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Evaluation of a Hypertensive Rat Model Using Peripheral Blood Neutrophil Activity, Phagocytic Activity and Oxidized LDL Evaluation

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Abstract. *Background/Aim: A system is being developed that can be used to easily evaluate the health condition of an individual with the help of trace amounts of a blood sample by focusing on xenobiotics. The system is called "Multimodal homeostasis evaluation system" (measurement of neutrophil activity, phagocytic activity of phagocytes and quantification of oxidized LDL (OxLDL)). To elucidate the possibility of using this system as an evaluation system for the health condition of an individual, clearly explaining the changes in various diseases is essential. In this study, evaluations were carried out using hypertensive model animals. Materials and Methods: Spontaneously hypertensive model rats SHR/NCrlCrlj and control rats WKY/NCrlCrlj were raised for 10 weeks from 6 to 16 weeks of age and their blood pressure was measured over time. Blood neutrophil activity (superoxide anion ($O_2^{\bullet-}$) generation and myeloperoxidase (MPO) activity) and phagocytic activity of phagocytes was measured by our developed apparatus (a simple prototype device under development). OxLDL was measured by an ELISA kit, and biochemical markers were measured using the blood sample. Results: Compared to WKY rats of the control group, systolic blood pressure, diastolic blood pressure, and mean blood pressure of SHR rats increased significantly with age. In SHR rats, there was a significant elevation in $O_2^{\bullet-}$ generation and MPO activity of neutrophils, alanine aminotransferase and triglycerides of blood, while phagocytic activity, OxLDL, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, total cholesterol and total-bilirubin decreased. Conclusion: In the hypertensive model, biochemical markers were found to have a relationship with $O_2^{\bullet-}$ generation, MPO activity, phagocytic activity of phagocytes, and OxLDL. This system is expected to be useful for clinical monitoring of hypertension diseases.*

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In recent years, the onset of chronic diseases, such as hypertension, arteriosclerosis, diabetes, and Alzheimer's disease are increasing due to lifestyle habits such as overeating, lack of exercise, and increased stress (1). It is considered that one of the onset mechanisms of lifestyle diseases is chronic inflammation caused due to the accumulation of *xenobiotics* such as viruses and bacteria, dead cells, denatured proteins, advanced glycation end products (AGEs), oxidized LDL (OxLDL), and amyloid-β within an organism (2). The following three factors are considered important to evaluate the health condition of an individual based on the accumulation of *xenobiotics*. 1. Oxidative stress participates in the production of *xenobiotics* such as OxLDL and AGEs (3-4). It is considered that measurement of neutrophil activity can be one of the methods to evaluate the status of oxidative stress that increases with aging and stress (5). 2. Decrease in the ability of phagocytes increases the accumulation of foreign substances. It is considered that the ability of phagocytes to eliminate *xenobiotics* can be evaluated through phagocytic activity (6). 3. OxLDL has high cytotoxicity, and accumulation of OxLDL induces chronic inflammation (7). Therefore, OxLDL is considered as one of the representative

molecules of *xenobiotics*, and quantification of OxLDL is useful in evaluating the accumulation status of *xenobiotics*.

Evaluating the health condition of an individual based on the above three aspects has so far presented problems such as the volume of a blood sample required is high, expensive, long evaluation time, and requirement of specialized personnel for measurement. For this reason, Hamamatsu Photonics K.K. (Shizuoka, Japan) developed a technology that simultaneously converts neutrophil activity into two types of optical information.The superoxide ($O_2^{\bullet-}$) production and myeloperoxidase (MPO) activity after stimulation with phorbol 12-myristate 13-acetate (PMA) in the hemolyzed blood sample was simultaneously determined with a real-time chemiluminescence and fluorescence monitoring system, comprised of a high speed on/off system with light emitting diode (LED) excitation light and a chemiluminescence/ fluorescence high sensitivity detector system (8). The instrument that measures the neutrophil activity can easily detect trace amounts of fluorescence with high sensitivity without hemolyzing erythrocytes (9). Making use of the fact that pH-sensitive particles cause pH to be reduced in phagocytes along with increased fluorescence intensity, an improved instrument to measure the fluorescence intensity quickly was developed (Prototype) (FL- T1200; Hamamatsu Photonics K.K., Shizuoka, Japan). In addition, an improved instrument was developed so that fluorescence intensity can be measured conveniently, utilizing the fact that fluorescence intensity increases as shedding particles are reduced inside the phagocytes. The establishment of a method to conveniently evaluate the mouse phagocytic activity of macrophage strain J744.1 and trace human peripheral blood using the improved instrument was reported (10-11). In addition, a system has been developed to detect OxLDL using silkworm-type biotinylated lectin-like OxLDL receptor-1 ligand recognition region (12).

