Regular transient limb ischemia prevents atherosclerosis progression in hypercholesterolemic rabbits

Yan Zhou[1], Nan-Rong Zhang[1], Zhi-Nan Zheng[1], Yi Yang[2], Bao-Feng Lyu[3], Hong-Li Wang[4], San-Qing Jin[1]

[1]Department of Anesthesia, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510655, China;
[2]Department of Urology, Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Shenzhen, Guangdong 518035, China;
[3]Department of Anesthesia, The First Affiliated Hospital, Zheng Zhou University, Zhengzhou, Henan 450052, China;
[4]Department of Anesthesia, The Fifth Central Hospital of Tianjin, Tianjin 300450, China.

Abstract

Background: Endothelial dysfunction, the initial pathogenic factor in atherosclerosis, can be alleviated via transient limb ischemia. We observed the effects of regular transient limb ischemia (RTLI) on atherosclerosis in hypercholesterolemic rabbits.

Methods: Twenty-eight rabbits were randomized to control, cholesterol, sham, ischemia groups ($n = 7$ each) between October 2010 and March 2011. They were fed a normal diet in the control group and hypercholesterolemic diet in other groups for 12 weeks. Six cycles of RTLI were performed once per day on the ischemia group. Serum samples were prepared to measure the total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) before the experiment (W0), at the end of weeks 4, 8, 12 (W4, W8, W12). The whole aorta was harvested at W12 and stained using Sudan IV to identify the plaque. The plaque area was measured using Image J. Results were analyzed by analysis of variance or rank sum test.

Results: Concentrations of TC in the cholesterol group were higher than those in the control group at W4 (29.60 [23.75, 39.30] *vs.* 1.00 [0.80, 1.55], $Z = -2.745$, $P = 0.006$), W8 (41.78 [28.08, 47.37] *vs.* 0.35 [0.10, 0.68], $Z = -2.739$, $P = 0.006$), W12 (48.32 [40.04, 48.95] *vs.* 0.61 [0.50, 0.86], $Z = -2.739$, $P = 0.006$). Similar results were obtained for HDL-C and LDL-C. Serum concentrations of TC, HDL-C, and LDL-C in the hypercholesterolemic groups had no differences (all $P > 0.05$). The percentage of plaque area in the cholesterol group was higher than that in the control group (47.22 ± 23.89% *vs.* 0, $Z = -2.986$, $P = 0.003$). Square root of the percentage of plaque area was smaller in the ischemia group than that in the cholesterol (0.44 ± 0.13 *vs.* 0.67 ± 0.18, $P = 0.014$) or sham groups (0.44 ± 0.13 *vs.* 0.61 ± 0.12, $P = 0.049$).

Conclusion: In hypercholesterolemic rabbits, RTLI might prevent atherosclerosis progression by reducing the percentage of plaque area.

Keywords: Atherosclerosis; Ischemic pre-conditioning; Hypercholesterolemia

Introduction

The complications of atherosclerosis are the main causes of death worldwide.[1] The formation of atherosclerosis is affected by many risk factors. However, since Ross demonstrated that various kinds of insults to the endothelium could result in plaque lesions through an inflammatory-fibroproliferative response, endothelial cell injury, and dysfunction, endothelial injury has been widely accepted as the initial factors that play critical roles in the pathogenesis of atherosclerosis.[2] Endothelial dysfunction is even found to precede the development of morphological atherosclerotic changes.[2,3] Therefore, restoration of the dysfunctional endothelium is a key target of interventions to reduce cardiovascular risk factors.[4-7]

Transient limb ischemia (TLI), defined as one or several episodes of brief occlusion followed by reperfusion of blood flow to a limb, can provide multiorgan protection against further prolonged episodes of ischemia, and it is derived from the idea of remote ischemic pre-conditioning (RIPC).[8] Kharbanda's study found that endothelial dysfunction might be prevented by TLI, which was able to attenuate the systemic activation of neutrophils.[9] A study has shown that TLI with three cycles of 5-min ischemia followed by 5-min reperfusion prevented ischemia-reperfusion-induced endothelial dysfunction in the contralateral arm in humans.[10] A study demonstrated that TLI augmented endothelium-dependent vasodilation in humans through an increase of nitric oxide (NO) production.[11] Liang's study demonstrated that long-term

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Correspondence to: Dr. San-Qing Jin, Department of Anesthesia, The Sixth Affiliated Hospital, Sun Yat-sen University, 26# Yuancunerheng Road, Guangzhou, Guangdong 510655, China
E-Mail: sanqingjin@hotmail.com

