischemic cardiovascular disease.1 Recently, a novel class of small noncoding RNAs, called microRNAs (miRNA), have been identified as important transcriptional and posttranscriptional inhibitors of gene expression that regulate the translational output of target messenger RNAs.2,3 Selective miRNAs have been identified in vascular endothelial cells, where they play key roles in regulating the angiogenic process.4,6 The ability to modulate key miRNAs involved in angiogenesis may lead to therapeutic applications for vascular diseases characterized by impaired angiogenesis.7-9 Thus, techniques to deliver miRNA-based therapies,4 targeted specifically to the vascular endothelium and using a noninvasive approach, would be an important step toward the development of a safe and effective strategy for miRNA delivery.

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Ultrasound-targeted microbubble destruction (UTMD) is a noninvasive method of targeted gene delivery using ultrasonic destruction of intravenously administered DNA-bearing carrier microbubbles.10,11 UTMD is more effective than direct intramuscular administration, where transfection occurs primarily within the endothelial surface of vessels, predominantly arterioles and capillaries, resulting in a more effective

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angiogenic response. The noninvasive nature of UTMD facilitates multigene delivery for therapeutic angiogenesis, which when given in a temporally separated fashion leads to more sustained and functional neovascularization. The advantages of (1) targeted vascular transfection, (2) minimal delivery to remote regions, and (3) noninvasive methodology makes UTMD uniquely suited to proangiogenic miRNA delivery.

One specific miRNA, miR-126-3p, has unique properties that make it an attractive therapeutic target. It is one of the only endothelial cell–restricted miRNA and has been shown to regulate vascular integrity and developmental angiogenesis. Deletion of the endothelial cell–specific miR-126-3p impairs maintenance of vascular integrity during embryogenesis and reduces angiogenesis after myocardial infarction in mice. The proangiogenic effect of miR-126-3p has been attributed to the repression of sprouty-related protein-1 (SPRED-1) and phosphatidylinositol-3-kinase regulatory subunit 2 (PIK3R2), negative regulators of vascular endothelial growth factor (VEGF) signaling, leading to enhanced VEGF action. More recently, the sister strand of miR-126-3p, miR-126-5p, has also been shown to have a biological effect, promoting endothelial proliferation and limiting atherosclerosis. The aims of our present study was to (1) develop and optimize UTMD techniques for miRNA therapeutics and (2) to test UTMD of miR-126-3p for therapeutic angiogenesis in the setting of chronic hindlimb ischemia. Our results demonstrate that miR-126-3p can be bound to cationic microbubbles, prolonging miRNA circulatory time in vivo after intravenous injection. UTMD of miR-126-3p results in knockdown of SPRED-1 and PIK3R2 along with positive modulation of angiopoietin-1 (Ang)-1 signaling, without effects on miR-126-5p. Finally, UTMD of miR-126-3p results in improved perfusion, increased vessel density and arteriolar to capillary ratio, and enhanced pericyte coverage in chronic hindlimb ischemia. This study provides strong evidence supporting UTMD as an effective noninvasive technique for miRNA delivery for therapeutic angiogenesis.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
miRNA-126-3p Transfection and Knockdown of SPRED1 and PIK3R2 In Vitro
We first tested the effects of miR-126-3p transfection (siPORT NeoFX) in human umbilical vein endothelial cells (HUVECs). MiR-126-3p levels by polymerase chain reaction in HUVECs showed peak expression at day 2 after transfection and was unchanged after transfection with scrambled miRNA (Figure 1A). MiR-126-3p–transfected HUVECs showed repression of SPRED-1 and PIK3R2, negative regulators of VEGF signaling, peaking 24 hours after transfection and persisting out to day 3 (Figure 1B). In comparison, scramble miRNA–transfected HUVECs showed no significant repression of either SPRED-1 or PIK3R2 (Figure 1B). When transfected with fluorescent miRNA, transfection in HUVECs was localized to cytoplasm and the nucleus (Figure 1C). This data suggest that exogenous miR-126-3p enters the cell (nucleus) and has strand-specific effects and is not a secondary effect.

In Vitro Stimulation With Ang-1 in miR-126-3p–Transfected HUVECs (Phosphorylated Tie2 Measurement)
The transfection of HUVECs with miR-126-3p had no effect on the levels of phosphorylated Tie2. However, under stimulation with Ang-1, miR-126-3p transfection led to increased levels of phosphorylated Tie2 protein (P<0.001 versus all other groups; Figure 1D). The increase in phosphorylated Tie2 was because of knockdown of PIK3R2 and not because of inhibition of SPRED1 (Figure 1E and 1F). This demonstrates the positive modulation of Ang-1 effect by miR-126-3p transfection and knockdown of PIK3R2, leading to enhanced phosphorylation of the Tie2 receptor.

In Vitro Migration and Tube Formation
In vitro endothelial cell function in response to miR-126-3p transfection was assessed by Matrigel Tube formation and standard Boyden Migration assays in our laboratory. Functional analysis of miR-126-3p–transfected HUVECs showed greater angiogenic potential by enhanced matrigel tube formation and migration studies in transfected cells when compared with control and scrambled miRNA–transfected HUVECs, with increased nodes and tubes (Figure 2A–2D). Next we performed additional in vitro matrigel angiogenesis experiments with specific inhibitors of VEGFR2 and Tie2 to determine the relative contributions of VEGF and Ang-1 to miR-126-3p–mediated neovascularization (Figure 2E–2F). This showed that the proangiogenic effect of miR-126-3p was suppressed by blockade of both VEGFR2 and Tie2, with a greater suppression by blockade of VEGFR2. These data provide in vitro evidence of the proangiogenic potential of miR-126-3p.

miR-126-3p Microbubble Conjugation and In Vivo Circulation
Cationic microbubbles were charge-coupled to a varying range of miR-126-3p under different conditions to optimize for a range of miR–microbubble binding conditions. After miR-126-3p and microbubble conjugation, binding of miR-126-3p to 1×10⁶ cationic microbubbles saturated at ≈60,000 miR-126-3p molecules per microbubble with the addition of 2.5 μg of miR-126-3p (Figure IA in the online-only Data Supplement). Upon intravenous administration to male Fischer 344 rats, greater concentrations of miR-126-3p were found in the plasma after injection of miR-126-3p conjugated microbubbles as compared
with miR-126-3p alone, microbubbles alone, or sham. Thus, conjugation to cationic microbubbles resulted in the stabilization and prolongation of miR-126-3p circulatory time in vivo compared with unbound miR-126-3p alone (Figure IB in the online-only Data Supplement).

