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Original Article

The hematologic effects of low intensity 650 nm laser irradiation on hypercholesterolemia rabbits

Hong Wang[1*], Juan Deng[1*], Wenjun Tu[2], Lin Zhang[3], Hongli Chen[1], Xiaoguang Wu[1], Yingxin Li[1], Hong Sha[1]

[1]Institute of Biomedical Engineering, Academy of Medical Science and Peking Union Medical College, Tianjin 300192, P. R. China; [2]Institute of Radiation Medicine, Academy of Medical Science and Peking Union Medical College, Tianjin 300192, P. R. China; [3]Dental Hospital of Tianjin Medical University, Tianjin 300070, P. R. China. [*]Equal contributors and co-first authors.

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Abstract: Purpose: To test the hematologic effects of low intensity 650 nm laser irradiation on hypercholesterolemia rabbits. Methods: Ten male big-eared rabbits were selected from hypercholesterolemia animal model-making and divided into model group and laser treatment groups. Five normal rabbits were selected as control group. Auricle root irradiation of 650 nm laser 100 mW in 30 min were applied on treatment group twice a day, treatment of 6 days in one week, 20 weeks course of treatment. Changes in blood lipid, microcirculation, rheological properties, and aggregation morphology of erythrocytes were observed every two weeks. Histopathological examination were performed in the end of experiments. Results: After 20 weeks' treatment, triglyceride (TG), cholesterol (CHO), high density lipoprotein (HDL) and low density lipoprotein (LDL) of serum in hypercholesterolemia groups showed less changes in the first 4 weeks, butdifferent decreasing trends were shownin the next 16 weeks' therapy. Erythrocytes aggregation of model groups showed rouleau state, while red blood cells in control group showed fine homodisperse, erythrocytes in treatment group performed better dispersion than model groups. Erythrocyte deformation index (DI) and blood flow value showed a statistic improvement in treatment group than control and model group ($P<0.01$). Value of DI in treatment group decreased after 18 weeks than values before therapy ($P<0.01$). Varying degrees of Aorta plaque formation was observed for individual difference in model and treatment groups, while no plaque was found in control group. Conclusions: low energy laser improve microcirculation, rheological properties and blood lipid that might be related with erythrocytes aggregation and deformability.

Keywords: Low intensity laser, hyperlipidemia, erythrocytes, blood microcirculation

Introduction

In the past decades, low intensity laser therapy (LLLT) was used in surgical anti-infection, rehabilitation physical therapy and photodynamic therapy, blood exposure was one of main media in the application of LLLT, which applied by intravenous and body-surface irradiation, Intravenous irradiation introduced laser light directly into the flowing blood by piercing the veins, which was firstly performed in the former Soviet Union about 25 years ago. Previous studies show LLLT of blood functioned in various diseases as modality in clinical, changes numbers of erythrocytes in elderly patients with coronary heart disease [1], activated superoxide dismutase (SOD) in ischemic heart disease [2, 3], increased oxygen content and stimulates microcirculation in blood of patients with pre-hypertension [4-7], enhanced anti-inflammation effect in chronic pancreatitis [8], nonspecific lung diseases and bronchial epithelial dysplasia [9, 10]. LLLT was applied combined with chemotherapy and made better therapeutic results than chemotherapy alone, and also applied as metabolic and health modulation in elderly people. Nowadays in china, a lot of elderly people used semiconductor laser wristwatch (650 nm) to enhance curative effect of their geriatric disease such as hyperlipidemia and get positive effect in partial of them at least. However, mechanism of LLLT was not clear as little research was reported. This article analyzed microcirculation, rheological properties, biochemical parameters, erythrocyte aggregation and lipoid constitutes in blood of hypercholesterolemia rabbits, discussed the possible theories about LLLT of blood.

