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Reactive Effect of Low Intensity He-Ne Laser upon Damaged Ultrastructure of Human Erythrocyte Membrane in Fenton System by Atomic Force Microscopy

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Abstract To find out the mechanism of modulating the deformability of erythrocytes with low intensity He-Ne laser action, we studied the effect of low intensity He-Ne laser on the ultrastructure of human erythrocyte membrane. Erythrocytes were treated with free radicals from a Fenton reaction system before exposing them to low intensity He-Ne laser. The ultrastructure of damaged erythrocyte membrane was examined by atomic force microscopy. The results showed that the erythrocyte membrane became very rough and the molecules on the surface of the membrane congregated into particles of different magnitudes sizes after treating with free radicals. Comparing the degree of congregation of the molecular particles in the non-irradiated group and the He-Ne laser irradiated (9 mW and 18 mW) group, we found the average size of molecular particles in the laser irradiated group was smaller than that in the non-irradiated group, indicating that the low intensity laser had repairing function to the damage of erythrocyte membrane produced by the free radicals.

Keywords low intensity He-Ne laser; erythrocyte membrane; ultrastructure; atomic force microscopy

There is a great significance for guiding clinical treatment by exploring the biological effects and mechanisms of action of laser irradiation [1]. Low intensity laser irradiation has been clinically investigated and used to treat various pathologic processes such as tissue repair [2], wound healing [3] and prevention of restenosis following coronary angioplasty [4]. In recent years blood therapy using low intensity laser irradiation has become popular in a variety of clinical applications because of its ability to modulate blood rheology [5], improve microcirculation [6], increase activity of multiple enzymes [7,8] and improve the deformability of erythrocytes [9]. A lot of studies have used biochemistry data to show the effect of low intensity laser radiation on erythrocyte rheology, such as changes in membrane fluidity and lipid peroxidation levels, the content of its metabolized products malondialdehyde (MDA), superoxide dismutase and ATPase, and the membrane cholesterol-membrane phosphatide ratio in the membrane [10–12]. Despite numerous reports on laser cellular effects, the interaction mechanisms of low intensity laser irradiation with erythrocytes and its components are still far from clear. There are few reports showing molecular images of normal or damaged erythrocytes after laser irradiation. Our understanding of how the interaction between laser and molecules leads to the structural response of the erythrocyte membrane *in vivo* remains poor. The normal structure and function of erythrocyte membrane is considered a major factor in carrying out its physiological function. Observing the changes of the molecular image in the erythrocyte membrane has great significance in revealing the function of the erythrocyte. Atomic force microscopy (AFM) is a powerful tool for studying changes in membrane components and the protein skeleton of erythrocytes. It also helps us to understand the pathologic mechanisms of erythrocytes. AFM is the most prominent instrument among the scanning probe microscopes. It has been widely applied to investigate biological processes and

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topographic structures of the cell surface and biopolymers, both in air and in physiological liquid environments [13]. AFM can provide high resolution images of the cell surface and capture 3-D images under native conditions.

Based on the previous work of our group [14] and in order to observe the effect of low intensity laser irradiation on erythrocytes after treatment with free radicals, in the current study we used AFM to scan the ultrastructure images of human erythrocytes. This is the first time the effect of low intensity laser irradiation in the erythrocyte has been discussed from the point view of molecular imaging, which was obtained by AFM.

Materials and Methods

Preparation of erythrocytes

Four healthy volunteers (two men and two women), aged between 23 and 27 years, gave their consent to participate in this study. Two milliliters of fresh whole blood, drawn from each volunteer by venipuncture into a Vacutainer (Guangzhou Improve Medical Instruments, Guangzhou, China) containing EDTA, was centrifuged at 750 *g* for 10 min at 20 ºC. After the yellowish supernatant containing plasma and the white fluffy coat on the pellet were discarded, the erythrocytes were resuspended and washed three times in PBS (140 mM NaCl, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 10 mM glucose, pH 7.4) with centrifugation. Then 50 μl extracted erythrocytes were resuspended in 950 μl of PBS for normal erythrocytes. Three hundred microliters of erythrocyte solution were extracted and incubated in 3 ml of Fenton reaction solution (3% H_2O_2, 450 μM $FeSO_4$ and 1.8% NaCl mixed with isotonic solution; pH 7.0) for 30 min at 37 ºC [15]. After incubation, the damaged erythrocytes were washed three times in PBS and resuspended in 5.7 ml of PBS again.

