

Non-invasive Serum Cholesterol Detection Using Near-infrared Light Transmission

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Abstract- This paper investigated whether a simple, non-invasive blood vessel visualization technique using near-infrared light is suitable for determining serum cholesterol levels. Six-week-old male Wistar rats were divided into 6 groups ($n = 10/\text{group}$) and were fed either control diet alone or control diet with 0.5%, 1.0%, 1.25%, 1.5%, or 2.0% cholesterol for 8 weeks. A cuff was subsequently placed on the proximal part of the tail (cuff pressure, 80 mmHg), the tail distal to the compression site was exposed to near-infrared light (wavelength, 850 nm), and transmitted light was photographed. Near-infrared transmission images were acquired before and after cuff inflation, and hemoglobin and haematocrit levels in veins distal to the compression site were determined. Increased dietary cholesterol levels resulted in increased total serum cholesterol levels. Before cuff inflation, rats that were fed the 2.0% cholesterol diet had significantly lower hemoglobin levels than those that fed the control diet ($P < 0.01$). There were no significant differences in hemoglobin and haematocrit levels at 5 min after cuff inflation. From the association between total serum cholesterol levels and differences in hemoglobin levels, the differences in hemoglobin levels were almost constant when total serum cholesterol levels increased to near 150 mg/dL, but the differences significantly elevated at total serum cholesterol levels >150 mg/dL ($r = 0.449$, $P < 0.001$). The correlation between total serum cholesterol levels and relative changes in near-infrared light transmission significantly adhered to that between total serum cholesterol levels and differences in hemoglobin levels ($r = 0.452$, $P < 0.005$). At total serum cholesterol levels >150 mg/dL, increased cholesterol levels affected erythrocyte membranes and probably reduced their deformability, resulting in decreased hemoglobin levels (anemia) with erythrocyte destruction in the spleen as the rate-determining factor. This decrease was detected as changes in near-infrared light transmission.

Keywords- Near-Infrared Light; Cholesterol; Hemoglobin; Cuff Inflation

I. INTRODUCTION

Diabetes mellitus, dyslipidaemia, and hypertension are the main causes of ischaemic diseases. There is an increased interest in preventing these lifestyle diseases with a particular focus on arteriosclerosis, as hypercholesterolaemia is the primary risk factor for arteriosclerosis [1]. In humans, hypercholesterolaemia is defined as a total serum cholesterol level of ≥ 220 mg/dL. Because cholesterol is only minimally soluble in water, it binds with proteins to form lipoproteins. Blood cholesterol is divided into 2 types: low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), of which LDL-C is closely linked to the onset of arteriosclerosis [2]. Early detection of arteriosclerosis is extremely important to prevent ischaemic diseases, thereby necessitating routine, non-invasive diagnosis of hypercholesterolaemia.

Measuring cholesterol levels in skin cells has been proposed as a simple, less-invasive screening method for arteriosclerosis, because the skin contains high cholesterol levels. Skin cholesterol tests use commercially available test pads, which are stuck onto the palm of the hand, to estimate skin cholesterol levels based on changes in skin color due to enzymatic reactions. Skin cholesterol levels estimated by this method have been demonstrated to significantly correlate (correlation coefficients, 0.29–0.38) with Framingham risk scores and total cholesterol, LDL-C and ICAM-1 levels [3]. These tests have revealed to be of possible use for predicting coronary artery diseases [4]. However, other studies did not find significant correlations between skin cholesterol and serum cholesterol levels [4–6]. Thus, the usefulness of skin cholesterol for estimating serum cholesterol levels is uncertain.

It is known that erythrocyte cell deformability decreases with an increase in blood cholesterol level [7, 8]. Deformability is determined by factors including cell structure, cell morphology, cytoplasmic viscosity, and cell membrane flexibility. Dyslipidaemia and hypercholesterolaemia increase blood viscosity and the levels of available cholesterol from which erythrocyte membranes are comprised, resulting in reduced erythrocyte membrane flexibility [9]. As erythrocyte deformability continues to decrease, there is also a greater probability of developing anemia [10]. Anaemia that results from abnormalities in the cholesterol capacity of erythrocyte membranes is called spur cell anemia [11]. Deformed erythrocytes are promptly destroyed in the spleen.