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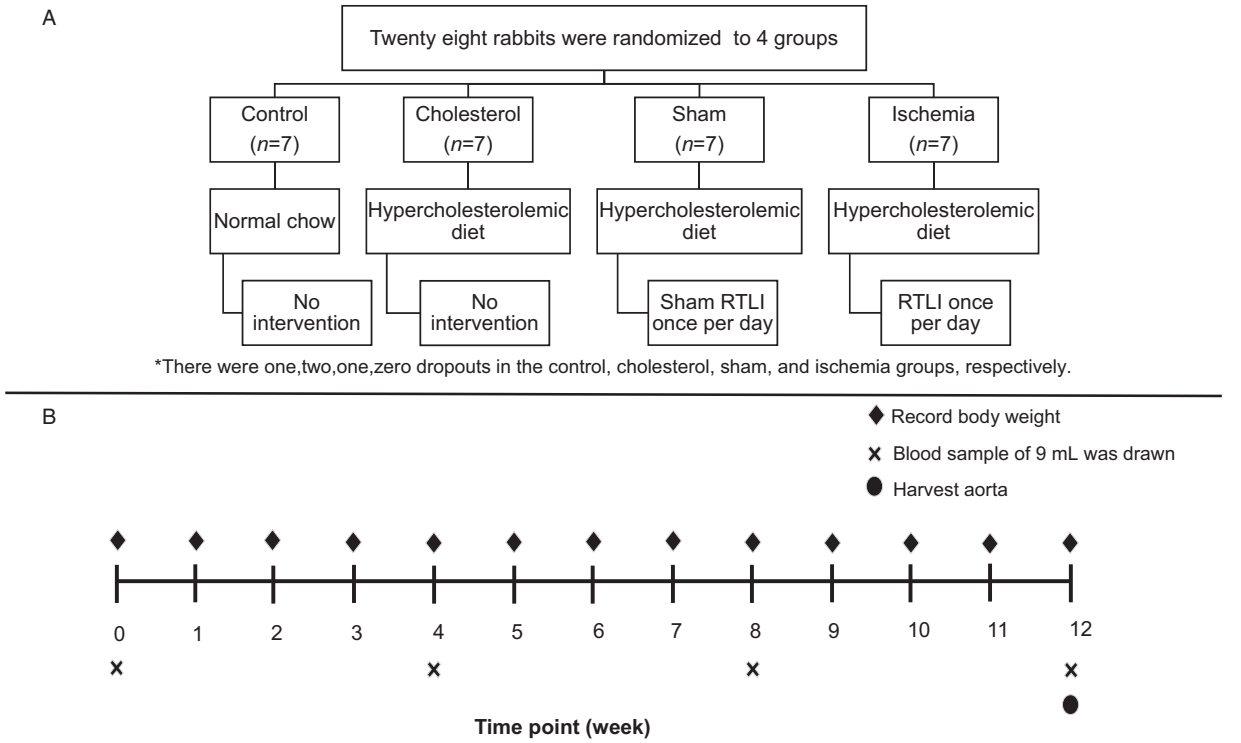

Figure 1: Flow chart of the experimental protocols of the study. (A) Animal grouping, diet, and intervention. Rabbits in the ischemia group were kept in rabbit hutches and subjected to the RTLI once per day in the afternoon during the 12-week period. Rabbits in the sham group were kept in rabbit hutches and had a deflated cuff on the left hind limb for 60 min accordingly. (B) The time points to record body weight; blood sample was drawn to prepare serum for lipids analysis and finally harvest aorta. RTLI: Regular transient limb ischemia.

RIPC improved endothelial function in patients with coronary heart disease.[12] Although the exact mechanism of endothelial protection by TLI remains unclear, TLI has been reported to provide endothelial protection.[13]

From the above studies, endothelial dysfunction can eventually lead to atherosclerosis, and TLI can protect endothelial function. We hypothesized that long-term regular TLI (hereinafter referred to as RTLI) might be an effective method to prevent progression of atherosclerosis mediated by protecting the endothelial function. This pilot study aimed to explore whether RTLI could prevent atherosclerosis progression in hypercholesterolemic rabbits.

Methods

Ethics

The study protocol was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University (No. LAEC-2010-0901), and the study complied with the Regulations for the Administration of Affairs Concerning Experimental Animals (Approved by the State Council on October 31, 1988, and promulgated by Decree No. 2 of the State Science and Technology Commission on November 14, 1988, China).

Animals and grouping

A total of 28 male New Zealand white rabbits from Medical Experimental Animal Centre of Guangdong Province, Guangzhou, China, with animal production license number SCXK ([Yue] 2008-0002), weighing 2.0 ± 0.2 kg, were randomly assigned to four groups ($n = 7$ each): control group, cholesterol group, sham RTLI as sham group, and RTLI as the ischemia group [Figure 1A].

Rabbits were maintained in individual stainless-steel cages with temperature-controlled air-conditioning (20°C) and 50% humidity, in a light-dark cycle of 12 *vs.*12 h.