Methods

Experiment animals

Animals experiments were performed under the permission of Chinese academy of medical sciences IACUC (Institutional Animal Care and Use Committee), Protocol number: ILAS-GLP-2013-012. Eighteen male big-eared rabbits were bought from Beijing KEYU animal breeding center, 12 of them were selected for hypercholesterolemia modeling, and allthese rabbits (5-6 months age) were 2.0-3.0 kg weights. Rabbits of model and therapy groups were fed with hyperlipid diet (cholesterol 1%, lard oil 5%, yolk power 5%, pig bile 0.2% and base feed 88.8%; 150 g/day), and their blood lipid parameter were detected every 2 weeks. Hyper lipid diet feeding were maintained until their blood lipid was continuous statistic difference ($P< 0.05$) for 2 weeks with that ofcontrol group, which was fed with base diet (formula feed: corn 30%, soybean cake power 23%, wheat bran 11.5%, rice bran 10%, grass meal 20%, bone meal 2%, salt 0.5% and vitamin feed additives 3%, Reference National standards of PRC: GB 14924.4-2001). Rabbits were feed separately in single cage (Length/Width/Height: 0.42 m/0.51 m/0.41 m), housing environment of rabbits was kept 16-26°C temperature, 40%-70% wet, mechanical ventilation 8 times one hour, working illumination ≥200 lx, animal illumination 100-200 lx, lighting 12 h and shade 12 h as night cycle control. In histopathological examination, experimental rabbits were administrated intraperitoneal injection of sodium pentobarbital solution 50-80 mg/kg and cut the femoral artery for mercy killing.

Low level laser irradiation

Because of its fur covered skin, rabbits auricle rootwere selected for irradiation in efficiency. Optical fiber output ports was fixed with ear of rabbit, while the rabbits were movement limited and locked into a special cage, about 1 centimeter distance from the optical fiberheadtorabbitsauricle root skin (with an angle of 45° with ears, area of contour about 4 cm^2). Semiconductor laser therapy equipment (SAS-XN, Zheng-An Med Co., China) was used, and 650 nm laser of output power 100 mw was employed for experiments. Calibration with a power meter (Gentec-EO, Canada) were performed before each therapy. Treatment of irradiation was given twice for 30 min (about 45 J/cm^2 irradiation energy) every day (8:00 AM and 5:00 PM), six days each week, totally 20 weeks.

Blood cytometry and biochemical assay

Blood samples were gotten from ear vein vessels. Before vein puncturing, kneaded rabbits' ears softly, sterilized with 75% alcohol on operating area, usingablood collection needle centripetally penetrated into terminal auricular artery in parallel direction, and blood flowed automatically into blood lancet. 0.5 mlblood was obtained from ear vessels was mixed with anticoagulation EDTA-K2, and prepared for blood cell count by using fully automatic hematology analyzer (France, ABX Pentra DX120), and 1.0 ml without anticoagulation were obtained for biochemical assay of blood serum every two weeks, using blood biochemical analyzer (Japan, Hitachi 7100).

Erythrocyte aggregation observation

To observe erythrocyte aggregation, a simply blood dropping technology was used for rough determination of erythrocyte aggregation. A drop of fresh blood (20 μl) were collected and immediately dropped onto slid glass, and let a cover slid horizontally fall down onto blood sample at a distance of 10 cm, then observed adhesive morphology of blood cells under microscope (OPTIPHOT-2, Nikon, Japan).

Hemorheology analysis

Erythrocyte sedimentation rate (ESR) were detected using Westergren tube method (0.4 ml sodium citrate, 1.6 ml fresh blood), then 1 ml blood sample of hypercholesterolemia rabbits with EDTA anticoagulant was prepared for hemorheology measurement (plasma viscosity, cut viscosity) by using an automatic blood rheology analysis instrument (Precil, LBY-N7500B, China). Twice detections were performed before and after therapy, After import data of ESR and Hematocrit, value of erythrocyte deformation and other indexes were calculated automatically by PC software of blood rheology analysis instrument.

Blood microcirculation

Blood flow measurement was performed by an instrument of full-field laser perfusion imager

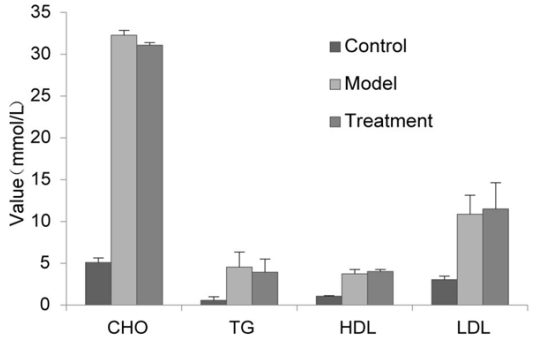

Figure 1. Blood lipid parameters (CHO, TG, HDL, LDL) of three groups after 4 weeks hypercholesterolemia animal molding.