**miRNA-126-3p Transfection by UTMD In Vivo in Normal Hindlimb**

UTMD of miR-126-3p produced targeted transfection in non-ischemic (normal) Fischer 344 rat hindlimb skeletal muscle, peaking early at 3 hours after delivery and lasting for 3 days (Figure 3A), and resulted in inhibition of SPRED-1 and PIK3R2 by polymerase chain reaction (Figure II in the online-only Data Supplement). Knockdown of SPRED1 and PIK3R2 mRNA levels was highest at 24 hours after delivery and persisted for 3 days in UTMD miR-126-3p–delivered groups (Figure 3B). Representative Western blots demonstrating reduced SPRED-1 and PIK3R2 at days 1 and 3 are shown in Figure 3C. There was minimal to no uptake in remote...
organs, liver, and spleen, with no significant knockdown of SPRED1 and PIK3R2 after UTMD of miR-126-3p (Figure III in the online-only Data Supplement).

**miRNA-126-3p Levels in Ischemic Hindlimb After UTMD**

First, we examined endogenous expression of miR-126-3p and miR-126-5p in our ischemic hindlimb model (Figure 4A and 4B). There was a similar trend \((P > 0.05)\) of increasing expression of both miR-126-3p and miR-126-5p after ischemia, with miR-126-3p levels being \(\approx 2\times\) that of miR-126-5p at all time points, both peaking at 2 weeks after left femoral artery ligation and stripping. Next, we performed UTMD \((1 \times 10^9\) cationic microbubbles charge-coupled with 2.5 \(\mu\)g of miR-126-3p or scrambled miRNA \(2.5\ \mu\)g\), ultrasound settings 1.3 MHz, transmit power of 0.9 W \([120 \text{ V}, 9 \text{ mA}, \text{ peak negative acoustic pressure} -2100 \text{ kPa at a pulsing interval of } 5\ \text{s}]\)\(^{11-13}\) in Fischer 344 rats at 2 weeks after left femoral artery ligation and stripping, with a subset of ischemic animals receiving 3 rounds of UTMD of miR-126-3p (day 14, 16, and 18 after ligation/stripping). In the setting of chronic ischemia, miR-126-3p–delivered animals showed higher
miR-126-3p levels in the delivered hindlimb, indicating effective transfection of miR-126-3p at the target site (P<0.05 versus control and scramble) at day 15 (24 hours after delivery) and remained elevated for 3 days (P<0.05 versus control and scramble at day 17; Figure 4C). By day 28 (14 days after delivery), miR-126-3p levels were back to basal levels, showing no difference to control and scrambled-delivered groups (Figure 4C).

Localization of miRNA Transfection: In Situ Hybridization and Fluorescent Microscopy
In situ hybridization showed robust miR-126-3p expression in miR-126-3p–delivered animals both at 24 and 72 hours after delivery, with both vascular and peri-vascular transfection (Figure 4D). There was detectable signal in both control and scrambled ischemic muscles consistent with endogenous miR-126 expression, but greater signal was seen (reddish-brown staining, black arrows) after UTMD of miR-126-3p within ischemic muscle. To further assess localization of miR transfection, we performed UTMD of fluorescent miRNA to ischemic hindlimb muscle. At day 1 post-UTMD of fluorescent miRNA, we found red fluorescent miRNA in ischemic muscle co-localized to endothelial cells, confirming targeted vascular transfection (Figure 4E).

miR-126-3p Induced Target Knockdown In Vivo in Ischemic Hindlimbs
UTMD of miR-126-3p to ischemic hindlimb muscle decreased protein expression of PIK3R2 and SPRED1 by Western blot. The greatest knockdown of both PIK3R2 and SPRED1 was at 24 hours and day 3 after delivery, with the knockdown effect of miR-126 diminished by day 28 (day 14 after UTMD). Protein expression levels of PIK3R2 and SPRED1 were no different across scrambled and nontreated control groups (Figure 5A and 5B). Triple delivery of miR-126-3p via UTMD to ischemic muscle showed knockdown of SPRED1 and PIK3R2 protein expression assessed by Western blotting, with robust knockdown of PIK3R2 at an early time point of day 19 (1 day after the final/3rd UTMD) that persisted until day 28 (Figure 5C). When compared with single miR-126-3p delivery, triple miR-126-3p UTMD delivery showed even greater knockdown of PIK3R2 and SPRED1 at the protein and mRNA level, with
Figure 4. Endogenous miR-126-3p and miR-126-5p after ischemia, and data after ultrasound targeted microbubble destruction (UTMD) of miR-126-3p to ischemic hindlimb muscle showing site-specific increase of miR-126-3p levels. Levels of endogenous miR-126-3p (A) and miR-126-5p (B) at increasing time points after ischemia. Bar graph demonstrating a similar trend of increasing expression (P>0.05) of both the miRs after ischemia. N=4 to 9 per group. C, Time course of miR-126-3p levels in ischemic hindlimb muscle at day 1, 3, and 14 after UTMD. MiR-126-3p levels peak at day 1 and remain elevated at day 3 (*P<0.05 vs control, scramble at day 1 and day 3, respectively; N=6 per time point for each group). Data expressed as mean±SEM. D, In situ hybridization images on delivered ischemic hindlimb muscle at 24 h and 72 h after delivery, showing miR-126-3p in ischemic muscle of all treatment groups, highest in the UTMD miR-126-3p animals. miR-126-3p is indicated by the reddish-brown staining (black arrow). Scale bar=100 μm. E, Representative fluorescent microscopic images of ischemic hindlimb muscle at day 1 after UTMD of fluorescent microRNA (miRNA; control had no UTMD). The red fluorescent miRNA is seen co-localized to endothelial cells (green, CD31) in miRNA UTMD-treated muscle (white arrows). Nuclei are stained blue (DAPI). Scale bar=100 μm.
more prolonged knockdown of PIK3R2 at day 28 compared with single delivery (Figure 5D–5F).