After 1 week of adaptation, all rabbits received restrictive amounts of rabbit chow for 12 weeks (150 g/day), that is, 70 g in the morning and 80 g in the evening. Rabbits in the control group were fed with normal rabbit chow. Rabbits in the other three hypercholesterolemic groups were fed with normal chow in the morning and hypercholesterolemic diet in the evening (80 g hypercholesterolemic chow consisted of 1.5 g cholesterol, 9 g of corn oil, and 69.5 g of normal chow). To prepare the hypercholesterolemic diet, cholesterol powder (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) was dissolved in heated corn oil (Wilmar International Co., Ltd, Shanghai, China) and mixed immediately with the normal rabbit chow. The food was packed in a container and allowed to cool down completely before use. This feeding protocol was adopted in accordance with the literature[14] and our pilot study. During our pilot study, we found that the rabbits would eat up the hypercholesterolemic chow with our feeding protocol, but not the chow consisting of 1% cholesterol and 6% corn oil given twice per day.[14] With our feeding regimen, the total percentage of cholesterol and corn oil

consumed by the rabbit every day equaled that in a previously described protocol.[14] All rabbits had free access to water during the experimental period. Body weight of all animals was recorded before the experiment and at the end of every week during the experimental period (in weeks, W0–W12).

Intervention procedures

The rabbits in the ischemia group were kept in rabbit hutches and allowed to rest at least 15 min before being subjected to RTLI. RTLI was induced by six cycles of ischemia and reperfusion by inflating a 4.5 cm-wide blood pressure cuff (Shenzhen Medke Technology Co., Ltd, Shenzhen, China) placed on the topmost segment of the left hind limb to 200 mmHg for 5 min followed by deflating the cuff for 5 min, and this protocol has been proven to be safe and reliable in our previous study.[15] This procedure was started at the beginning of the 12-week hypercholesterolemic diet feeding. The rabbits in the ischemia group were subjected to the above RTLI once per day in the afternoon during the 12-week period, while the rabbits in the sham group were kept in rabbit hutches and had a deflated cuff on the left hind limb for 60 min accordingly. All procedures were performed without anesthesia, and the rabbits were calmed down by caressing.[15] The rabbits in the control and cholesterol groups were only fed in the cages with their respective diet; no intervention was performed on them [Figure 1A].

Analysis of serum lipids

Blood (9 mL) was drawn from the central ear artery in the morning after a 14-h fast before the beginning of the experiment (W0), at the end of the fourth, eighth, and twelfth week of the experiment (W4, W8, W12, respectively), and it was centrifuged (1500 × *g* for 15 min at 4°C) to separate the serum. Then, the serum samples were stored at –80°C until analysis. Serum concentrations of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride were measured by the enzymatic method using an automatic biochemical analyzer (ADVIA-2400, Bayer, Germany) [Figure 1B].

Aorta harvesting

At the end of the 12-week period, the rabbits were euthanized with an overdose of sodium pentobarbital (120 mg/kg; Sigma-Aldrich, Saint Louis, MO, USA) intravenously. Then, a thoracoabdominal incision was made, and the whole aorta, including the arch and thoracic and abdominal aorta, was dissected from the dorsal wall, and all adipose tissues attached were removed gently; then it was fixed in 10% neutral formalin (Guangzhou Aifa Biomedical Technology Engineering Co., Ltd., Guangzhou, China) at room temperature for 24 h for *en face* analysis of atherosclerotic lesions [Figure 1B].

En face quantification of plaque area in the whole aorta

The extent of atherosclerosis was expressed as the percentage of plaque area in the aorta, which was determined by *en face* quantification as described in the literature.[16] Briefly, after fixation with 10% neutral formalin, the aorta was cut longitudinally, unfolded, and rinsed in 70% ethanol (the volume of absolute ethyl alcohol [Sigma-Aldrich]: the volume of distilled water- = 7:3) for 3 min. Then, the aorta was stained with Sudan IV solution, which consisted of 0.1% Sudan IV (AMRESCO, Solon, OH, USA), 35% ethanol, and 50% acetone (Sigma-Aldrich) for 3 to 5 min, followed by destaining in 80% ethanol for 3 to 5 min. Thereafter, the stained aorta was splayed and pinned flat on a white plate. Images were captured using a digital camera (Canon Inc, Tokyo, Japan) in a fixed parameter and transferred to the computer. The atherosclerotic lesion area stained by Sudan IV (red stain) and total aortic surface area were measured using a computer-assisted image analysis system (NIH, Bethesda, Maryland, USA).

Statistical analysis

Continuous normal distributed variables with homogeneity variance were presented as mean ± standard deviation, repeated values were analyzed using the analysis of variance (ANOVA) for repeated measures, and other values were analyzed using the two-tailed one-way ANOVA. Comparison among normal distributed variables was done using multiple comparisons of the least significant difference. Non-normal distributed variables or normal distributed variables with heterogeneity variance were presented as median (interquartile range) and analyzed using a Mann-Whitney *U* rank sum test.