Our group carried out a study using arteriosclerosis model mice to examine the usefulness of the three aspects that focus on *xenobiotics* (13). Hypertension, a lifestyle disease was used in this study. The incidence of hypertension is high, and hypertension is known to be associated with the onset of stroke and cardiovascular diseases (14). On the other hand, there are many people who maintain their health despite being diagnosed as suffering from hypertension. Many people cannot be directly connected as having hypertension diseases despite suffering from hypertension. The usefulness of these three viewpoints were verified using spontaneously hypertensive model rats considering that if the indices evaluating the health condition changes, then the indices can be useful in finding a method to cope with hypertension.

Materials and Methods

Animals and treatments. Male 6-week-old SHR/Crij (Spontaneously Hypertensive Rat) rats and WKY/Crlj were obtained from Charles River Laboratories Japan (Yokohama, Japan). The care and handling of the animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals at Kagawa University and were approved by the Kagawa University Institutional Animal Care and Use Committee. The rats were housed under conditions of controlled temperature and humidity with a 12-h light/dark cycle and unrestricted access to food and water. A low-fat diet (LFD; 16.1 kJ/g, 10% of energy as fat, D12450B) was purchased from Research Diets, Inc. (New Brunswick, NJ, USA).

After 1 week of prefeeding, the rats were divided into the following groups (n=3 per group): WKY group and SHR group. Each rat was then fed a low-fat diet for 10 weeks. Systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean blood pressure (MBP) were monitored by tail-cuff methods (BP-98A, Softron Co., Tokyo, Japan) at 6 weeks old, 10 weeks old, 13 weeks old, and 16 weeks old. At 6 weeks old and 16 weeks old, blood samples were collected for $O_2^{\bullet-}$ production and MPO activity from the tail vein. At 16 weeks old, plasma was collected from the heart and obtained by centrifugation. All surgeries were performed under diethyl ether anesthesia, and rats were euthanized by diethyl ether inhalation.

Measurement of biochemical markers. The level of plasma biochemical markers (triglyceride (TG), total cholesterol (T-CHO), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), aspartate transaminase (AST), alanine aminotransferase (ALT) and total-bilirubin (T-BIL)) were measured by Oriental Yeast Co. Ltd.

Simultaneous measurement of $O_2^{\bullet-}$ production and MPO activity by real-time monitoring of chemiluminescence and fluorescence (CFL-P2200; Hamamatsu Photonics K.K., Shizuoka, Japan). Thirty μl of peripheral blood was hemolyzed with hemolysis buffer (RBC Lysis Buffer, Bay bioscience, Kobe, Japan), and the cell sample was collected. Cell pellet was suspended in Ringer-Hepes buffer (154 mM NaCl, 5.6 mM KCl, and 10 mM Hepes, pH 7.4) and pre-incubated with 1 mM $CaCl_2$, 0.5 μM MCLA (6-(4-methoxyphenyl)-2-methyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one hydrochloride; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and 10 μM aminophenyl fluorescein (APF; Goryo Chemical, Inc., Hokkaido, Japan) on a glass slide (total volume: 500 μl) for 2 min at 37˚C. Here, MCLA was used as an $O_2^{\bullet-}$ -sensitive chemiluminescence probe and APF was used as a hypochlorous acid-sensitive fluorescence probe. The chemiluminescence intensity was calculated as relative chemiluminescence units (RCU), and the fluorescence intensity as relative fluorescence units (RFU) at a sampling rate of (0.5 sec). The sample was continuously stirred at 37˚C during the measurements. After 150 sec, PMA (1 μM) was injected into the sample by an auto injector. The integration fluorescence intensity (iFI) and integration chemiluminescence intensity (iCI) were calculated as the peak intensity minus the basal value after PMA-induced responses.