Given recent data on the biological effects of the sister strand of miR-126-3p, we quantified miR-126-5p expression in all 3 treatment groups. We did not observe any significant changes in miR-126-5p levels at day 15 and day 28 after ligation (day 1 and day 14 after UMTD) across all 3 groups (Figure IVA in the online-only Data Supplement). Specifically, there was no downregulation of miR-126-5p after UMTD of miR-126-3p. Given the effect of miR-126-5p in suppressing Notch1 inhibitor delta-like 1 homolog, we examined delta-like 1 homolog expression in all treatment
miR-126-3p treatment showed improvement in normalized perfusion to 0.79±0.03 (P<0.05 versus perfusion at day 14, pre-UTMD). A subset of animals underwent repeated (3×) UTMD of miR-126 (day 14, day 16, and day 18), where the resulting angiogenic response was even greater, with normalized microvascular perfusion at day 28 increasing even further to 0.88±0.02 (Figure 6C).

**Fluorescent Microangiography**

Microvascular density and neovessel characterization was assessed using fluorescent microangiography, a method of postmortem vascular casting. Representative fluorescent microangiographic images of the hindlimb muscle showed increased vessel density in miR-126-3p–treated animals compared with control and scramble-treated groups. Through analysis of the vasculature in the ischemic hindlimb of animals, both the total vessel length and vascular density were significantly greater compared with control and scramble treatment groups. By branch
order analysis, miR-126-3p delivery by UTMD resulted in a higher arteriolar/capillary ratio compared with scramble and control groups at day 28 (Figure 6G).

Immunohistochemistry and Tie2 Expression

Immunostaining was performed on hindlimb skeletal muscle, using antibodies against von Willebrand Factor (endothelial cells) and Desmin (pericytes). After UTMD of miR-126-3p, there was a significant increase in desmin-positive arterioles and capillaries compared with scrambled-treated and control ischemic muscle (Figure 6H and 6I). Desmin is a marker of pericytes, with increased pericyte coverage suggesting greater vessel maturation and stabilization in miR-126–treated muscle compared with scrambled miRNA-treated and control ischemic muscle. Improved pericyte coverage is a marker of an enhanced Ang-1 effect mediated by UTMD of miR-126-3p. In keeping with an enhanced Ang-1 effect and our in vitro data, UTMD of miR-126-3p to ischemic hindlimb muscle showed significant upregulation of phosphorylated Tie2 and Tie2 levels at the protein level at day 28 after ligation (Figure V in the online-only Data Supplement).

Discussion

Current evidence indicates that miRNAs are important regulators in many cardiovascular diseases and, coupled with their ability to specifically target key cellular pathways, makes the possibility of exploiting miRNAs to develop novel therapeutic strategies extremely attractive. Several miRNAs, termed angiomiRs, have been identified that regulate vascular development, angiogenesis, and endothelial cell function. The identification of these angiomiRs as key regulators of angiogenesis has opened a new avenue for cardiovascular therapeutics for ischemic cardiovascular diseases. Although many techniques exist, the optimal delivery method for proangiogenic miRNA therapies remains to be determined. Our present study demonstrates that ultrasound-mediated miR-126-3p delivery results in targeted vascular miR-126-3p transfection, increased vascular density, and improved microvascular perfusion with minimal to no uptake in remote organs, and as such is a promising noninvasive technique for proangiogenic miRNA therapeutics.

There are several important advantages to miRNAs as therapeutic agents. Their small size and conserved sequence across species make them more amenable to translation to product development for human application. In addition, the ability of one miRNA to target multiple mRNAs that function in the same or closely related intracellular pathway or signaling cascade is an advantage over targeting one specific gene. A major challenge in the development of miRNA-based therapeutics concerns the potential for systemic delivery into targeted sites, such as a specific organ, like the heart, or cell type within an organ, like the vascular endothelium. Although native circulating miRNAs are stable in plasma because of protection by exosomes, microvesicles, or lipoprotein complexes, exogenously administered synthetic miRNAs are subject to RINase-dependent degradation by endogenous ribonucleases. To overcome this limitation, chemical modifications have been performed to stabilize them in vivo and increase binding efficacy and cellular uptake. For example, conjugation of miR122 antagomiRs to cholesterol can facilitate cellular uptake and biological action within the liver in vivo, leading to reduced plasma cholesterol levels. Similarly, chemical modifications to anti–miR-33 oligonucleotides increase metabolic stability and tissue half-life, leading to a reduction in atherosclerotic plaque burden in LDL receptor knockout mice. Alternatively, miRNA mimics (synthetic RNA duplexes in which one strand is identical to the miRNA sequence, designed to mimic the function of the endogenous miRNA) may lead to downregulation of target genes. We showed that conjugation of miR-126 to cationic lipid microbubbles (≈60,000 miRNA molecules per microbubble), similar to binding to lipoproteins or microvesicles, prolongs their circulatory time in vivo. Although unbound miRNA was rapidly cleared, microbubble conjugation allowed the miRNA to persist in the systemic circulation where they can undergo site-targeted transfection by focused externally applied ultrasound.

Several other challenges remain for the successful development of miRNA therapeutics, including (1) lack of tissue specificity, (2) lack of optimal delivery methods, (3) risk of systemic or off-target effects, and (4) poor tissue/organ uptake. Although intravenous administration of antimiRs and miRNA mimics have been the primary method of systemic delivery to date, uptake is preferentially targeted to the liver, spleen, and kidney. Thus, efficient and targeted delivery strategies for miRNA are needed, with several currently under investigation, including (1) conjugation to a targeting molecule, such as peptides or antibodies, to promote homing of the antimiR/miRNA mimic to the target tissue, (2) encapsulation strategies (eg, liposome-based methods) to enhance tissue uptake, and (3) direct delivery techniques, such as catheter-based intracoronary injections. A noninvasive method of miRNA delivery that results in site-targeted transfection, with minimal systemic/off-target effects, would be an important advance to the field of miRNA therapeutics.