(MoorFLPI-2, Moor Instruments Ltd, UK). Two regions of interest (ROI) in each rabbit's ear were selected for measurement using a setting of Exposure Time: 20 ms, Time Constant: 3.0 s Mode: Temporal, Filter: 25 frames, Sample Interval: 1000 ms, Image Resolution: 752 × 580. Mean flux were calculated by the software moorFLPI Full-Field Laser Perfusion Imager Review V4.0.

Pathological observation

Heart, liver, spleen and kidney were collected after mercy killing and fixated in 40 g/l formaldehyde solution immediately, after paraffin embedding, section and hematoxylin-eosin staining, checking biological tissue denaturation and lipid deposition were observed under microscope. Aorta between initial sites to 6 cm under aorta arch was token out and vertically cut to observe the pathology changes by visual observation, and then took photos with a camera and calculated area of plaque and aorta using software Image J V1.47. Plaque area ratio was finally measured. The aortic arch, thoracic aorta, abdominal aorta, femoral artery, carotid artery and organs like heart, liver, kidney and spleen were collected and observed the changes of vessel wall using paraffin section.

Statistical analysis

All data were analyzed using PC software SPSS 13.0. Serum biochemical changes, deformation index and aorta plaque area ratio results were analyzed by *t*-test methodof differences in mean values, it were considered significant when *P* values were<0.05.

Results

Result of hypercholesterolemia animal molding

Twelves rabbits were treated for hypercholesterolemia animal mold. After 4 weeks hyperlipid diet feeding, onewas dead in the third week, ten of successful model rabbits were selected for treatment and model groups, 5 rabbits each group. Values of blood lipid parameters of rabbits before irradiation experiment were showed in **Figure 1**. Hypercholesterolemia rabbits of model and treatment groups showed a significant difference with control groups (*P<0.05*), and no statistic difference between model and treatment groups (*P>0.05*).

Result of blood cytometry and serum biochemical assay

Model and treatment groups were fed with hyperlipid diet in the first 4 weeks from laser irradiation treatment beginning, and then changed with normal diet for 16 weeks laser irradiation treatment. Erythrocytes, leukocyte, thrombocyte and hemoglobin were recorded every two weeks, no observably difference were found between model and therapy groups. Serum biochemical changes were detected and results of Triglyceride (TG), cholesterol (CHO), high density lipoprotein (HDL) and low density lipoprotein (LDL) in serum were shown in **Figure 2**. Less changes were found in the first 4 weeks, while fast decreasing of serum lipids level in model and treatment groups were shown during therapy, different decreasing trends were shown in the next 16 weeks' therapy.

Observation of erythrocytes aggregation

Erythrocytes aggregation were observed using microscopes after 20 weeks LLLT, and different morphological aggregation were checked. Blood samples from model groups showed rouleau state, while red blood cells in control group showed fine homodisperse, erythrocytes in treatment group performed better dispersion than model groups. The images token from microscope and HCT value which detected simultaneously were shown in **Figure 3**.