Laser irradiation protocol

The damaged erythrocyte suspension was divided into three aliquots, two for laser irradiation and the third for non-irradiation as the control. Blood samples were placed into plastic Petri dishes. The beam from a 632.8 nm He-Ne laser (Guangzhou Laser Technology Institute, Guangzhou, China) was used. The power of the laser was 9 mW and 18 mW, which did not induce damage to erythrocytes [16,17]. A laser ergometer (LM-200A; Optics Department, Chinese Calculation Research Institute, Guangzhou, China) was used to regulate the output power of the He-Ne laser. The precision of the laser ergometer was 0.01 mW. The diameter of the light spot was 25 mm and the irradiation time was 30 min. The energy density of the laser was 3.294 J/cm^2 and 6.588 J/cm^2. With the exception of the irradiation protocol, all groups were treated under the same conditions with the same sample handling. After irradiation, both irradiated and non-irradiated samples were diluted with PBS. The hematocrit of the solutions was approximately 0.25%.

AFM sample preparation and image collection

All erythrocyte samples were deposited on freshly cleaved mica surfaces and fixed with 2.5% glutaraldehyde for 5 min. The fixed cells were washed five times with distilled water in order to avoid the disturbance of salinity, then air-dried at room temperature. AFM (AutoProbe CP; Veeco Instruments, Santa Barbara, USA) was carried out in air and imaged in contact mode. Microfabricated silicon cantilevers with a constant force of approximately 2.8 N/m were used. AFM images were planar leveled using Proscan Image Processing Software Version 2.1 (Thermo Microscopes) provided with the instrument in order to eliminate low frequency background noise in the scanning direction.

Statistical analysis

Student's *t*-test for paired data was used to determine the differences among experimental groups. Results were expressed as the mean±SD. All statistical calculations and analyses were carried out with Origin 7.0 software (OriginLab, Northampton, USA). $P<0.05$ was considered significant.

Results

Effect of free radicals on erythrocyte membrane

The AFM images of whole erythrocytes revealed some differences between normal [**Fig. 1(A,B)**] and damaged erythrocytes [**Fig. 1(C,D)**]. A low magnification (10×10 μm^2 square scanning) view of both normal and damaged whole erythrocytes showed an image of a disk with a central depression. There was no difference in the whole shape aspect of the damaged erythrocyte and the normal one, except the depth of the central concave area; the depth of the central concave area of the damaged one was shallower than that of the normal one. The diameters of the two kinds of cell were both approximately 8 μm. However, there was a significant difference in the cell surface between normal and damaged erythrocytes. The

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surface of the normal erythrocyte membrane was smooth, whereas the surface of the damaged erythrocyte was very rough.

Effect of He-Ne laser irradiation on damaged erythrocyte membrane

Fig. 2 shows the AFM 2-D and 3-D topographic images of laser irradiated erythrocytes and non-irradiated erythrocytes, all of them previously treated with free radicals (scan size 2 μm×2 μm). From the images, it can be seen that the surface of the erythrocytes featured the mountain-ridge-like structure, similar to those reported by others [18,19]. Based on the previous studies, the particles on the erythrocyte surface formed clusters of membrane proteins protruding from the membrane surface, including peripheral proteins and integral proteins. The dark areas or gorges corresponded to the phospholipids membrane bilayer below the cytoskeleton. **Fig. 2(A,D)** shows that the particles of non-irradiated erythrocytes, treated with free radicals, aggregated and some particles that existed alone were cross-linked. It is clear that two dark holes have formed due to heavy proteins joining together.