UTMD is a noninvasive method of gene delivery that has been studied for viral vector, plasmid, and siRNA delivery. Although other studies have used high-power ultrasound and DNA-bearing carrier microbubbles to deliver plasmid vectors encoding miRNA to the kidney and liver, our study is one of the first to investigate UTMD of miRNA and the first application for cardiovascular therapeutics, specifically aimed at therapeutic angiogenesis in chronic hindlimb ischemia. In addition, we closely detailed the in vivo spatial and temporal aspects of UTMD for miRNA delivery, including localization, timing of transfection, and target knockdown, as well as the benefits of multiple deliveries to improve therapeutic effect. Similar to plasmid delivery, UTMD results in targeted transfection of miR-126 to the vascular endothelium, as shown by in situ hybridization and fluorescent microscopy, and may be a key advantage of UTMD over other miRNA delivery techniques for therapeutic angiogenesis. Transfection occurs rapidly within 3 hours and is transient, lasting 3 days, with target knockdown also occurring rapidly within 24 hours after UTMD, which may be beneficial for miRNA therapies for acute processes, such as acute myocardial ischemia–reperfusion injury. Target knockdown was transient and lasted out to day 3, and so for treatment of chronic ischemia, we...
used repeated deliveries (3 UTMD deliveries every 2 days) to prolong the therapeutic effect, finding multiple deliveries to produce a more sustained target knockdown, and a greater angiogenic response compared with single delivery. Use of miRNA mimics or plasmid vectors encoding miR-126 for UTMD may also prolong transfection and target knockdown, further increasing therapeutic effect, and is worthy of future studies. Importantly, remote uptake was minimal after UTMD of miR-126, with no significant target knockdown in the liver or spleen. Thus, the advantages of (1) targeted vascular transfection, (2) minimal delivery to remote regions, (3) noninvasive delivery method that can be easily repeated make the technique of UTMD uniquely suited to endothelial cell–specific proangiogenic miRNA delivery.

Several studies have investigated therapeutic aspects of miR-126-3p. Zernecke et al demonstrated that delivery of miR-126-3p in endothelial cell apoptotic bodies resulted in plaque stabilization in mouse models of atherosclerosis via increased production of CXCL12.19 We did not find any changes in CXCL12 with UTMD of miR-126-3p in our study, which may reflect differences in disease model and mode of delivery. Similarly, Jansen et al showed that endothelial microparticles released from apoptotic endothelial cells promote vascular re-endothelialization by transfer of miR-126-3p into recipient endothelial cells, an effect that is blunted in the presence of hyperglycemia.30 Although miR-126-3p is the dominant strand and the subject of most studies of miR-126, there is increasing interest in the sister strand, miR-126-5p. Schober et al16 demonstrated that miR-126-5p suppresses Notch1 inhibitor delta-like 1 homolog, maintaining the regenerative capacity of endothelial cells and limiting atherosclerosis. Given the single miR-126-3p strand could theoretically act as an antisense RNA for miR-126-5p, we quantified miR-126-5p expression in vivo in all 3 treatment groups. We found no significant change in miR-126-5p expression either (1 day) or late (14 days) after UTMD of miR-126-3p. This suggests that the increased angiogenesis observed was mediated only via exogenous miR-126-3p and that miR-126-3p delivery did not lead to suppression of endogenous miR-126-5p.

Our present study showed that miR-126-3p increased tubule formation and migratory capacity of endothelial cells in vitro and that UTMD of miR-126-3p in the setting of chronic hindlimb ischemia in vivo resulted in increased microvascular perfusion and vascular density, with knockdown of known targets SPRED1 and PIK3R2,14,15 both negative regulators of VEGF signaling. Interestingly, although miR-126-3p transfection and target knockdown was relatively short-lived, the observed proangiogenic effect persisted for 2 weeks after delivery. Analysis of the neovascularure revealed increased arteriolar/capillary ratio and improved pericyte coverage, suggesting enhanced vessel maturation. This data coupled with our in vitro data showing miR-126-3p–mediated downregulation of PIK3R2 accounts for the increased Tie-2 phosphorylation under the stimulation of Ang-1 in endothelial cells is in keeping with the findings of Sessa et al who showed that miR-126–induced suppression of PIK3R2 also promotes the action of Ang-1 on neovessel stabilization and maturation.38 Thus, by simultaneously targeting both the VEGF pathway to stimulate early angiogenesis and Ang-1 signaling to promote vessel stabilization, miR-126 therapy results in a more robust angiogenic response, consistent with observations seen with studies of combination VEGF and Ang-1 gene delivery.13,39

Conclusions

We conclude that ultrasound-mediated gene delivery of miR-126-3p–bearing carrier microbubbles to chronically ischemic skeletal muscle results in improved tissue perfusion, vascular density, arteriolar formation, and neovessel maturation, with no significant off-target effects in remote organs. With advantages of targeted vascular transfection and ease of repeated deliveries, UTMD is a promising noninvasive technique for miRNA delivery for therapeutic angiogenesis.

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Disclosures

None.