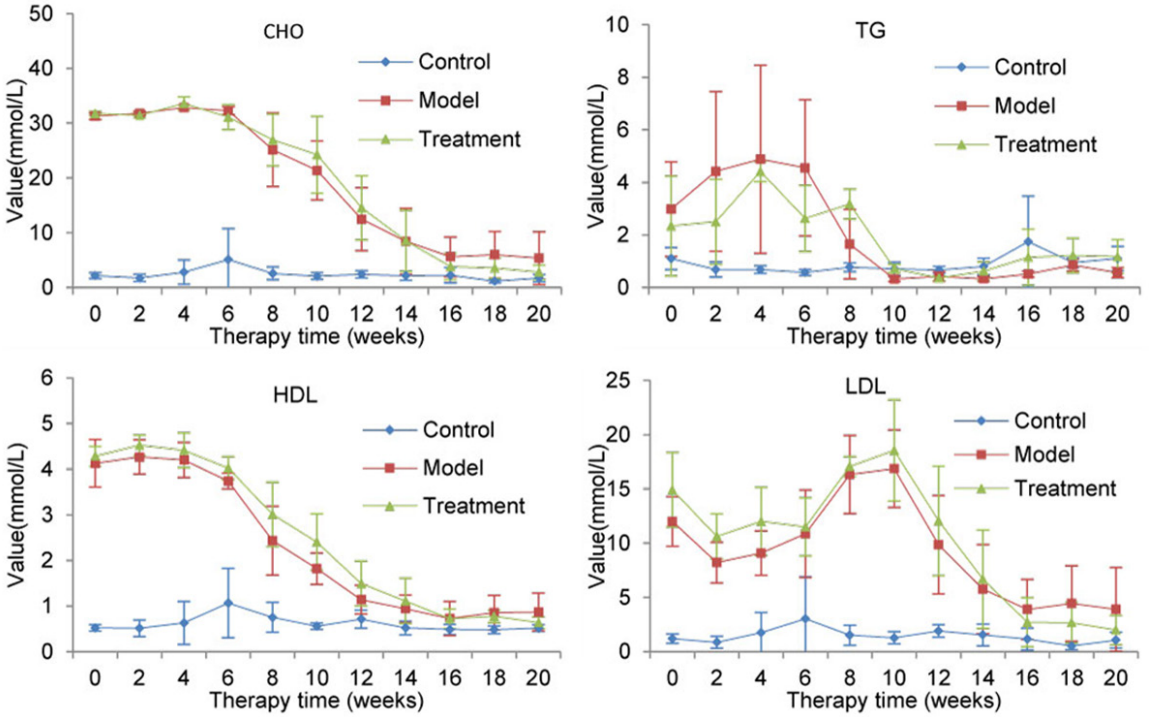

Figure 2. Serum lipid parameter CHO, TG, HDL and LDL changes during Low level laser therapy in hypercholesterolemia rabbits and control groups.

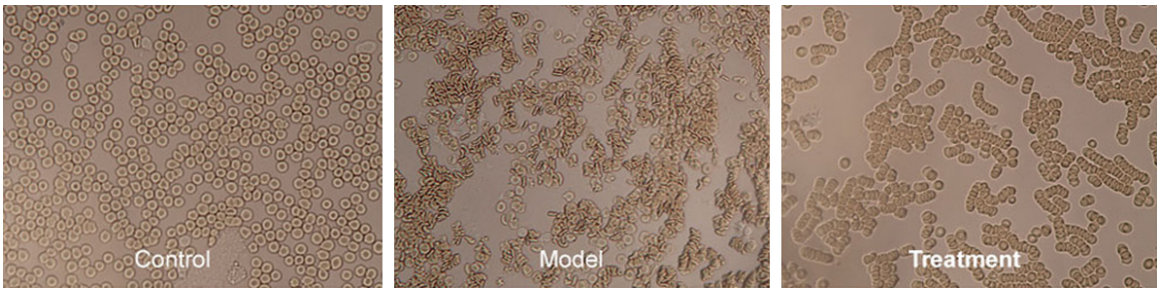

Figure 3. Erythrocytes aggregation observation in three groups. Value of HCT in three groups after 16 weeks therapy were 0.363±0.023, 0.387±0.018, and 0.365±0.044 separately.

Erythrocyte deformation index and microcirculation status in rabbit ears

To find the rheological changes of LLLT in hyperlipid rabbits, blood rheology assay and laser Doppler flowmetry were employed in the experiments. The compared results were listed in **Table 1**, compared with control group, other two groups were statistic difference both in erythrocyte deformation index (DI) and values of blood flow (*P<0.01*). DI and blood flow value showed a significant improvement in treatment group than that in control and model group (*P<0.01*). Value of DI in treatment group decreased after 18 weeks LLLT, and showed a statistic difference (*P<0.01*) than that before therapy. Although median value of microcirculation blood flow increased after 18 weeks LLLT in control and treatment groups, but no statistic difference were found between treatment and model groups after therapy (*P>0.05*). Three Regions of interest (ROI) that contained more capillary and less branch vein in ears superficial tissues were selected during blood flow assessment using software moor FLPI, as **Figure 4** showed.