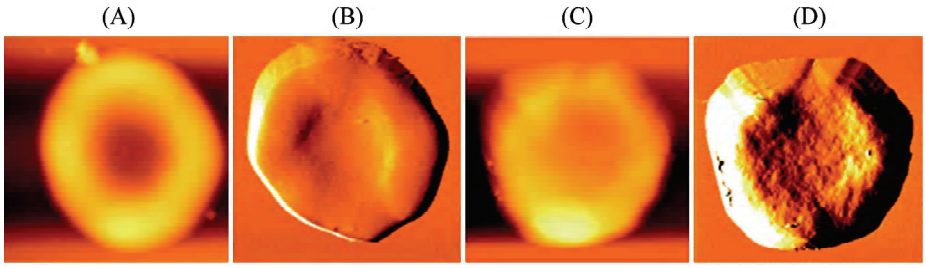

Fig. 1 Atomic force microscopy images of whole erythrocytes (scan size 10 μm×10 μm)

(A) Topographic image of a normal erythrocyte. (B) Error-signal mode image of a normal erythrocyte. (C) Topographic image of an erythrocyte treated with free radicals of the Fenton system. (D) Error-signal mode image of a damaged erythrocyte.

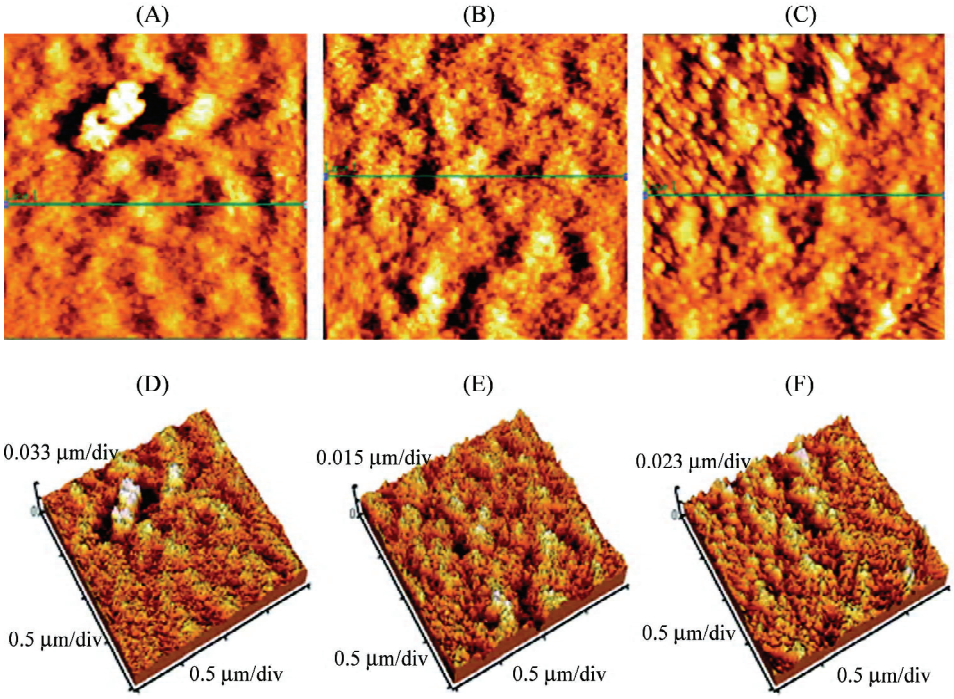

Fig. 2 Atomic force microscopy images of the ultrastructure of the membrane of damaged erythrocytes treated with free radicals of the Fenton system (scan size 2 μm×2 μm)

(A) Topographic image of damaged erythrocytes treated with free radicals and not irradiated. (B, C) Small scan size topographic images of damaged erythrocytes irradiated by 9 mW and 18 mW He-Ne laser, respectively, after treatment with free radicals. (D–F) 3-D images corresponding to panels A–C. div, minor tick labels of axis.