This study was designed as two levels of factorial design, after the comparison between the control and the cholesterol groups to identify the cholesterol effect, the three hypercholesterolemic diet groups were compared to identify the RTLI effect. When the plaque area among the three groups was compared, a paradox result (there was no statistical significance between the cholesterol and sham groups, and between the sham and ischemia groups; however, there was a statistically significant difference between the cholesterol and ischemia groups) was obtained, so they were compared again after transformation of the original data to square root. Statistical analysis was performed using SPSS version 16.0 statistical software package (SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

Results

General data

As shown in Figure 2, no significant differences in terms of weight gain were found among the four groups over the 12-week period ($F = 0.352$, $P = 0.788$). There were one, two, one, and zero dropouts in the control, cholesterol, sham, and ischemia groups, respectively, due to death before the experiment finished.

Lipid profiles

As shown in Table 1, the concentrations of TC in the cholesterol group were higher than those in the control

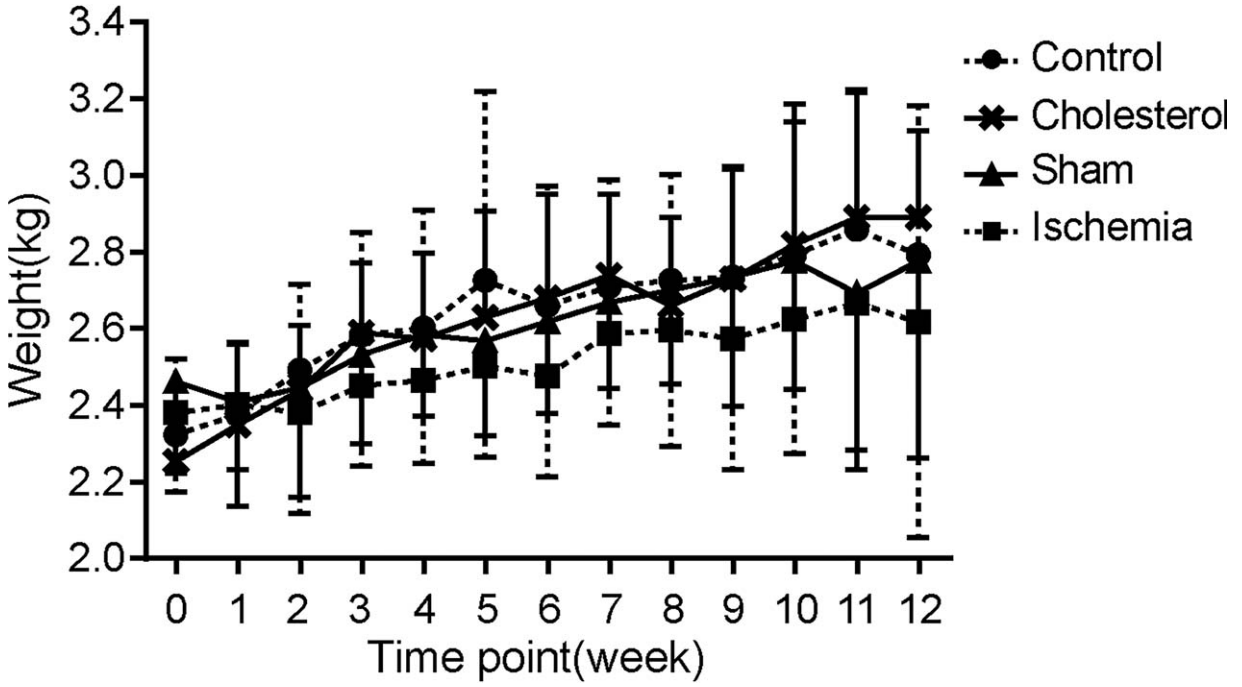

Figure 2: Weight gain of the rabbits in the control, cholesterol, sham, and ischemia groups over the 12-week period. Data are presented as mean ± standard deviation, $n=6$, 5, 6, and 7 in the control, cholesterol, sham, and ischemia groups, respectively. No differences were found among groups ($P=0.788$). kg: Kilogram; W0: Before the experiment; W1 to W12: End of the first week to the 12th week.

Table 1: Serum concentrations of TC, HDL-C, LDL-C, and triglyceride in rabbits according to the test points in the control and cholesterol groups.