Balkan *et al.* [12] fed rabbits with a high cholesterol diet and determined erythrocyte haemolysis. Haemolysis was found to be significantly increased compared with that in control rabbits. Haematocrit and hemoglobin levels were also significantly lower in rabbits fed the high cholesterol diet than in control rabbits. These findings suggest that erythrocyte counts in blood were decreased because of anemia caused by a high cholesterol diet [13, 14].

Near-infrared (NIR) light has a high transmittance in biological tissues and a high absorbance by hemoglobin [15]. Numerous commercial products exploit these characteristics. For example, exposing the palm of the hand to NIR light and photographing the reflected and transmitted light is used to non-invasively acquire a palm vein pattern, which can be used for

personal identification [16]. Furthermore, because of the different NIR light absorption characteristics of reduced and oxidised hemoglobin, active regions in the brain can be determined by measuring changes in arterial and venous blood [17]. The authors also compressed the upper arms of human subjects using a cuff to cause blood congestion in distal finger veins and measured the resulting reduction in NIR light transmission [18]. This paper anticipates that this technique could be useful for non-invasive determinations of vascular stiffness (vascular compliance).

Based on the above findings, the authors hypothesized that increased serum cholesterol levels would induce reduced hemoglobin levels and that measuring NIR light transmission under these circumstances would make it possible to determine differences from the normal physiological state. Thus, in this study, the authors investigated whether a simple, non-invasive technique of blood vessel visualization using NIR light was suitable for determining serum cholesterol levels. To achieve this, they compressed the tails of rats with induced hypercholesterolaemia to create caudal vein congestion and measured NIR light transmission.

II. METHODS

A. Experimental Animals

Six-week-old male Wistar rats (Clea Japan, Inc., Tokyo, Japan) were used for this study. Throughout the feeding period, rats were housed in cages (2 rats/cage), and water and food was provided *ad libitum*. Room temperature was maintained at 24 °C and humidity was set at 30–60%. Lights were turned on at 09:00 h and turned off at 21:00 h.

After the preliminary feeding period, rats were divided into 6 groups (10 rats/group). Rats in these groups were fed either control diet (CE-2, Clea Japan, Inc.) alone or control diet mixed with 0.5%, 1.0%, 1.25%, 1.5%, or 2.0% cholesterol (w/w) (Wako Pure Chemical Industries Ltd., Osaka, Japan). Feed mixed with cholesterol also contained 0.5% sodium cholate (w/w) (Wako Pure Chemical Industries Ltd.) [19]. This feeding period was continued for 8 weeks. This study was approved by the Utsunomiya University Animal Research Committee.

B. Rat Tail Caudal Vein Imaging Using NIR Light

Fig. 1 shows a schematic of the equipment used to image rat tail caudal veins using NIR light. An inhalation anesthesia system was used to anesthetize the rats by the inhalation of isoflurane with 1.0% concentration at a flow rate of 450 mL/min. Because rat tail caudal veins are located on the lateral side, rats were secured on the measuring platform in the left lateral decubitus position. The measuring platform had a slit measuring 10 mm in the tail length direction and 5 mm in the tail width direction; the tail was placed above this slit.