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Compared with the non-irradiated group, the degree of aggregation of the protein molecular particles of both laser irradiated groups had been improved [**Fig. 2(B,C,E,F)**]. The interspaces among particles of laser irradiated erythrocytes were larger than that of the non-irradiated one. From **Fig. 2(B,C)**, we can see some small single particles, which might be the cross-linked protein polymers disassembled into monomers. There is no hole on the erythrocyte membrane. The average size of the particles on damaged erythrocyte membranes was 66.41±3.25 nm (**Fig. 3**). But the sizes of particles of laser irradiated erythrocyte membranes were smaller than those of the control. The average size of particles of 9 mW laser irradiated erythrocytes was 62.50±1.78 nm (P<0.05), and that of the 18 mW laser irradiated erythrocytes was 54.69±3.22 nm (P <0.01), which is close to the size of particles that are not larger than 50 nm on normal erythrocyte membrane [20]. There was also a significant difference between the 18 mW and 9 mW groups (P<0.05). The result implies that laser irradiation has a positive effect on the modulation of erythrocyte membrane. There is a repair function for laser irradiation, in that the reactive effect of He-Ne laser makes the damaged erythrocytes turn to the normal ones.

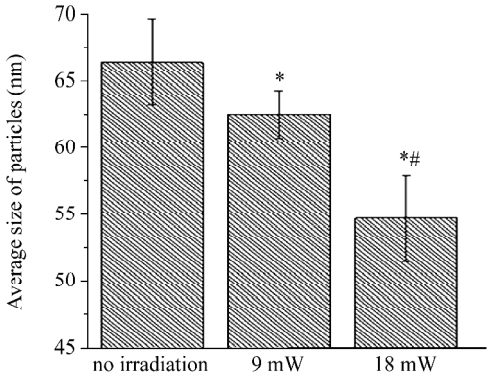

Fig. 3 Effect of laser irradiation on average size of damaged erythrocytes (n=6)

* P<0.05 versus non-irradiated erythrocytes; # P<0.05 versus 9 mW laser irradiated erythrocytes. Data are presented as mean±SD.

Effect of laser irradiation on fluctuations of erythrocyte membrane

Comparing the line scans from randomly chosen sites from the three kinds of cells [**Fig. 2(A–C)**], there are some differences among them (**Fig. 4**). The fluctuation of the non-irradiated erythrocyte membrane [**Fig. 4(A)**] was larger than that of laser irradiated ones [**Fig. 4(B,C)**]. The curves of the irradiated groups were much flatter than that of the non-irradiated. The distance between peaks in the irradiated samples was smaller than in the non-irradiated sample. Moreover, the distance between the highest and lowest points for the non-irradiated cell was 24.7 nm, whereas the corresponding distance for 9 mW and 18 mW laser irradiated cells was 22.24 nm [**Fig. 4(B)**] and 18.68 nm [**Fig. 4(C)**], respectively. The possible explanation for this observation is that laser irradiation can lead to an increase in the fluidity of the molecules and the erythrocyte membrane, improving the function of the erythrocyte membrane. The congregative form of the proteins is extended. A possible explanation could be that the cross-linked protein polymers disassembled into monomers as a result of laser irradiation, therefore the curves of the irradiation groups were much flatter and the distances between the highest point and the lowest point were smaller.

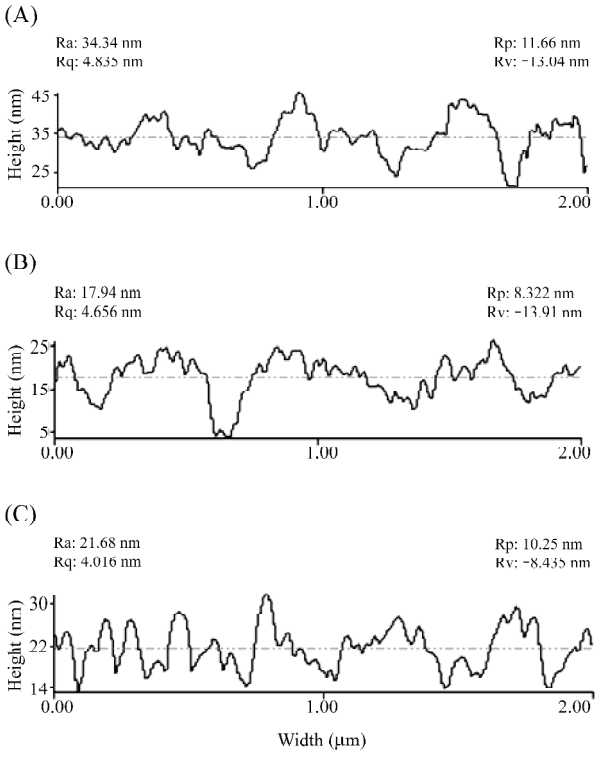

Fig. 4 Sectional curves corresponding to the lines in Fig. 2 show the relative flatness of the ultrastructure images