Pathological changes of aorta in anatomic experiments after LLLT

Aorta plaque area ratio were calculated by software Image JV1.47, varying degrees of plaque

Table 1. Result of erythrocytes deformation index (DI) and microcirculation measured from Laser Doppler Flowmetry (Camera Gain: 72, Exposure Time: 20 ms, Time Constant: 3.0 sec Mode: Temporal, Filter: 25 frames, Sample Interval: 1000 ms, Image Resolution: 752 × 580)

	Time (weeks)	Control	Model	Treatment
Erythrocyte Deformation Index	0	0.52±0.16	0.68±0.21	0.69±0.26
	16	0.58±0.27	0.72±0.14	0.67±0.11
Median value of blood flow in ROIs	0	193.45±17.73	176.45±26.12	173.28±7.6
	16	246.57±25.08	165.89±55.29	180.91±36.5

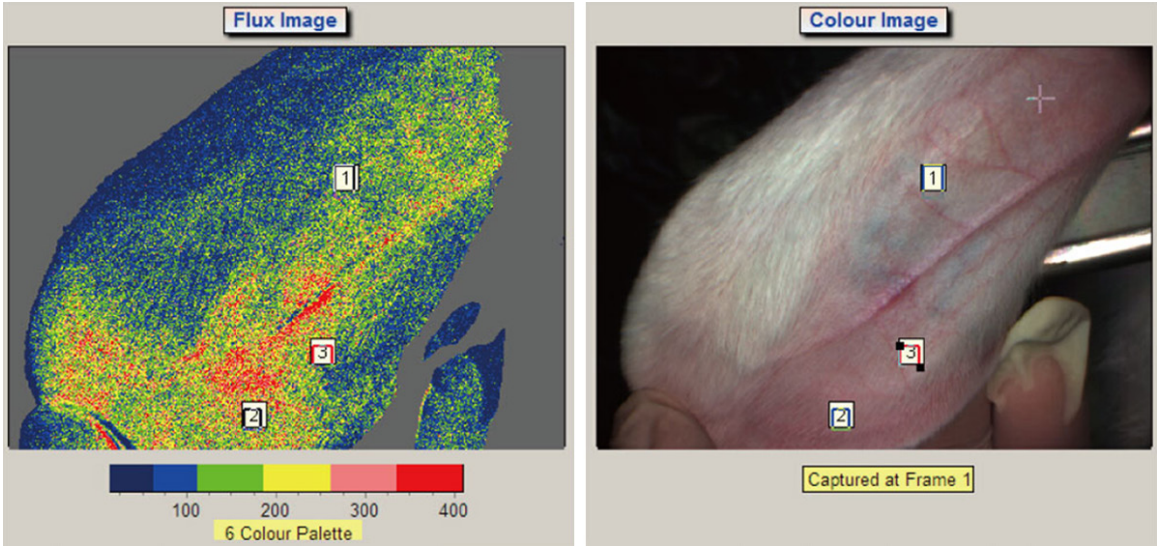

Figure 4. Regions of interest in superficial tissue of rabbit ears in blood flow assessment using PC tools moor FLPI. GIF image were list in the right.

Table 2. Aorta atheromatous plaque ratio in anatomic experiments in hypercholesterolemic rabbits after low laser therapy. No statistic difference was found between model and treatment groups (*P>0.05*)

Group	Individual Value (%)	Mean±Sd (%)
Control	0, 0, 0, 0, 0	0.00
Model	17, 100, 0, 24, 79	44±43.09
Treatment	86, 95, 16, 5, 0	40.4±46.21

formation was observed individually in model and treatment groups, while no plaque was found in control group. Results were shown in **Table 2**, as significant difference in individuals, no statistic difference was found between model and treatment groups (*P>0.05*). Pathological results showed that arteries fromhyperlipid animal appeared intima thickening, proliferation of foam cells, lipid accumulation and medial membrane calcification. In addition, inflammatory cell infiltration, hepatocyte steatosis, renal tubules dilation and cardiovascular thickening were found from pathological examination of anatomic organs (shown in **Figure 5**). Results in **Table 3** gave the statistics of pathological phenomena as listed above.