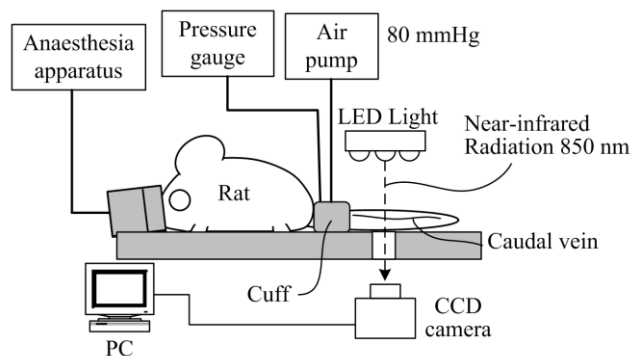


Fig. 1 A schematic of our experimental set-up

The experimental set-up included a site to compress the rat tail and a site to capture transmission images of rat tail veins. The compression site consisted of a rat tail cuff (BP-98E Cuff Sensor no. 12, width; 15 mm, Softron, Tokyo, Japan; hereafter referred to as cuff), a manual bulb for inflating the cuff and a mercury pressure gauge for measuring the pressure inside the cuff. The cuff was placed on the proximal part of the tail and blood vessel images were photographed at 40 mm distal to the cuff compression site. The imaging site consisted of a NIR LED light (LMC-61×61-10IR, Aitec System Co. Ltd., Yokohama, Japan) with a center wavelength of 850 nm and a black-and-white CCD camera (MC-781P, Texas Instruments Japan Ltd., Tokyo, Japan) that could acquire photographs in the NIR light wavelength range (700–1,200 nm). This camera had a peak sensitivity wavelength of 720 nm and the relative sensitivity at a wavelength of 850 nm was approximately 0.7. A macro lens (VS-LD35, VS Technology Ltd., Tokyo, Japan) with a focal length of 35 mm was attached to this camera. The relative sensitivity of this lens to visible light was approximately 0.9 at a wavelength of 700 nm and approximately 0.8 at a wavelength of 1,000 nm. The gain of the CCD camera was fixed for all measurements.

NIR light was transmitted through the tail from the right side and captured by the CCD camera at intervals of 1 s as a transmission image of the blood vessels in the slit area. The periods of photographing before, during and after cuff compression were 60, 300, and 60 s, respectively. It is known that cuff pressure has an effect on transmission imaging of blood

vessels. Because the systolic blood pressure in rats (14-weeks-old) was generally 130–135 mmHg, the cuff pressure was set at a lower pressure of 80 mmHg. The imaging area was approximately 9 mm × 5 mm (164 × 76 pixels) and the total pixel count was 12,464.

C. Hemoglobin and Haematocrit Measurements

After caudal tail imaging, a rat was placed in the lateral position under anesthesia, and a 0.4-mm needle (27 G) was inserted into a lateral tail caudal vein before cuff compression and at 5 min after cuff inflation. Blood that flowed from the puncture was collected in a microcuvette (10 µL, B-Hb, Hemocue AB, Ängelholm, Sweden) to measure the hemoglobin level and a haematocrit capillary tube to determine the haematocrit level. Hemoglobin levels were measured using a whole blood hemoglobin measuring system (Hemoglobin 201+ photometer, Hemocue AB) and haematocrit levels were determined using a haematocrit centrifuge (Centech 3220, Kubota Co., Tokyo, Japan).

D. Total Serum Cholesterol Measurements

After measuring hemoglobin and haematocrit levels, blood was collected from the rat's inferior vena cava under anesthesia. This blood was left standing for 3 h and then spun in a centrifuge (CT15RE, Hitachi Koki Co. Ltd., Tokyo, Japan) at 500 rpm for 15 min at 4 °C to separate serum from the clot. Serum was collected using a micropipette and total serum cholesterol was measured using a cholesterol kit (Cholesterol E-Test Wako, Wako Pure Chemical Industries Ltd.) and a spectrophotometer.

III. RESULTS

A. Total Serum Cholesterol, Hemoglobin and Haematocrit Levels

Table 1 shows the effects of the amount of dietary cholesterol on total serum cholesterol, hemoglobin and haematocrit levels. Scheffé's multiple comparison test was used to compare the total serum cholesterol levels in the different rat groups. Rats that were fed the 1.5% and 2.0% cholesterol diets had significantly higher total serum cholesterol levels than those fed the control diet ($P < 0.01$), with values being as much as 4-times higher for the 2.0% cholesterol diet. Fat deposits were visually confirmed in the livers of rats fed the 2.0% cholesterol diet. For humans, a total serum cholesterol level of ≥ 220 mg/dL is used to diagnose hypercholesterolaemia.

As a result of Scheffé's multiple comparison test, before cuff compression, rats that were fed the 2.0% cholesterol diet had significantly lower hemoglobin levels than those that were fed the control diet ($P < 0.01$). There were no significant differences in hemoglobin levels at 5 min after cuff inflation. The hemoglobin levels at 5 min after cuff inflation were significantly higher compared with those before cuff compression for all diets ($P < 0.01$) in the t-test. A difference in hemoglobin levels was determined by subtracting the hemoglobin levels before cuff compression from that at 5 min after cuff inflation. Rats that were fed the 2.0% or 1.5% cholesterol diet had significantly higher differences in hemoglobin levels than those that were fed the control diet or 0.5% cholesterol diet ($P < 0.01$).