Evaluation of phagocytic activity of rat peripheral blood using the Prototype (FL-T1200; Hmamastu Photonics K.K). A 1500 μl phosphate-buffered saline (Sigma-Aldrich, St.Loius, MO, USA) containing 30 μg of pH-sensitive fluorescent particles (pHrodo, Green- *Escherichia coli*; Molecular probes, Carlsbad, CA, USA) was heated to 37˚C with a block heater. During the measurement, 10 μl of rat peripheral blood was added and the fluorescence intensity was measured immediately with the Prototype for 50 counts (0.5 sec/count). These values were defined as the negative

control (the values without phagocytosis). After phagocytosis for 2 h at 37˚C, the fluorescence intensity was again measured with the Prototype for 50 counts (0.5 sec/count). The difference in the fluorescence intensity obtained by subtracting the negative control value from the second measurements were defined as the phagocytic fluorescence intensity (PFI).

Measurement of plasma OxLDL. The levels of plasma OxLDL were measured using an ELISA kit (Kamiya Biomedical Company, Seattle, WA, USA), according to the manufacturer's instructions.

Statistical analysis. All results are expressed as the means±SEM. If the difference based on *t*-test or two-way analysis of variance (ANOVA) was significant, Tukey's multiple comparison test was used for paired comparisons using GraphPad Prism for Windows (GraphPad Software, Inc., San Diego, CA, USA). The results were considered to be significantly different at $p<0.05$.

Results

Changes in blood pressure during 6 to 16 weeks. At 6 weeks old, SBP, DBP and MBP showed no differences between the SHR group and the WKY group. From 10 weeks old, SBP, DBP and MBP were significantly higher in the SHR group than in the WKY group (Figure 1A-C).

Analysis of Biochemical marker. The levels of plasma LDL-C, HDL-C, T-CHO and T-BIL were significantly lower in the SHR group than in the WKY group (Figure 2A-D), and ALT and T-BIL were significantly higher in the SHR group than in the WKY group (Figure 2E and F), but ALT were no differences between the SHR group and the WKY group (Figure 2G).

Change of the blood PFI, $O_2^{\bullet-}$ production and MPO activity and the plasma OxLDL. At 6 weeks old, both $O_2^{\bullet-}$ production and MPO activity were no different between the SHR group and the WKY group. However, at 16 weeks old, both $O_2^{\bullet-}$ production and MPO activity were significantly higher in the SHR group than in the WKY group (Figure 3A-B). At 6 weeks old, PFI were significantly higher in the SHR group than in the WKY group. However, at 16 weeks old, PFI was significantly lower in the SHR group than in the WKY group (Figure 3C). At 16 weeks old, OxLDL was significantly lower in the SHR group than in the WKY group (Figure 3D).