Parameters	Time (week)	Control ($n=6$)	Cholesterol ($n=5$)	*Z*	*P*
TC (mmol/L)	0	1.15 (0.90, 1.45)	1.10 (0.75, 1.55)	−0.459	0.647
	4	1.00 (0.80, 1.55)	29.60 (23.75, 39.30)	−2.745	0.006
	8	0.35 (0.10, 0.68)	41.78 (28.08, 47.37)	−2.739	0.006
	12	0.61 (0.50, 0.86)	48.32 (40.04, 48.95)	−2.739	0.006
HDL-C (mmol/L)	0	0.50 (0.37, 0.54)	0.52 (0.50, 0.75)	−1.192	0.233
	4	0.54 (0.40, 0.63)	5.94 (3.56, 7.20)	−2.739	0.006
	8	0.40 (0.23, 0.54)	2.11 (2.05, 3.55)	−2.745	0.006
	12	0.42 (0.27, 0.45)	3.38 (3.20, 3.98)	−2.739	0.006
LDL-C (mmol/L)	0	0.32 (0.27, 0.50)	0.28 (0.14, 0.44)	−1.006	0.314
	4	0.57 (0.35, 1.21)	41.25 (31.78, 49.98)	−2.739	0.006
	8	0.32 (0.17, 0.57)	17.85 (14.51, 24.84)	−2.739	0.006
	12	0.23 (0.16, 0.43)	25.95 (22.52, 28.39)	−2.739	0.006
Triglyceride (mmol/L)	0	0.80 (0.70, 0.95)	0.80 (0.60, 1.65)	−0.282	0.778
	4	0.94 (0.73, 1.24)	1.19 (1.04, 2.84)	−1.826	0.068
	8	0.65 (0.50, 0.95)	1.80 (1.30, 2.80)	−2.745	0.006
	12	0.40 (0.38, 0.55)	1.80 (1.10, 3.05)	−2.764	0.006

All values are presented as median (P25, P75). Control, the group in which the rabbits were fed with normal chow without any intervention; Cholesterol, the group in which the rabbits were fed with hypercholesterolemic chow without any intervention. There were one and two dropouts in the control and cholesterol groups, respectively. HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TC: Total cholesterol.

group at W4 (cholesterol *vs.* control, 29.60 [23.75, 39.30] mmol/L *vs.* 1.00 [0.80, 1.55] mmol/L, $Z=-2.745$, $P=0.006$), at W8 (41.78 [28.08, 47.37] mmol/L *vs.* 0.35 [0.10, 0.68] mmol/L, $Z=-2.739$, $P=0.006$), and at W12 (48.32[40.04, 48.95] mmol/L *vs.* 0.61[0.50, 0.86] mmol/L, $Z=-2.739$, $P=0.006$). The concentrations of HDL-C in the cholesterol group were higher than those of the control group at W4 (cholesterol *vs.* control, 5.94 [3.56, 7.20] mmol/L *vs.* 0.54 [0.40, 0.63] mmol/L, $Z=-2.739$, $P=0.006$), W8 (2.11 [2.05, 3.55] mmol/L *vs.* 0.40 [0.23, 0.54] mmol/L, $Z=-2.745$, $P=0.006$), and W12 (3.38 [3.20, 3.98] mmol/L *vs.* 0.42 [0.27, 0.45] mmol/L, $Z=-2.739$, $P=0.006$). The concentrations of LDL-C in the cholesterol group were higher than those of

Table 2: Serum concentrations of TC, HDL-C, LDL-C, and triglyceride in rabbits according to the test points in the cholesterol, sham and ischemia groups.

Parameters	Time (week)	Cholesterol ($n=5$)	Sham ($n=6$)	Ischemia ($n=7$)	Chi-squared	*P*
TC (mmol/L)	0	1.10 (0.75, 1.55)	1.10 (0.90, 1.53)	1.10 (0.90, 1.40)	0.278	0.870
	4	29.60 (23.75, 39.30)	33.80 (29.48, 39.13)	38.00 (27.50, 38.40)	0.683	0.711
	8	41.78 (28.08, 47.37)	46.81 (39.09, 47.24)	44.89 (43.34, 46.69)	0.711	0.701
	12	48.32 (40.04, 48.95)	46.02 (34.34, 48.32)	46.08 (36.78, 48.13)	2.028	0.363
HDL-C (mmol/L)	0	0.52 (0.50, 0.75)	0.57 (0.54, 0.77)	0.49 (0.33, 0.74)	2.220	0.330
	4	5.94 (3.56, 7.20)	6.63 (5.85, 7.04)	6.39 (5.36, 7.40)	0.905	0.636
	8	2.11 (2.05, 3.55)	2.91 (2.53, 3.43)	3.00 (2.55, 3.17)	0.905	0.636
	12	3.38 (3.20, 3.98)	3.60 (3.05, 3.95)	3.50 (2.75, 4.18)	0.051	0.975
LDL-C (mmol/L)	0	0.28 (0.14, 0.44)	0.35 (0.23, 0.45)	0.27 (0.26, 0.33)	0.407	0.816
	4	41.25 (31.78, 49.98)	44.74 (39.17, 51.47)	42.59 (33.88, 54.49)	0.263	0.877
	8	17.85 (14.51, 24.84)	29.05 (20.50, 31.88)	27.43 (24.60, 30.98)	4.910	0.086
	12	25.95 (22.52, 28.39)	25.83 (20.45, 27.47)	26.43 (20.25, 30.95)	0.157	0.924
Triglyceride (mmol/L)	0	0.80 (0.60, 1.65)	0.85 (0.78, 1.20)	0.80 (0.70, 0.80)	1.491	0.474
	4	1.19 (1.04, 2.84)	1.75 (0.89, 2.76)	1.56 (1.23, 2.18)	0.147	0.929
	8	1.80 (1.30, 2.80)	1.70 (1.18, 4.43)	1.70 (1.40, 3.90)	0	1.000
	12	1.80 (1.10, 3.05)	1.60 (0.80, 6.75)	1.25 (1.05, 1.65)	1.585	0.453