References

MicroRNAs (miRNAs) are small noncoding RNAs that regulate the expression of large number of genes, making them an attractive therapeutic target. As such, effective and safe delivery strategies for miRNA are needed. One specific endothelial miRNA, miR-126-3p, is a promising target for proangiogenic miRNA therapy. We used ultrasound-targeted microbubble destruction to deliver miR-126-3p in a rodent model of chronic hindlimb ischemia, showing improved perfusion and vessel density. Furthermore, we showed that the proangiogenic action of miR-126-3p is mediated by positive modulation of VEGF to stimulate early angiogenesis and Ang-1 signaling to promote vessel stabilization. Our findings suggest that ultrasound-targeted microbubble destruction may be a safe and effective method for targeted miRNA delivery for therapeutic angiogenesis.
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Supplementary Figure I. miR-126-3p bound to microbubbles (MB) remains more stable in the systemic circulation. A) Binding of miR-126 to cationic microbubbles ($1 \times 10^9$) at increasing miRNA incubation doses. Maximal plateau binding occurred at 2.5 µg of miR-126-3p, with ~60,000 miR-126-3p molecules per microbubble. B) Time course of miR-126-3p circulation in plasma after intravenous injection showing a prolonged miR half-life when bound to microbubbles. (N=6 per group). Data expressed as mean ± SEM.
Supplementary Figure II. Time course of A) SPRED1 and B) PIK3R2 expression by quantitative PCR after UTMD of scrambled miRNA to normal skeletal hindlimb muscle, compared to scrambled miRNA alone with or without bubbles and ultrasound (U/S). There was no change on SPRED1 or PIK3R2 levels at any time point after UTMD of scrambled miRNA. (N=6 per group at each time point). Data expressed as mean ± SEM.
Supplementary Figure III. Data on remote organ transfection of miR-126-3p, and knockdown of targets SPRED1 and PIK3R2 in liver and spleen by quantitative PCR. No significant differences were observed versus control untreated liver/spleen, suggesting no remote off-target effects of UTMD of miR-126-3p to ischemic hindlimb muscle. (N=5-6 per group). Data expressed as mean ± SEM.
Supplemental Figure IV: Effect of miR-126-3p delivery via UTMD on miR-126-5p, Dlk1 and CXCL12 (by PCR) at various time points following ischemia. **A**) There were no significant changes observed in miR-126-5p levels at day 15 and day 28 post surgery (Day 1 and Day 14 post UTMD). Specifically there was no downregulation of miR-126-5p after miR-126-3p transfection. **B**) Dlk1 levels did not change after miR-126-3p transfection by UTMD vs control. Dlk1 levels increased early after scrambled miRNA delivery by UTMD, but normalized by day 28. **C**) No changes were observed in CXCL12 levels in any treatment group. Specifically, there was no change in CXCL12 expression by UTMD of miR-126-3p. (N=7-8 for each group) *p<0.05 vs day 28.
Supplementary Figure V. UTMD of miR-126-3p to ischemic hindlimb muscle showed a significant upregulation of pTie2 and Tie2 levels at the protein level (western blotting) at day 28 post-ligation (day 14 after UTMD). *p<0.05 vs control and scramble. N=8-10 per group.
MATERIALS and METHODS

miR-126-3p Preparation and In Vitro HUVEC Transfection

MicroRNA-126 (miR-126-3p, Eurofins MWG Operon) was synthesized based on the following sequence 5’- UCGUACCGUGAGUAAUAUGCG -3’. A microRNA with a scrambled sequence with approximately equal GC content to miR-126-3p, was synthesized and served as the negative miRNA control for all experiments - mirVana™ miRNA Mimic, Negative Control #1 (Catalog number: 4464061) - https://www.thermofisher.com/order/catalog/product/4464061.

To determine the effect of miR-126-3p on specific angiogenic transcript and protein concentrations, human umbilical vein endothelial cells (HUVECs) were transfected and assayed using quantitative real-time polymerase chain reaction (qRT-PCR). HUVECs were cultured and transfected with miR-126-3p according to the manufacturer’s instructions using the siPORT NeoFX Transfection Agent (Applied Biosystems Inc.).

Briefly, HUVECs were grown in DMEM with 10% Fetal Bovine Serum (CellGro) to 80% confluence at 37°C and 5% CO₂. Adherent cells were washed and trypsinized. Trypsin was inactivated by re-suspending the cells in DMEM with 10% FBS (Invitrogen). The SiPORT NeoFX transfection agent was diluted in Opti-MEM I medium (Life Technologies) and incubated for 10 minutes at room temperature. miR-126-3p was diluted into 50µL Opti-MEM I medium at a concentration of 30nM. Diluted microRNA and diluted siPORT NeoFX Transfection agent were mixed and incubated for another 10 minutes at room temperature to allow transfection complexes to form and subsequently dispensed into wells of a clean 6-well culture plate. The HUVEC suspension was overlaid onto the transfection complexes and gently mixed to equilibrate. Transfected cells were incubated at 37°C and 5% CO₂ for 24 hours.

Fluorescent miRNA In Vitro Transfection and UTMD In Vivo

To determine the transfection efficiency of miRNA in vitro, fluorescent miRNA BLOCK-iT™ Alexa Fluor® Red (Invitrogen) was transfected in HUVECs cells according to manufacturer’s recommendations. At 24 hours after transfection, cells were washed twice with PBS and new medium was added. Transfection efficiency was confirmed from fluorescent microscopy images of cells taken at 24 hours post transfection. In vivo transfection of fluorescent miRNA (2.5µg) to the hindlimb of ischemic Fischer rats was achieved through ultrasound-targeted microbubble destruction (UTMD). At 24 hours after UTMD, animals were sacrificed and hindlimb muscles were embedded in OCT (Sakura Finetek) and cryosectioned at a thickness of 18um, fixed with 2% paraformaldehyde. Immunostaining with CD31 and DAPI nuclear counterstaining was done with the protocol described in the immunostaining staining section. In vivo transfection of the fluorescent miR was visualized with confocal imaging of the stained tissue sections.