R_a, arithmetic average of the absolute values of the surface height deviations measured from mean line, expressed as $R_a=\frac{1}{N}\sum_{j=1}^{N}|Z_j|$, Where Z_j is the current Z value, and N is the number of the point. R_q, root mean square average of hight deviation take from the mean image date line, expressed as $R_q=\sqrt{\frac{\sum(Z_j)^2}{N}}$, Where Z_j is the current Z value, and N is the number of the point. R_v, minimum peak height within the analyzed area with respect to the mean date line. R_p, maximum peak height within the analyzed area with respect to the mean date line.

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Discussion

Free radical is the active substance, which contains not pair-matching electrons for the required physiological process. Free radicals are necessary to physiological process. However, producing free radicals in excess or at inapposite positions in cells will lead to pathological changes in the organism. It can produce a great amount of ·OH by the Haber-Weiss reaction ($Fe^{2+}+H_2O_2 \rightarrow \cdot OH+OH^-+Fe^{3+}$). ·OH is one of the strongest oxidative free radicals. Free radicals lead to changes of molecular chemical structure and conformation by damaging the hydrogen bond of membrane protein molecules, decreasing the content of membrane hydrosulfuryl bases, which are oxidized into S-S, peroxidating the hydrophile and hydrophobe areas of the phosphatide membrane molecule [15]. Furthermore, these changes could induce the destruction of the normal configuration and interaction of molecules. There are a lot of macromolecular polymers and MDA and the cross-linking of amidogen between the membrane proteins and phosphatides of the damaged erythrocyte membrane, which increases its rigidity [21]. Finally, it leads to the decrease of the fluidity of the erythrocyte membrane and deformability of the erythrocyte. Our outcomes in the current study show that the erythrocyte membrane was damaged by free radicals.

There are a lot of biostimulatory effects of low intensity laser irradiation that can provide energy for erythrocytes at a low energy state and for metabolic enzymes. The chemical activity and the stability of erythrocyte membrane is increased after low intensity laser irradiation. The results of our study showed that proteins could be prevented from cross-linking and congregating by laser irradiation. Free radicals could weaken the binding force of hydrogen bonds between proteins, and break other non-covalently bonds of protein binding as well. But molecules of the erythrocyte membrane have absorbed He-Ne laser photons, which were able to provide energy of 1.96 eV. The energy was used to break off the binding of abnormal non-covalent bonds (for example, the energy of a hydrogen bond is approximately 0.087 V–0.22 V, and that of a Van der Waals bond is approximately 0.043–0.13 V) induced by the change of conformation of membrane proteins which were damaged by free radicals and to recover the binding of the normal bonds. Then the binding force in membrane proteins or between proteins was increased. Furthermore, the revolving movement and chain swing of protein molecules themselves, which were damaged by free radicals, were reduced. The vibratory and rotational kinetic energy of membrane molecules can be provided by the low intensity He-Ne laser photons, which leads to an increase in the revolving movement and resonance movement of molecules [22], then the fluidity of the membrane increases and the function of erythrocyte membrane improves. The cross-linking of membrane proteins leads to conformational changes of membrane proteins [23]. Conformational changes of membrane proteins, especially the band 3, can modify the relation between the area of the outer membrane leaflet relative to the inner one, which in turn can lead to a change of the overall shape of the cell [24]. The biostimulatory effect of low intensity laser irradiation stems from the fact that its energy can normalize the conformation of several kinds of proteins, leading to an increase in the interaction among proteins. Furthermore, low intensity laser irradiation not only can improve the activity of several kinds of antioxidant enzymes, and increase the activity of superoxide dismutase, which can effectively eliminate the free radicals from the erythrocyte membrane, but can also reduce the level of lipid peroxidation and the content of its metabolized product MDA [25]. The deformability of erythrocytes depends on the supply of ATP, which plays an important role in maintaining the nature of cytoskeleton molecules [26,27]. Laser photons might play the role of the enzyme, making the progress in glycolysis successfully. Providing energy by He-Ne laser, photons ensure enough energy for the erythrocyte, to preserve its shape and deformability