All values are presented as median (P25, P75). Cholesterol, the group in which the rabbits were fed with hypercholesterolemic chow without any intervention; Sham, the group in which the rabbits were fed with hypercholesterolemic chow with sham regular transient limb ischemia; Ischemia, the group in which the rabbits were fed with hypercholesterolemic chow with a six-cycle 5-min ischemia and 5-min reperfusion on one hind limb per day. There were two, one and zero dropouts in the cholesterol, sham and ischemia groups, respectively. HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TC: Total cholesterol.

the control group at W4 (cholesterol *vs.* control, 41.25 [31.78, 49.98] mmol/L *vs.* 0.57 [0.35, 1.21] mmol/L, $Z=-2.739$, $P=0.006$), W8 (17.85 [14.51, 24.84] mmol/L *vs.* 0.32 [0.17, 0.57] mmol/L, $Z=-2.739$, $P=0.006$), and W12 (25.95 [22.52, 28.39] mmol/L *vs.* 0.23 [0.16, 0.43] mmol/L, $Z=-2.739$, $P=0.006$). The concentrations of triglyceride in the cholesterol group were higher than those of the control group at W8 (cholesterol *vs.* control, 1.80 [1.30, 2.80] mmol/L *vs.* 0.65 [0.50, 0.95] mmol/L, $Z=-2.745$, $P=0.006$) and W12 (1.80 [1.10, 3.05] mmol/L *vs.* 0.40 [0.38, 0.55] mmol/L, $Z=-2.764$, $P=0.006$).

As shown in Table 2, no differences in the concentrations of TC, HDL-C, LDL-C, and triglyceride were observed among the three high-cholesterol diet groups at W0, W4, W8, and W12 (all $P>0.05$).

Aortic intima appearance and plaque area

The aortic intima of the rabbits in the control group was smooth and had no red-stained lipid-rich lesions. In contrast, obvious red-stained lipid-rich lesions were observed in the aortic intima of the other three groups; the lesions were flat or slightly protruding into the lumen. In the cholesterol and sham groups, some lesions were fused into a large plaque, which spread along the aortic arch and thoracic aorta. However, the main location of scattered plaques in the ischemia group was in the aortic arch; a few lesions were located in the thoracic aorta, and plaque size looked smaller than that in the cholesterol and sham groups [Figure 3].

The data in the control group and the cholesterol group were analyzed by a Mann-Whitney *U* rank sum test. The original percentage of the plaque area in the cholesterol group was higher than that in the control group (cholesterol *vs.* control, $47.22\pm23.89\%$ *vs.* 0, $Z=-2.986$, $P=0.003$).

The original percentages of the plaque area in the aorta of the cholesterol, sham, and ischemia groups were analyzed using the two-tailed one-way ANOVA ($F=3.987$, $P=0.041$). There was no statistical significance between the cholesterol and sham groups (cholesterol *vs.* sham, $47.22\pm23.89\%$ *vs.* $37.88\pm13.74\%$, $P=0.373$) and between the sham and ischemia groups (sham *vs.* ischemia, $37.88\pm13.74\%$ *vs.* $20.45\pm12.90\%$, $P=0.082$); however, there was a statistically significant difference between the cholesterol and ischemia groups (cholesterol *vs.* ischemia, $47.22\pm23.89\%$ *vs.* $20.45\pm12.90\%$, $P=0.016$) [Figure 4A]. The percentages of the plaque area of the cholesterol, sham, and ischemia groups after square root transformation were analyzed again using ANOVA ($F=4.385$, $P=0.032$): the square root of the percentage of plaque area in the aorta was smaller in the ischemia group than that in the cholesterol (ischemia *vs.* cholesterol, 0.44 ± 0.13 *vs.* 0.67 ± 0.18, $P=0.014$) or sham groups (ischemia *vs.* sham, 0.44 ± 0.13 *vs.* 0.61 ± 0.12, $P=0.049$) [Figure 4B].