As shown in Table 1, before cuff compression, rats that were fed the 2.0% cholesterol diet had significantly lower haematocrit levels than those that were fed the control diet ($P < 0.01$). There were no significant differences in haematocrit levels at 5 min after cuff inflation. The haematocrit levels at 5 min after cuff inflation were significantly higher compared with those before cuff compression for all diets ($P < 0.01$) in the t-test.

TABLE 1 EFFECTS OF DIETARY CHOLESTEROL ON TOTAL SERUM CHOLESTEROL, HEMOGLOBIN, AND HAEMATOCRIT LEVELS (*-**: $P < 0.01$, †-‡: $P < 0.01$)

Cholesterol ratio in diet, %	Serum cholesterol level, mg/dL	Hemoglobin level, g/dL			Haematocrit level, %		
		Before compression [†]	After compression [‡]	Difference (after - before)	Before compression [†]	After compression [‡]	Difference (after - before)
Control	53.7 (7.5)*	15.8 (0.3)*	17.1 (0.4)	1.2 (0.3)*	53.2 (0.8)*	58.7 (1.3)	5.5 (1.2)
0.5	138.3 (14.4)	15.7 (0.4)	16.9 (0.5)	1.2 (0.3)*	50.6 (1.5)	56.0 (1.5)	5.4 (2.3)
1.0	165.9 (13.2)	15.5 (0.5)	17.2 (0.4)	1.8 (0.5)	50.1 (1.2)	55.7 (2.5)	5.6 (2.6)
1.25	184.1 (14.2)	15.4 (0.4)	17.3 (0.7)	1.9 (0.5)	50.3 (2.5)	56.0 (3.0)	5.7 (1.9)
1.5	204.8 (13.9)**	15.2 (0.4)	17.4 (0.4)	2.2 (0.3)**	50.9 (2.5)	56.7 (2.0)	5.8 (2.1)
2.0	226.2 (16.8)**	15.0 (0.4)**	17.2 (0.4)	2.2 (0.3)**	49.2 (1.7)**	57.4 (3.8)	8.2 (3.4)

B. Effects of Dietary Cholesterol on Blood Vessel Transmission Images

Fig. 2 shows representative blood vessel transmission images for a control rat. The left sides of the images are the direction towards the top of the tail. The center of the images is darker than the top and bottom as caudal tail veins are present in these areas. Overall, the blood vessel images were darker at 5 min after cuff inflation compared with those before compression. Compressing the proximal part of the tail occluded the veins at the compression site, and blood congestion occurred at the site distal to the compression site. These results suggest that the congestion caused the promoted absorption of NIR light and,

therefore, its decreased transmission, which resulted in darker over all images. These results were also observed in finger vein images taken when the upper arms of human subjects were compressed [18]. The mean brightness of blood vessel transmission images was calculated by averaging the gray scale values for all pixels in the images. NIR LED light output was adjusted so that the mean brightness before compression was approximately 170.

Fig. 2 shows the representative time course of mean brightness from initiation of the experiment for the rat that was fed the control diet or 2.0% cholesterol diet. There was very minor change in mean brightness before compression (0–60 s). Once compression was initiated, the mean brightness decreased and converged to a certain value. After terminating compression (360–420 s), the mean brightness rapidly increased, and recovered to its pre-compression value. These changes over time were the same as those observed for finger vein images taken when the upper arms of human subjects were compressed.

Change in mean brightness was determined by subtracting the mean brightness at the end of compression from that at the start of compression and then divided by the mean brightness at the start of compression to determine relative change in mean brightness.

Fig. 3 shows the effects of the amount of dietary cholesterol on relative change in mean brightness. Scheffé's multiple comparison test showed that rats that were fed the 2.0% cholesterol diet had a significantly higher relative change in mean brightness compared with rats that were fed the control diet and those that were fed the 0.5% cholesterol diet ($P < 0.05$). Rats that were fed the 1.5% cholesterol diet also had a significantly higher relative change in mean brightness compared with those that were fed the control diet ($P < 0.05$).