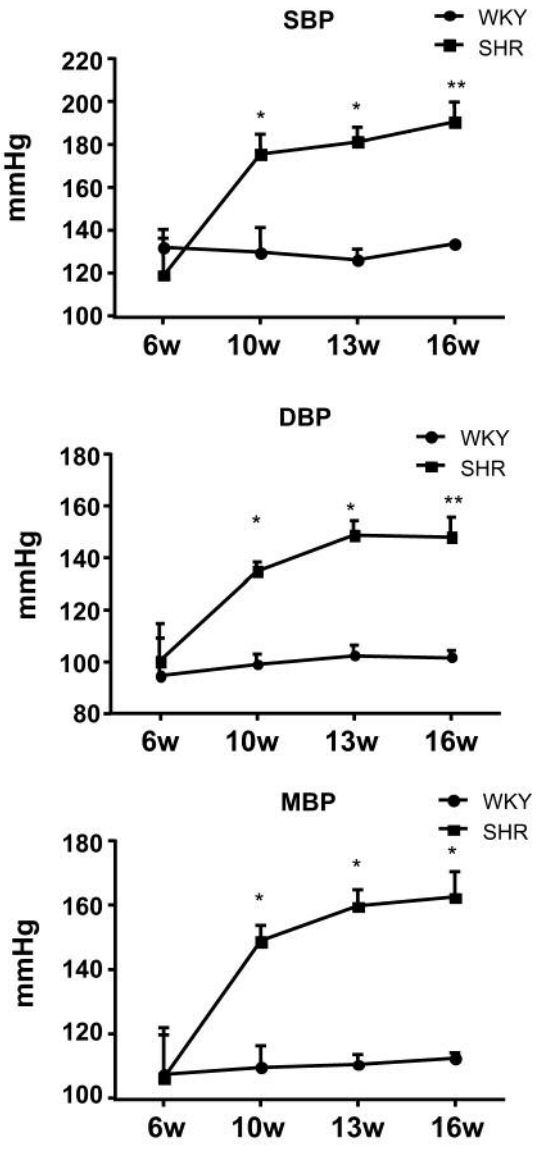

Figure 1. *Changes in blood pressure over time between the SHR group and WKY group. Systolic blood pressure (SBP) (A), diastolic blood pressure (DBP) (B), and mean blood pressure (MBP) (C) were measured at 6 weeks old, 10 weeks old, 13 weeks old and 16 weeks old rats. Black circle: WKY group; black square: SHR group. The data are presented as the means±SEM and were obtained from 3 rats per group. *p<0.05, **p<0.01, ***p<0.001 compared to the WKY group.*

Correlation between $O_2^{\bullet-}$ production and MPO activity, PFI, OxLDL and biochemical markers. The correlation coefficient showing the correlation between the four indices and each biochemical marker is summarized in Table I. $O_2^{\bullet-}$ production showed a positive correlation with SBP (Pearson's correlation coefficient (r=0.270), ALT (r=0.717), AST (r=0.122) and TG (r=0.672), and a negative correlation with T-CHO (r=–0.699), LDL-C (r=–0.564), HDL-C (r=–0.649) and T-BIL (r=–0.058). MPO activity showed a positive correlation with SBP (r=0.859), ALT (r=0.656) and AST (r=0.351) and a negative correlation with AST (r=–0.287), T-CHO (r=–0.705), LDL-C (r=–0.762), HDL-C (r=–0.715) and T-BIL (r=–0.776). PFI showed a positive correlation with SBP (r=0.429), T-CHO (r=0.841), LDL-C (r=0.738), HDL-C (r=0.775) and T-BIL (r=0.675), and a negative correlation with ALT (r=–0.879), AST (r=–0.059) and TG (r=–0.784).