Our results show the beneficial effect of low intensity laser irradiation on the function of erythrocyte membrane damaged by free radicals, and the effect of 18 mW laser irradiation on damaged erythrocytes is better than that of 9 mW laser irradiation. A possible explanation is that 18 mW laser can provide more photons and energy than 9 mW laser in the same timeframe. Absorbing more photons would recover the functions of the erythrocyte and erythrocyte membrane more quickly. The possible mechanisms by which low intensity laser irradiation improves the deformability of erythrocytes damaged by free radical are: (1) enhancing the activity of the ATPase and thus enhancing the metabolic function of erythrocytes and the repairing function of erythrocyte membrane; (2) increasing the activity of several kinds of antioxidant enzymes and their ability to eliminate free radicals from erythrocytes; and (3) recovering the damaged bonds and abnormal molecular conformation of the erythrocyte membrane.

AFM can provide nanometer resolution images of cell surfaces. An additional advantage of AFM is that the images can be achieved with very simple sample preparation. In contrast, transmission electron microscopy requires the

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use of ultra-thin sections, and scanning electron microscopy requires that the cells be coated with a metal. AFM has been used in blood cells, but can also be used to image other biological systems, such as endothelial cells, DNA, proteins and tumor cells. Furthermore, AFM can be used to probe nanomechanical and other fundamental properties of sample surfaces by collecting so-called force curves over a point on the sample surface. From the point of view of molecular images, AFM could be applied widely in cell biology and pathology testing. The current study showed the repair function of He-Ne laser irradiation on erythrocytes damaged by free radicals. However, this is a preliminary study and more in-depth research is recommended. In order to transfer the positive effect of low intensity laser irradiation therapy into clinical applications, the effects of blue laser and other low intensity laser irradiation on erythrocytes, revealed by AFM, could be studied in the future. Moreover, lower doses of He-Ne laser irradiation than those used in the current study could be tried and optimal doses could be determined.

References

1 Karu T. The science of low-power laser therapy. Amsterdam: Gordon and Breach Science Publishers 1998

2 Lundeberg T, Malen M. Low power He-Ne laser treatment of venous leg ulcers. Ann Plast Surg 1991, 27: 535–537

3 Lagan KM, Clements BA, McDonough S, Baxter GD. Low intensity laser therapy (830 nm) in the management of minor postsurgical wounds: A controlled clinical study. Lasers Surg Med 2001, 28: 27–32

4 Derkacz A, Bialy D, Protasiewicz M, Pawlik E, Abramski K, Grobelny A, Palasz Z *et al*. Photostimulation of coronary arteries with low power laser radiation: Preliminary results for a new method in invasive cardiology therapy. Med Sci Monit 2003, 9: 335–339

5 Siposan DG, Lukacs A. Effect of low-level laser radiation on some rheological factors in human blood: An *in vitro* study. J Clin Laser Med Surg 2000, 18: 185–195

6 Korolevich AN, Dubina NS, Vecherinsky SI. Investigation of the influence of low-intensity laser radiation on human blood microcirculation. Proc Soc Photo Opt Instrum Eng 2000, 3923: 175–179

7 Kujawa J, Zavodnik L, Zavodnik I, Buko V, Lapshyna A, Bryszewska M. Effect of low-intensity (3.75–25 J/cm^2) near-infrared (810 nm) laser radiation on red blood cell ATPase activities and membrane structure. J Clin Laser Med Surg 2004, 22: 111–117

8 Piasecka A, Leyko W, Krajewska E, Bryszewska M. Effect of combined treatment with perindoprilat and low-power red light laser irradiation on human erythrocyte membrane fluidity, membrane potential and acetylcholinesterase activity. Scand J Clin Lab Invest 2000, 60: 395–402