Discussion

Therapy of blood irradiation using low-level laser was beginning from last century in former Soviet Union, numbers of study reports showed its positive therapeutic effect, but it still was uncertain in usual manner, especially using by intravenous irradiation. In this research, authors discussed the LLLT effect in hypercholesterolemia using experimental animal. Rabbits were used due to its wide use as hypercholesterolemia model animal [11-13]. In agreement of previous study, CHO, TG, HDL and LDL increased after high cholesterol diet fed (**Figure 1**), especially in CHO and TG. During high cholesterol diet fed, one of them died while one

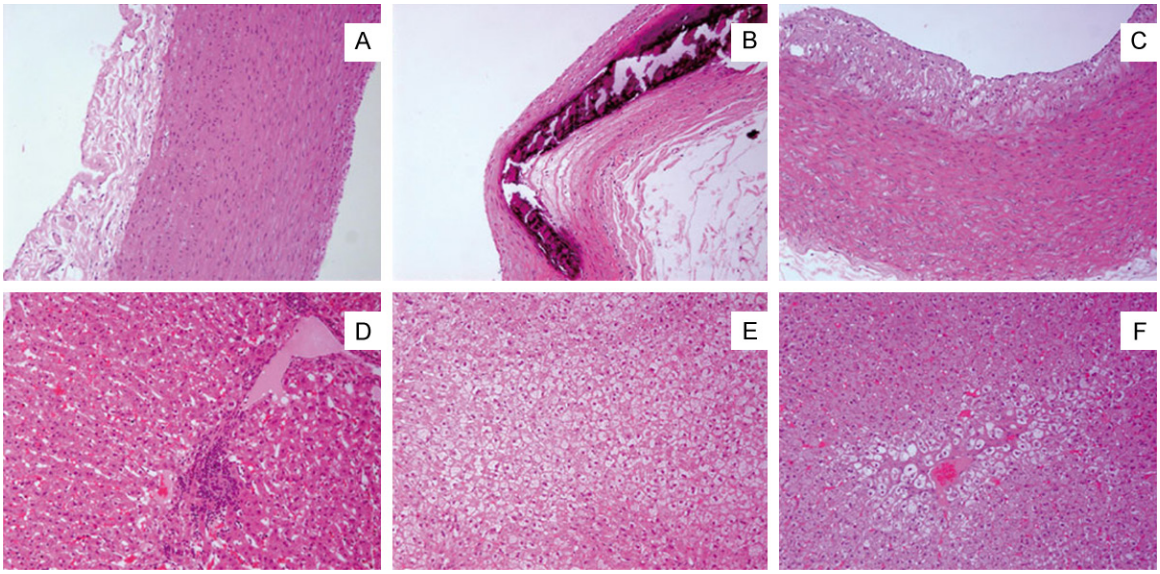

Figure 5. Pathological examination in model, treatment and control groups: A. The aortic arch in control group; B. The medial membrane calcification of aortic arch were found a rabbit of model group; C. Proliferation of foam cells and lipid accumulation in treatment group; D. Focal inflammatory cell infiltration in liver tissue from rabbits of model group; E. Hepatocyte steatosis from model group; F. Local steatosis from treatment group.

Table 3. Statistics results of Pathological abnormal phenomena (artery intima thickening, proliferation of foam cells, lipid accumulation and medial membrane calcification; organic inflammatory cell infiltration, hepatocyte steatosis, renal tubules dilation and cardiovascular thickening)

	Aortic arch	Carotid artery	Thoracic aorta	Abdominal aorta	Femoral artery	Heart	Liver	Kidney	Spleen
Model group	5 (2*)	2 (1*)	5 (2*)	2	1	1	5	2	1
Treatment group	4	2	2 (1*)	2 (1)*	2	3	2	2	0

*Number of rabbits with aorta calcification observed.

showed not significant changes in blood test, which might cause by individual tolerance.