Quantitative real time-PCR

A qScript microRNA cDNA Synthesis kit (Quanta Biosciences) was used for reverse transcription (RT) reactions. The RT reaction consisted of two separate reactions: 1) poly(A) tailing reaction and 2) first-strand cDNA synthesis. Poly(A) tailing
reaction contained 2 µL of poly(A) tailing buffer (5x), 1µg of total RNA, and 1 µL of poly(A) polymerase and was completed in following steps: 37 °C for 60 min followed by 70°C for 5 min. First-strand cDNA synthesis reaction contained 10 µL from the poly(A) tailing reaction, 9 µL of microRNA cDNA reaction mix and 1 µL of qScript reverse transcriptase together incubated at 42 °C for 20 min followed by 85 °C for 5 min. PerfeCta Universal PCR primer® and miR-126-3p primers (UCGUACCGUGAGUAAUAAUGCG) and miR-126-5p (CAUUAUUACUUUGGUACGCG) were used to detect miR-126-3p and miR-126-5p levels with ViiA 7 RT-PCR (Life technologies). Each reaction was carried out in a total volume of 10µL containing: 1 µL RT product, 0.2 µL of universal primer, 0.2 µL of miR-126-3p primer, 5µL of iScript SYBR Green reagent (Bio-Rad), and 3.6 µL nuclease free water. Real time PCR was completed at the following temperatures: activation at 95°C for 2 minutes followed by 40 cycles at 95°C for 5 seconds and 60°C for 30 seconds. The melt curve was developed based on 95°C for 15 seconds, 60°C for 30 seconds and 95°C for 5 seconds. The relative expression level of each individual miRNA after normalization to miR-RNU-6B was calculated using the 2-ΔΔCT method.

Quantitative RT-PCR for SPRED1, PIK3R2, DLK-1 and CXCL12 was performed using standard techniques in our laboratory. Briefly, hindlimb tissue was homogenized in TRIzol (Invitrogen) and sonicated for 10 seconds. Subsequently, total RNA was isolated using RNA isolation kit (BioRad) and quantified by absorbance. Total RNA was reverse-transcribed in 20µL volumes using cDNA Synthesis Kit (BioRad). qPCR was performed using Perfecta SYBR Green FastMix kit (Quanta Biosciences) following the manufacturer's standard protocol. The expression levels were normalized to controls using the 2^ΔΔCT method or expressed as absolute copy number (based of standard curve).

**Western Blot Analysis for PIK3R2 and SPRED1**

Knockdown of miR-126-3p targets (PIK3R2 and SPRED1) in hindlimb tissue of normal and ischemic groups was assessed by western blotting as previously described. Tissue samples were cut into appropriately sized sections and lysed in RIPA buffer (Sigma) by sonication. Protein concentrations of each samples were determine by Bradford Assay (Bio-Rad) and 50µg of protein was used for loading. Gel electrophoresis was performed at 120V. Transfer was performed at room temperature at 80 V for 2 hours. After transfer, the membrane was stained with Ponceau Red for 30-60seconds to detect bands and confirm the transfer. The membrane was then kept in blocking buffer for 1 hour and rinsed with washing buffer. Primary antibodies were added (anti-SPRED1 Abcam ab77079; 1:1,000, anti- PIK3R2 Abcam ab131067; 1:5,000, anti-GAPDH Santa Cruz sc-25778) and incubated overnight at 4°C. The membrane was rinsed again and anti-Rabbit HRP secondary antibody (1:5,000, Promega) was added to detect SPRED1, PIK3R2 and GAPDH. The membrane was incubated with secondary antibodies for 45 minutes at room temperature. After final washes, the blot was developed with ECL detection kit (Amersham, GE Healthcare) on a photo film (Kodak). Densitometry readings were obtained using the Quantity One software and the sample protein concentrations were normalized to the intensity of GAPDH.
In Vitro Endothelial Cell Functional Analysis

*In vitro* endothelial cell function in response to miR-126-3p was assessed by standard Boyden Migration and Matrigel Tubule formation assays. For migration assays, VEGF (100 ng/mL) was placed in each well of the Boyden companion plate. An 8µm (pore size) insert was placed in each well containing the HUVEC suspension. After incubation, each Boyden chamber insert was gently washed, and non-adherent cells were removed. Cells were fixed and stained using DiffQuik (Sigma) and allowed to dry overnight. The membrane was removed and mounted on a slide for quantification using light microscopy with a 20X objective.

*In vitro* angiogenesis/matrigel capillary tube formation assay was performed to assess the effects of miR-126-3p on capillary-like network formation in a 96-well plate. 60 µl of Matrigel™ (Becton Dickinson) was added to each well of the 96-well tissue culture plate (Corning) and allowed to polymerize at 37°C, 5% CO₂ for 45 minutes under sterile conditions. 1x10⁴ HUVECs transfected with scramble miR and miR-126-3p were suspended in 100 µl Medium 199 (Gibco, Life Technologies Inc) enriched with growth factors and serum and plated onto the surface of the matrigel. All the wells were stimulated with VEGF (50 ng/ml) to facilitate *in vitro* tube formation. Control group was also included which did not undergo any transfection, receiving the same conditions of VEGF stimulation. The capillary-like network formation was observed at regular intervals until 28 hours and photomicrographs were recorded at each time interval for quantification. The total number of nodes and ends of the tubes were quantified. Tube number was quantified using the formula, tubes = (number of nodes + number of ends)/2. Data was expressed as mean ± SD.

**Effect of miR-126-3p on Ang1 signalling**

To determine the effect of miR-126-3p and Ang1 on pTie2 levels, HUVECs were transfected with miR-126-3p and stimulated with Ang1 and assayed through western blot. On the day of transfection, 90pmol of miR was used for transfection of each well of the 6-wells plates with Lipofectamine RNAiMax Transfection Agent (Life Technologies). BLOCK-i™ Alexa Fluor® Red Fluorescent Control (Life Technologies) was used for transfection of positive controls. Briefly, Lipofectamine RNAiMax and miRNA were diluted separately with Opti-MEM I medium (Applied Biosystems Inc.). Diluted miRNA and lipofectamine RNAiMax were mixed and incubated for 5 minutes at room temperature and subsequently added to the corresponding wells. At 6 hours after transfection, Ang1 (200µg/mL) was added to the corresponding wells. Ang1 was not added in the control groups. At 24 hours after transfection, cells were washed twice with phosphate-buffered saline (PBS) and lysed with RIPA buffer (Sigma) by sonication. Western blotting was performed as described earlier with anti-pTie2 (Abcam ab78142; 1:5000). We further transfected HUVECs with siSPRED1 and siPIK3R2 (both from Life Technologies) to investigate the involvement of downstream targets in Ang1 signaling following the transfection protocol mentioned above. Subsequently, cell lysates were collected after 48 hours for protein extraction and subjected to western blotting as previously described.