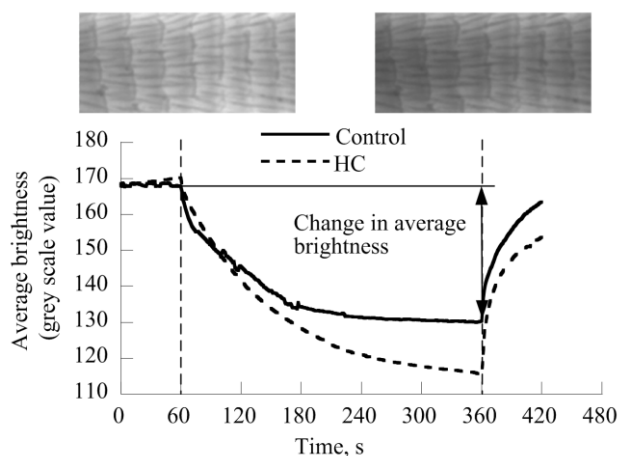


Fig. 2 NIR images of lateral tail veins in a control rat before and after compression at 80 mmHg cuff pressure, and representative data for mean brightness (gray scale values). HC means data measured from a rat that was fed the 2.0% cholesterol diet

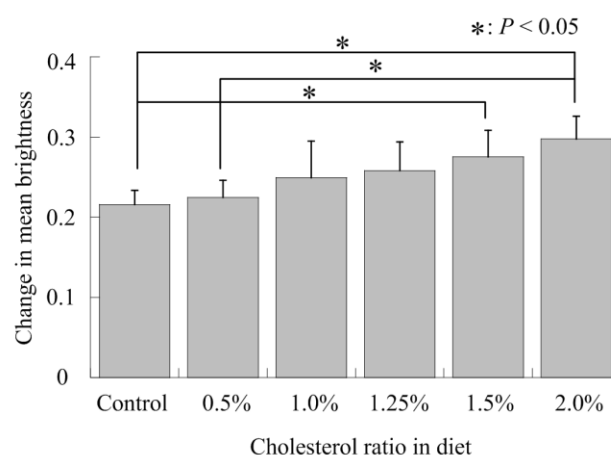


Fig. 3 Relationship between change in mean brightness and dietary cholesterol ratio

C. Associations between Total Serum Cholesterol Levels and Differences in Hemoglobin Levels and Relative Changes in Mean Brightness

Fig. 4 shows a scatter plot for differences in hemoglobin levels against total serum cholesterol levels for all rats. The differences in hemoglobin levels remained approximately the same when total serum cholesterol levels ranged from approximately 50 mg/dL (total serum cholesterol in rats fed the control diet) to 150 mg/dL, but increased significantly at those >150 mg/dL (Pearson correlation coefficient $r = 0.449$, $P < 0.001$, SPSS ver. 17.0).

Fig. 5 shows a scatter plot for relative changes in mean brightness against total serum cholesterol levels for all rats. As similar to results shown in Figure 4, relative changes in mean brightness remained approximately the same at total serum cholesterol levels <150 mg/dL, but increased significantly at those >150 mg/dL (Pearson correlation coefficient $r = 0.452$, $P < 0.005$, SPSS ver. 17.0).

These results indicated that the association between total serum cholesterol levels and differences in hemoglobin levels was similar to that between total serum cholesterol levels and relative changes in mean brightness.

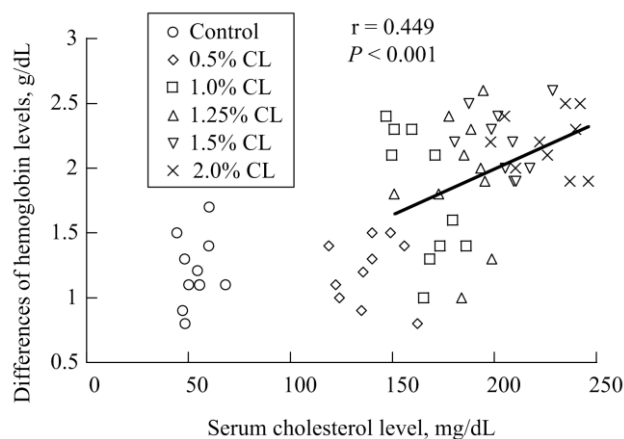


Fig. 4 Association between serum cholesterol levels and differences in hemoglobin levels. Difference in hemoglobin levels was determined by subtracting the hemoglobin levels before cuff compression from those at 5 min after cuff inflation. There was a significant correlation at hemoglobin levels >150 mg/dL