Discussion

This study demonstrated that RTLI could greatly decrease the percentage of plaque area in the aorta in a hypercholesterolemic rabbit model. The present study showed a promising result that RTLI could effectively prevent atherosclerosis progression.

We applied TLI as a long-term intervention (12 weeks; herein, RTLI) to prevent atherosclerosis. There is no report

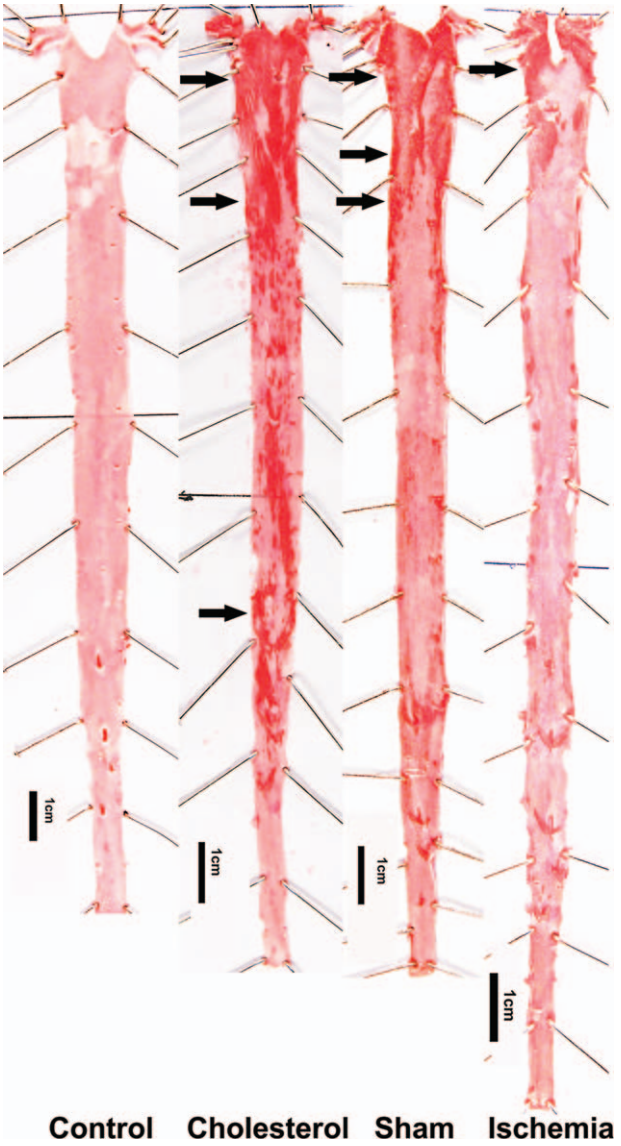

Figure 3: Representative images of the aorta by Sudan IV staining to identify the plaque lesions in the control, cholesterol, sham, and ischemia groups after 12 weeks. The plaque lesions are stained by Sudan IV as red color. There is an absence of lesions in the control group. Obvious lesions are seen in the other groups, especially in the cholesterol and sham groups. The lesions are fused and protruding out of the intima. "→" indicates the plaque lesions.

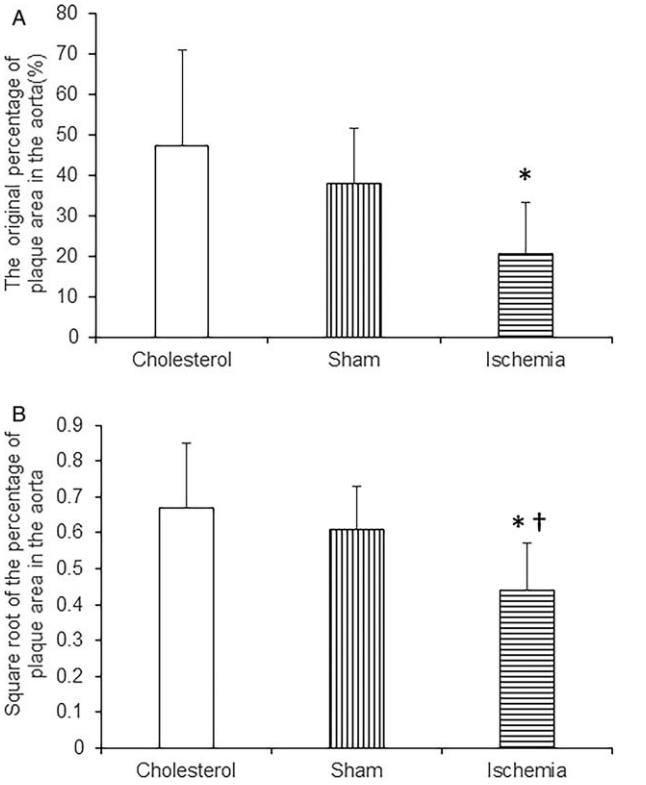

Figure 4: The plaque area in the whole aorta of the cholesterol, sham, and ischemia groups after 12 weeks. Data are presented as mean ± standard deviation. $n = 5, 6, 7$ in the cholesterol, sham, and ischemia groups, respectively. (A) The original percentages of the plaque area in the aorta. (B) Square root of the percentage of the plaque area in the aorta. $^{*}P < 0.05$ *vs.* the cholesterol group. $^{\dagger}P < 0.05$ *vs.* the sham group.