**Effect of VEGFR-2 / Tie-2 inhibition on miR-126-3p mediated *in vitro* angiogenesis**
In vitro angiogenesis/matrigel capillary tube formation assay was performed in HUVECs as described above using Matrigel™. MiR-126-3p transfected HUVECs were further treated with VEGFR2 inhibitor and Tie2 inhibitor (both Santa Cruz Biotechnology) and were used at concentrations according to manufacturer’s instructions. The capillary-like network formation was observed at regular intervals until 24 hours and photomicrographs were recorded at each time interval for quantification. The total number of nodes and ends of the tubes were quantified. Tube number was quantified using the formula, tubes = (number of nodes + number of ends)/2. Data was expressed as mean ± SD.

Microbubble Preparation
Cationic lipid microbubbles were created by sonicating an aqueous dispersion of 1 mg·ml⁻¹ polyethyleneglycol-40 stearate (Sigma), 2 mg·ml⁻¹ distearoyl phosphatidylcholine (Avanti) and 0.4 mg·ml⁻¹ 1,2- distearoyl-3-trimethylammoniumpropane (Avanti) with decafluorobutane gas.³-⁵ These microbubbles have a zeta potential of +60 mV.⁴ For perfusion imaging, lipid-shelled octafluoropropane gas microbubbles (Definity™, Lantheus Medical Imaging) were used. Microbubble concentrations were determined using a Coulter Multisizer IIe (Beckman-Coulter), prior to intravenous administration.

MicroRNA-Microbubble Conjugation Assay
Cationic microbubbles were charge-coupled to a varying range of miR-126-3p under different conditions to optimize for a range of miR-microbubble binding conditions. That is, miR-microbubble binding was optimized for binding solution, molarity of solution, length of incubation for binding, concentration of miR-126-3p, and concentration of microbubbles.

For miR-microbubble binding assays, cationic microbubbles were prepared as described above and washed 2-4 times with up to 10mL of 0.16M PBS. Cationic microbubbles and miR-126-3p were conjugated by adding 0µg, 1µg, 2.5µg, 5µg or 10µg of miR-126-3p to 1x10⁹ microbubbles in a solution containing 1mL 0.16M PBS in a 2mL microcentrifuge tube. The miR126-microbubble solutions were incubated for 30 minutes on a flat rocker (VWR) to facilitate miR-microbubble interaction.

Following miR-microbubble binding, the solution containing unbound miR126 was aliquoted and reserved. The remaining microbubble-miR126 mixture was then washed using 0.16M PBS to completely remove unbound miR from the microbubble solution. Microbubble concentrations were determined using a Beckman Coulter Multisizer (Beckman-Coulter) to standardize concentrations and monitor microbubble loss during the binding and washing process. The microbubble-miR126 mixture was vortexed to completely disrupt the microbubbles and the bound miR-126-3p concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) at 260nm.

The concentration of miR-126-3p was then normalized for the number of microbubbles in solution to determine the number of miR-126-3p molecules associated with each microbubble at the different starting miR-126-3p concentrations. The data was modelled to the equation y=(Bmax)x/(Kd + x) to determine Bmax (maximum
number of miR-126-3p molecules bound per microbubble) and Kd (the dissociation constant) by computer software (GraphPad).

**Microbubble Clumping Analysis**

To assess the risk of clumping/embolization *in vivo*, cationic microbubbles were incubated with increasing concentrations (0, 1, 2.5, 5 µg/mL) of miR-126-3p. After incubation, conjugated microbubbles were assessed for clumping using a Nikon Eclipse inverted microscope (Nikon Inc.) and phase contrast, pre- and post-miRNA binding, to visually assess microbubble associations. Microbubble concentrations and size distribution were then determined using a Beckman Multisizer (Beckman Coulter).

**Animal Preparation**

The study protocol was approved by the Animal Care and Use Committee at the Keenan Research Centre in the Li Ka Shing Knowledge Institute, St. Michael’s Hospital, University of Toronto. Male Fischer 344 rats (Charles River) were used for miR-126-3p/microbubble circulation studies and miR-126-3p UTMD studies in normal animals. For miR-126-3p UTMD studies in chronic ischemia, hindlimb ischemia was induced by ligation and stripping of the left femoral artery. For *in vivo* studies, rats were anesthetized with 2% inhaled isoflurane, and an intravenous catheter was placed in a jugular vein for miR-126-3p and microbubble administration. Analgesia (Anafen 5mg/kg sc.) was given as required.

**Time Course of miR-126-3p Circulation**

Time course of circulating miR-126-3p concentration following intravenous injection was assessed using qRT-PCR (as described above), with and without microbubble conjugation at various time points after intravenous injection in 24 rats. Rats were anesthetized and an intravenous catheter was inserted for microbubble administration. Prior to exogenous miR-126-3p and microbubble administration, 500µL of blood was sampled from the tail vein and stored in EDTA-containing BD vacutainers (BD Biosciences) to determine the endogenous baseline concentration of miR-126-3p in peripheral venous blood. Rats (n=6 per group) were subsequently injected with either microbubble (MB) alone, miR-126-3p alone, miR-126-3p-microbubble (MB + miR-126-3p) or received no injection (control). Circulating levels of miR126 were assessed at 0, 15, 30, 60, and 180 minutes after administration by sampling 500ul of venous peripheral blood as previously described. A minimum of 200 µL of plasma was isolated from peripheral venous blood by centrifugation at 1600g for 15 minutes at 4°C. Total RNA was extracted from plasma with TRIzol LS reagent (Invitrogen) in a 3:1 ratio for acid-phenol:chloroform extraction. The aqueous phase was transferred to a new 2ml microcentrifuge tube, mixed with 70% ethanol in a 1:1 ratio and passed through a RNA purification column as previously described using the Aurum Total RNA Mini Kit (Bio-Rad).