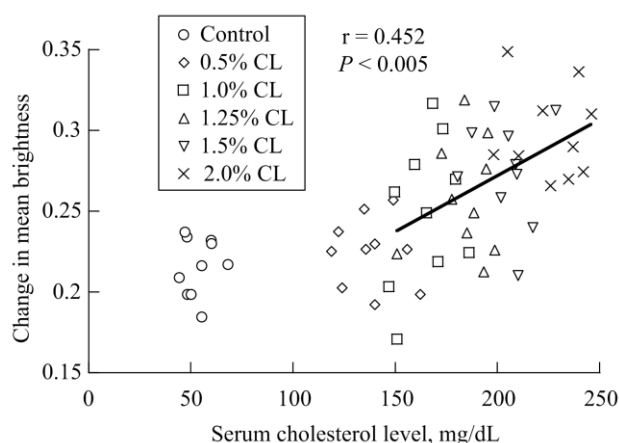


Fig. 5 Association between serum cholesterol levels and changes in mean brightness. Change in mean brightness was determined by subtracting the mean brightness at the end of compression from the mean brightness at the start of compression. Change in mean brightness was then divided by the mean brightness at the start of compression to determine the relative change in mean brightness. There was a significant correlation at hemoglobin levels >150 mg/dL

IV. DISCUSSION

In Table 1, before cuff compression, rats that were fed the 2.0% cholesterol diet demonstrated significantly lower hemoglobin and haematocrit levels than those that were fed the control diet ($P < 0.01$), but there were no differences in hemoglobin or haematocrit levels at 5 min after cuff inflation. It is known that the erythrocyte membrane cholesterol content is affected by serum cholesterol levels, and elevated serum cholesterol levels increase the erythrocyte membrane cholesterol content and distort the appearance of erythrocytes. The significant differences observed in hemoglobin and haematocrit levels before cuff compression were considered to have occurred because of the reduction in erythrocyte flexibility [9, 20]. After cuff compression, when blood congestion occurs in rat tail caudal veins, it is considered that the plasma flows out of the vessel through the vascular wall as the intravascular pressure increases. This indicates that erythrocytes remained within these congested veins and the significant differences in hemoglobin and haematocrit levels observed before cuff compression disappeared. These reasons raised a possibility of the cause of the significant differences in hemoglobin levels.

However, the results in Fig. 4 revealed that the association between the total serum cholesterol levels and differences in hemoglobin levels is not linear. The total serum cholesterol levels required >150 mg/dL to affect the differences in hemoglobin levels. Matawari *et al.* [21] showed that the serum cholesterol levels significantly increased in rats that were fed the diet containing 0.5% cholesterol and 0.15% sodium cholate; however, hematological parameters were not determined in these rats. Westerman *et al.* [22] measured hematological parameters after feeding rabbits a diet containing 5% cholesterol and found that blood conditions were biphasic before and after 5 weeks of feeding. Before 5 weeks of feeding (initial phase), serum

cholesterol levels rapidly increased, and erythrocyte cholesterol contents and erythrocyte counts were normal. However, after 5 weeks of feeding (second phase), the erythrocyte cholesterol contents increased and erythrocyte counts decreased as the serum cholesterol levels increased. The decreased affinity of serum lipoproteins was proposed as a primary cause for this phenomenon [23]. Rogausch *et al.* [24] measured hematological parameters and erythrocyte filterability in rabbits after 2, 6, and 10 weeks of feeding on the diet containing 1% cholesterol. They found that serum cholesterol levels significantly increased after 2 weeks, while hemoglobin and haematocrit levels decreased only after 10 weeks of feeding. Erythrocyte filterability also decreased only after 10 weeks of feeding. Thus, between 2 and 10 weeks of feeding, serum cholesterol levels were significantly elevated, whereas hemoglobin and haematocrit levels were normal, and the erythrocyte flexibility was normal although the shape was transformed. In this study, it is suggested that erythrocyte flexibility was unaffected by total serum cholesterol levels <150 mg/dL. It is considered that at total serum cholesterol levels >150 mg/dL, erythrocytes with reduced flexibility due to increased erythrocyte membrane cholesterol contents could not pass through the slit-like stomata in the sinuses of the spleen; thus, underwent phagocytosis by macrophages and other phagocytes, which resulted in decreased hemoglobin levels.