concerning the preventive effect of long-term limb ischemia on the progression of atherosclerosis; however, there are few studies reporting the effects of long-term limb ischemia on the other aspects. In 2012, Meng *et al*[17] found that 300 consecutive days of brief repetitive bilateral arm ischemic pre-conditioning reduced the incidence of recurrent stroke at 90 and 300 days and improved cerebral perfusion status. However, they had not investigated the direct effects of limb ischemic pre-conditioning upon atherosclerosis. Our result might partly interpret the mechanism of Meng *et al*'s[17] result. Kimura *et al*[11] also conducted a 4-week study, assessing endothelial function in healthy humans, and found that RTLI was associated with improved endothelial function, and increased endothelial progenitor cells, vascular endothelial growth factor, and NO levels. Neutrophil function changes (including decreased adhesion and suppressed phagocytosis) and cytokine changes, associated with modulation of the systemic inflammatory response, have been identified in a 10-day human RTLI study by Shimizu *et al*.[18]

Plaque area is an important index in many studies concerning protective intervention and the related mechanism of atherosclerosis progression.[19,20] In Johansson's study,[21] the plaque area in the thoracic aorta was increased 22 to 30 times in Angiotensin II-infused apolipoprotein E-deficient mice regardless of diet. Calkin demonstrated that Gemfibrozil significantly attenuated the plaque area of the aorta in streptozotocin-induced diabetic apolipoprotein-deficient mice, while no significant effect of gemfibrozil was observed on cholesterol in these diabetic mice.[22] To date, there has been no effective treatment strategy for atherosclerosis, which is the fundamental pathogenic factor of lethal diseases such as stroke and coronary heart disease. The present study found that RTLI, once per day for 12 weeks, can prevent progression of atherosclerosis in hypercholesterolemic rabbits by greatly reducing the percentage of plaque area in the aorta. To the best of our knowledge, this study is a rare report to verify that RTLI can decrease the percentage of plaque area in the aorta by a large amplitude in a hypercholesterolemic rabbit model *in vivo*. The technique in the present study might have the potential for clinical application as a novel and

effective adjunct intervention in atherosclerotic diseases and may be worthy of further intensive studies.

Our intermittent hypercholesterolemic chow-feeding regimen may be more suitable for establishing the hypercholesterolemic rabbit model. When rabbits are fed with high cholesterol, supplemented with a high-fat diet, the plaque area in the aorta is more easily visible.[14] However, as mentioned in our method, we had to change the feeding protocol, and we found that the rabbits would eat up the whole chow. Our protocol may be termed as intermittent hypercholesterolemic chow. Serum concentrations of TC, HDL-C, LDL-C, and triglyceride in the cholesterol group were higher than that in the control group. Obvious red-stained lipid-rich lesions were observed in the three hypercholesterolemic chow groups, but no lesion was found in all animals of the control group. Besides, there was no significant difference in weight gain among all groups. We concluded that the animal model of atherosclerosis could be established successfully with the feeding protocol of intermittent hypercholesterolemic chow.

The present study is to observe the effects of RTLI on atherosclerosis, and there is no data online to refer to in deciding the sample size, so we designed the sample size as seven rabbits in each group (routine animal experiments usually used $n = 5–10$ per group). The statistical analysis results showed that the sample size may be slightly smaller. However, the original data of the plaque area had shown statistical significance; and especially after the data transformation, the statistical significance was more powerful.

Although the present study found a promising result, that is, RTLI could greatly reduce plaque area in hypercholesterolemic rabbits, it still had some limitations. As a pilot study about the effects of RTLI on atherosclerosis, it was only a superficial step to observe the phenomenon and did not investigate the underlying mechanism of the atherosclerosis preventive effect of RTLI. Tissue micrographs of aortic with hematoxylin and eosin staining were not investigated, which was another limitation. In addition, we did not investigate the effect of other intensities, including the cuff inflating pressure, duration, and cycles of RTLI on atherosclerosis. The optimal intensity needs to be investigated. Finally, the causes of rabbit deaths before the end of the experiment were not investigated; however, it is common knowledge that almost all animal experiments will inevitably have some dropouts. Majority of these limitations will be investigated in our future research.

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Conflicts of interest

The patent to invent an RTLI machine for preventing and treating diseases related to atherosclerosis is declared and preserved by the corresponding author.

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