**Perfusion Imaging: Contrast-enhanced Ultrasound (CEU)**

Microvascular blood flow was measured in the ischemic hindlimb muscles (gastrocnemius/soleus group) using CEU pulse inversion imaging (HDI 5000, Philips Ultrasound) at a mechanical index of 1.0 and a transmit frequency of 3.3 MHz during
continuous intravenous infusion of perfusion microbubbles (1x10^7 min⁻¹).³–⁵ Data were recorded digitally, saved to magnetic-optical disk and transferred to a computer workstation for off-line analysis. Background images were acquired prior to microbubble infusion. Intermittent imaging was then performed by progressive prolongation of the pulsing interval (PI) from 0.2 to 40 seconds, using an internal timer. Averaged background frames were digitally subtracted from averaged contrast-enhanced frames at each PI. PI versus signal intensity (SI) data were fit to the function, y = A (1-e⁻βt), where y is SI at the pulsing interval t, A is the plateau SI which is an index of microvascular blood volume (MBV), and β is the rate constant reflecting microvascular blood velocity. Microvascular blood flow (MBF) was calculated by the product of A and β.⁶

**Ultrasound-Targeted Microbubble Destruction (UTMD)**

For UTMD, ultrasound was transmitted over the ischemic muscle using a phased array transducer (Sonos 5500, Philips Ultrasound) at 1.3 MHz and a transmit power of 0.9W (120 V, 9 mA, peak negative acoustic pressure -2100 kPa) at a pulsing interval of 5 s.³–⁵ Cationic microbubbles (1.5 mL; 1x10⁹) charge-coupled with 2.5 µg of miR-126-3p or scrambled miRNA (2.5 µg) was infused over 10 minutes intravenously during ultrasound transmission. To allow a wider field of delivery, ultrasound was transmitted during a slow sweep along the length of the ischemic hindlimb muscle, for a total of 20 minutes.

**Experimental In vivo Protocol**

For initial in vivo UTMD studies, non-ischemic Fischer 344 rats underwent UTMD of miR-126-3p or scrambled miR to the left hindlimb muscles (n=48). The right hindlimb muscles served as control for miRNA alone (without UTMD). Control animals (n=6) received no treatment. A subset of animals (n=6 for each time point) was sacrificed at 3 h, 24h, day 3 or day 7 after UTMD. Hindlimb skeletal muscle was removed for PCR and western blotting.

For chronic hindlimb ischemia studies, the left femoral artery was ligated and stripped at day 0. After 2 weeks (to allow for endogenous angiogenesis), baseline CEU perfusion was measured. UTMD of miR-126-3p (n=33) or scrambled miRNA (n=26) was performed. Control untreated animals (n=25) received no therapy. CEU perfusion was reassessed at day 28 post-ligation. Animals were sacrificed at day 17 and at day 28 post-ligation to collect tissue for post-mortem analysis. A subset of animals (n=23) underwent fluorescent microangiography at day 28 to assess microvascular density.

**Fluorescent Microangiography**

Fluorescent microangiography (FMA), a technique of post-mortem vascular casting, was performed as previously described.³–⁵ ⁷ A series of stacked images (4µm slices) were taken and the middle 25 slices (100 µm total thickness) were projected in order to quantify vascular parameters. Vessel density was calculated from the total length of vessels per tissue volume, and total length of a vascular tree in 0.036cm³ of tissue quantified. Capillary and arteriolar density was calculated by branch order analysis using Neurolucida Software package (MBF Bioscience), as previously
Capillaries were considered to be order 0 branch points while order 1 and order 2 branch points were considered to be arterioles.

In Situ Hybridization

10µm thick sections were cut from hindlimbs of animals which received miR-126-3p 24 and 72 hours before sacrifice, mounted on a clean, non-contaminated, SuperFrost®Plus slides and air-dried for 15min. Slides were submerged in 10% Neutral buffered Formalin (NBF) and left over night at room temperature. All buffers were prepared the day of staining and in situ hybridization was performed according to manufacturers protocol (Exiqon). Briefly slides were incubated with Proteinase-K (15µg/ml) at 37°C for 10min, dehydrated and hybridized at 55°C for 60min with the miR-126-3p probe (40nM with a RNA Tm of 85°C). Nuclear Fast Red was used as a nuclear counter stain and tissue samples were subsequently imaged by light microscopy.

Immunohistochemical Staining

Immunostaining was performed on hindlimb skeletal muscle, with antibodies against von Willebrand Factor (Santa Cruz) (endothelial cells) and Desmin (Abcam) (pericytes). Nuclei were counterstained with DAPI (Vector Laboratories) (blue). Briefly sections were embedded in OCT (Sakura Finetek) and cryosectioned at a thickness of 18um, fixed with 2% paraformaldehyde (Sigma-Aldrich) for 10min and washed with PBS. Sections were blocked with Donkey Serum (Abcam, 2%) and primary antibodies applied overnight (1:75 for vWF, 1:100 for Desmin). After overnight incubation sections were washed 3 times and secondary antibodies applied (Abcam, 1:200,) PE conjugated for vWF, and (Abcam, 1:200) FITC conjugate for Desmin. Secondary antibodies were incubated for 1 hour, washed with washing buffer containing Tween 20 (Thermal Fisher Scientific, 0.1%). Finally DAPI was applied as a nuclear counter stain. Sections were covered with a cover slip, glued and visualized under confocal microscopy the same day.

Statistical Methods

Data are expressed as mean ± standard error of the mean, unless otherwise specified. Differences among groups are analysed by one-way analysis of variance (ANOVA) followed by post hoc analysis (Bonferroni correction). GLM Univariate Analysis of variance (two-way ANOVA) followed by Bonferroni correction was applied for multiple comparisons when there were more than two factors influencing a variable. Differences were considered significant when P < 0.05. (GraphPad Prism5, version 5.0a).