Fig. 3 shows that there were significant differences in changes in brightness between the high cholesterol groups (2.0% and 1.5% cholesterol diets) and the low cholesterol groups (control diet and 0.5% cholesterol diet). As shown in Table 1, before cuff compression, the caudal tail vein blood hemoglobin levels decreased because of anemia in the high cholesterol groups, but both groups exhibited similar levels after compression. In other words, the differences in hemoglobin levels were significantly greater in the high cholesterol groups than in the low cholesterol groups. Because NIR light is absorbed by hemoglobin, the amount of transmitted light decreases and hemoglobin levels increases. Therefore, the authors considered that the variations in the differences in hemoglobin levels between the 2 groups (high vs. low cholesterol diets) were reflected by changes in brightness shown in Fig. 3.

Fig. 5 shows that relative changes in brightness were essentially stable at total serum cholesterol levels <150 mg/dL, but they increased at total serum cholesterol levels >150 mg/dL. Because the same trend was observed for differences in hemoglobin levels shown in Fig. 4, NIR light transmission was probably affected by differences in hemoglobin levels. Therefore, at total serum cholesterol levels >150 mg/dL, serum cholesterol may affect erythrocyte membranes and reduce their deformability. This would be a cause of reduced hemoglobin levels (anemia), with erythrocyte destruction in the spleen as the rate-determining factor. This hemoglobin level decrease was detected as change in brightness of NIR transmitted light. The results for relative changes in brightness shown in Fig. 5 were obtained using a non-invasive technique. Therefore, this non-invasive method was considered to enable the determination of total serum cholesterol levels.

The effects of blood cholesterol on physical vascular changes must be taken into consideration. It is known that increased blood cholesterol promotes arteriosclerosis [1]. However, because arteriosclerosis commonly occurs at sites where blood vessels curve and branch, there is less possibility that increased blood cholesterol is directly involved in this pathology, although it is possible that it has an effect on vascular stiffness. The main cause of vascular stiffness is aging [25, 26]. The vascular wall consists of elastin and collagen [27]. Ageing is known to result in decreased elastin content due to deficient elastin synthesis, which results in collagen deposition [28, 29]. These factors cause reduced vascular compliance. However, because the rats used in this study were relatively young, it can be assumed that there were no effects due to aging.

If the findings of this study are to be applied to humans, it will be necessary to consider factors that might affect the transmission of NIR light. Factors that affect hemoglobin levels include genetic erythrocyte disorders, such as sickle cell disease [30], hereditary spherocytosis [31] and others, and hypocholesterolaemia [32]. Because these conditions are associated with anemia, relative changes in NIR light transmission may be increased even though total serum cholesterol levels may not increase. In addition to aging, another factor that can affect the vascular wall is hypertension [33]. Hypertension decreases vascular compliance in the same manner as aging and also reduces vascular distensibility during fluid congestion. Therefore, relative change in NIR light transmission may not be sufficiently increased even though the total serum cholesterol levels are high.

V. CONCLUSIONS

Few changes in the differences in hemoglobin levels were observed at total serum cholesterol levels <150 mg/dL. However, at >150 mg/dL, the differences increased with an increase in total serum cholesterol levels. Changes in NIR light transmission depended on the differences in hemoglobin levels, namely, the non-invasive technique of blood vessel visualization was considered to enable the determination of serum cholesterol levels. The findings of this study indicate that the use of NIR light transmission could be highly beneficial for simple, non-invasive diagnosis of hypercholesterolaemia, thereby reducing the incidence of atherosclerosis.

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