In the first 4 weeks of therapy, LLLT seemed making no effect on level of serum cholesterol while giving high-cholesterol diet during therapy. From the fourth therapy week, model and treatment group were fed with low-cholesterol diet same as control group, levels of serum cholesterol and other lipid content decreased accordingly, showed being prone to levels of control group until 16 weeks treatment of LLLI. However, different decent rate of cholesterol was found between model and treatment groups. Slower decent rate were found in the beginning, whilebecamefaster in last few weeks than model group, that seemed interesting (**Figure 2**). So, how did laser irradiation make effect on cholesterol of blood serum? Firstly, cholesterol that adhered to membrane of erythrocytes was one of main factors of maintaining increased red blood cells adhesiveness/aggregation [14], balance of cholesterol in serum and erythrocytes rouleau might be upset by laser irradiation in high-cholesterol blood, and showed higher level of cholesterol in therapy group. Then, persistent irradiation of laser led an efficient consumption of cholesterol in blood, and finally resulted in lower level of serum cholesterol.

As it related above, LLLT might functioned on blood lipid and break the aggregation of red blood cell, released the cell overstake (**Figure 3**), it was easily being understood for its corresponding with the value of blood HCT. Besides, amount of ATP increasing, oxidation stress action and other theories might be also considered for better understanding of result from **Figure 3**.

In the results, erythrocyte deformation index and microcirculation were also observed, as it was performed in previous reports, for example, Vasil'ev et al. [15] using LLLI made positive changes in blood lipid spectrum and improvement of microcirculation in patients having CHD with hypercholesterolemia. And microcirculation studies were also been found in other LLLT application such as patients with rheumatoid arthritis [16] and chronic pancreatitis [17]. However, how could LLLT function in high-cholesterol blood? As we know, erythrocytes have unique flow-affecting properties namely aggregation capabilities, deformability and adherence to endothelial cells, which play a major role in blood flow [18]. Erythrocytes aggregation causes rheological obstruction of microcirculation in high-cholesterol model rabbits. In this study, deformability index (DI) was assessed using a rotational viscosimeter, andblood flow of microcirculation was also valued using Laser Doppler Perfusion Imaging (LDPI). DI in model and treatment groups were statistical higher than control group before and after therapy ($P<0.05$), after 20 weeks therapy, slightly increasing in control and model group was found, while slightly descend were found in treatment group (**Table 1**). In addition, blood flow of ROI in ears was assessed (**Figure 4**), which showed an agreement results with DI, increasing blood flow in control group might be related with the increasing of age, but interestingly, microcirculation of rabbits in treatment group improved while worse in model group (**Table 1**). It suggested that LLLT made an effect on erythrocyte DI and microcirculation, which might be related with erythrocytes aggregation.

To see the effect of LLLT on atheromatous plaque, this study also calculated the atheromatous plaque ratio in aorta which was collected in anatomic experiment. No statistic difference were found between model and treatment groups ($P>0.05$) (**Table 2**), obvious difference in individual value showed variance sensitive to high lipid diet in atherosclerosis. In addition, pathological examination of aortas and tissues of organs such as heart, kidney, liver and spleen indicated that high lipid diet induced different levels of pathologic changes, after comparison of model and treatment groups, aorta arch, thoracic aorta and liver tissue in model group showed 100% pathological changes, and more rabbits with aorta calcification were observed in model group than that in treatment group. Effect of LLLT on blood serum and erythrocytes aggregation, might be responsible for the conversion of stable atherosclerotic lesion to rupture-prone plaque, because of that diet-induced increases in cholesterol content of erythrocyte membranes (CEM) contribute to lipid core expansion [19]. Nevertheless, further studies of relationship between LLLT and atherosclerosis would be needed.

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Disclosure of conflict of interest

None.

Address correspondence to: Hong Sha, Institute of Biomedical Engineering, Academy of Medical Science and Peking Union Medical College, No. 236, Baiti Road, Tianjin 300192, P. R. China. Tel: +86-022-87891583; Fax: +86-022-87891583; E-mail: grossmetes29@126.com

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