9 Mi XQ, Chen JY, Liang ZJ, Zhou LW. *In vitro* effects of helium-neon laser irradiation on human blood: Blood viscosity and deformability of erythrocytes. Photomed Laser Surg 2004, 22: 477–482

10 Liu SX, Hou JB, Zhou LY, Zhu H. Influence of ILIB on metabolism of membrane-lipid etc. in patients with cerebral infarction. J Optoelectronics·Laser 2000, 11: 646–648

11 Xu F, Hu L, Wang XW, Zeng Z. Effects of low power He-Ne laser irradiation on rheological characteristics of human erythrocytes. Chinese Journal of Laser Medicine & Surgery 2001, 15: 98–101

12 Chen M, Luo QM. Biologic effect of low level laser and its improving action on red blood cell deformability. Acta Laser Biology Sinica 2002, 11: 55–57

13 Zhang PC, Bai C, Huang YM, Zhao H, Fang Y, Wang NX, Li Q. Atomic force microscopy study of fine structures of the entire surface of red blood cells. Scanning Microsc 1995, 9: 981–989

14 Zhao YP, Liu SH, Sun JB, Luo GY, Hua R, Liu QQ. Clinical research on erythrocyte deformability with different doses He-Ne exposure in patient with ischemia disease. SPIE 2005, 5630: 864–873

15 Huang YM, Zhao H, Yu X, Zhong W, Guo JL, Sun SQ, Li Y *et al*. Damaging mechanism of free radical to the membrane molecules of red blood cell. Acta Biophys Sin 1997, 13: 315–323

16 Mi XQ, Cen Y, Zhou ZY, Zhou LW, Chen JY. The *in vitro* effects of low power laser irradiation on animal erythrocyte rheology. Chinese Journal of Lasers 2004, 31: 888–892

17 Mi XQ, Chen JY, Cen Y, Liang ZJ, Zhou LW. A comparative study of 632.8 and 532 nm laser irradiation on some rheological factors in human blood *in vitro*. J Photochem Photobiol B 2004, 74: 7–12

18 Takeuchi M, Miyamoto H, Sako Y, Komizu H, Kusumi A. Structure of the erythrocyte membrane skeleton as observed by atomic force microscopy. Biophys J 1998, 74: 2171–2183

19 Liu F, Mizukami H, Sarnaik S, Ostafin A. Calcium-dependent human erythrocyte cytoskeleton stability analysis through atomic force microscopy. J Struct Biol 2005, 150: 200–210

20 Cheng Y, Liu M, Li R, Wang C, Bai C, Wang K. Gadolinium induces domain and pore formation of human erythrocyte membrane: An atomic force microscopic study. Biochim Biophys Acta 1999, 1421: 249–260

21 Chiu D, Qiang Li. Lipid peroxidated of human blood cell. Int J Blood Transfusion Hematology 1990, 13: 270–272

22 Berki T, Nemeth P, Poto L, Nemeth A. Effects of photosensitization and low-power helium-neon laser irradiation on liposomes and cell membranes. Scanning Microsc 1991, 5: 1157–1164

23 Wang X, Wu ZZ, Song GB, Wang HB, Long M, Cai SX. Effect of modification of membrane proteins thiol groups in hypoxanthine xanthine oxidase system on membrane viscoelasticities of erythrocytes. Acta Biophys Sin 1998, 14: 367–372

24 Betz T, Bakowsky U, Muller MR, Lehr CM, Bernhardt I. Conformational change of membrane proteins leads to shape changes of red blood cells. Bioelectrochemistry 2007, 70: 122–126

25 Zhu J, Nie F, Zhang MJ, Zhou L, Bao XQ, Zhang HG, Shi HM *et al*. The effects of ILLLI on SOD, MDA in peripheral blood in treatment of psoriasis. Applied Laser 2002, 22: 37–41

26 Zhou XN, Geng Zl. Laser irradiation blood therapy and laser irradiation magnetized blood therapy. Acta Laser Biol Sin 2001, 10: 153–160

27 Ohta Y, Otsuka C, Okamoto H. Changes in surface roughness of erythrocytes due to shear stress: Atomic force microscopic visualization of the surface microstructure. J Artif Organs 2003, 6: 101–105

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