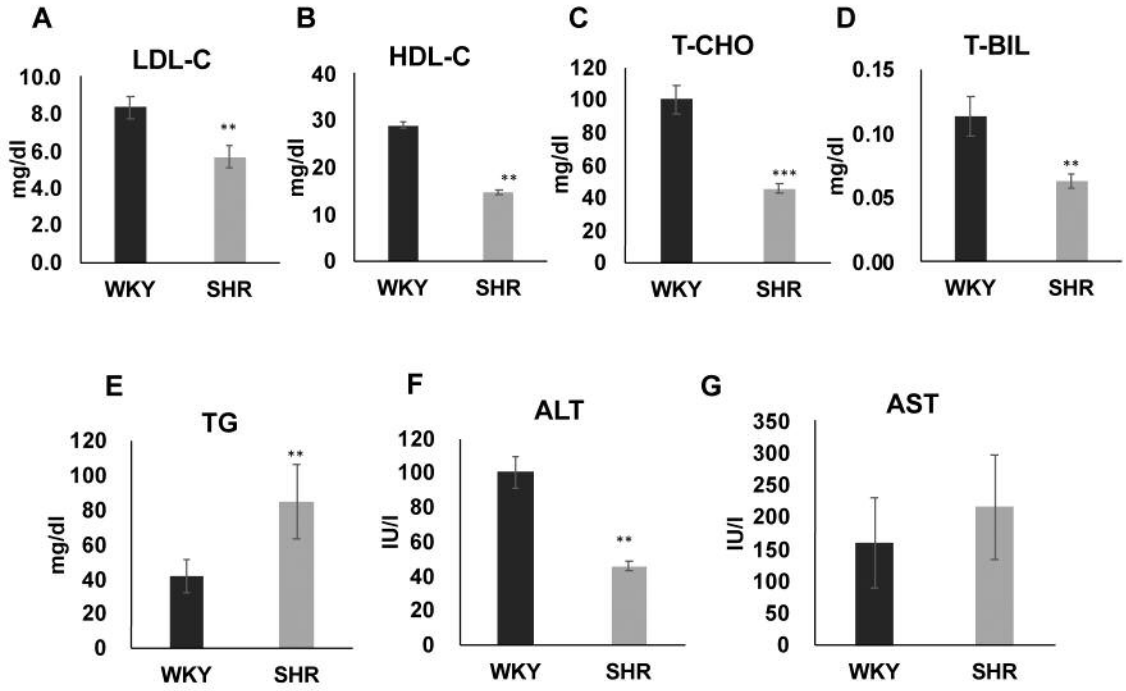

Figure 2. *The level of plasma biochemical markers between the SHR and WKY group. LDL-C: Low density lipoprotein cholesterol (A), HDL-C: high density lipoprotein cholesterol (B), T-CHO: total cholesterol (C), TG: triglyceride (D), ALT: alanine aminotransferase (E), T-BIL: total bilirubin (F). Black column: WKY group; gray column: SHR group. The data are presented as the means±SEM and were obtained from 3 rats per group. **p<0.01, ***p<0.001 compared to the WKY group.*

OxLDL showed a positive correlation with AST (r=0.025), T-CHO (r=0.745), LDL-C (r=0.737), HDL-C (r=0.630) and T-BIL (r=0.592), and a negative correlation with SBP (r=–0.756), ALT (r=–0.794) and TG (r=–0.691).

Discussion

Hypertension, diabetes, aging, dyslipidemia have been epidemiologically proven to be risk factors for stroke and cardiovascular diseases (15-17). Commonly used SHR hypertensive model rats are also known to have lipid metabolism abnormality along with spontaneous hypertension (18). In this study, it was confirmed that, SBP, DBP and MBP of SHR rats increase significantly with age (in weeks) when compared to the WKY group (Figure 1). In SHR rats, LDL-C, HDL-C, and T-CHO decreased significantly, while there was a significant increase in TG (Figure 2A-C and E). Hypertensive model rats have also been confirmed as having elevated blood pressure and lipid metabolism abnormality.

In this study, it was identified how the four indices (phagocytic activity, $O_2^{\bullet-}$ generation amount, MPO activity, and OxLDL amount) obtained from the peripheral blood, change with the increase in SBP, DBP and MBP (Figure 3). Compared to the WKY group, the amount of $O_2^{\bullet-}$ generation and MPO activity significantly increased in SHR group, while phagocytic activity significantly decreased, and there was hardly any change in the amount of OxLDL produced. Hypertension in older people has been reported to correlate with 8-isoprostane, a type of oxidative stress marker. In this study, the neutrophil activity ($O_2^{\bullet-}$ production amount and MPO activity) which is considered an index of oxidative stress, was found to be elevated. It is thought that in high blood pressure state, foreign bodies in the living body tend

Table I. *Correlation coefficient list between 4 indicators ($O_2^{\bullet-}$, MPO, PFI and OxLDL) and biochemical markers.*

R^2	O_2^- (iCI)	MPO (iFI)	PFI	OxLDL (pg/ml)
SBP (mmHg)	0.270	0.859	0.429	–0.756
ALT (IU/l)	0.717	0.656	–0.879	–0.794
AST (IU/l)	0.122	–0.287	–0.059	0.025
T-CHO (mg/dl)	–0.699	–0.705	0.841	0.745
TG (mg/dl)	0.672	0.351	–0.784	–0.691
LDL-C (mg/dl)	–0.564	–0.762	0.738	0.737
HDL-C (mg/dl)	–0.649	–0.715	0.775	0.630
T-BIL (mg/dl)	–0.058	–0.776	0.675	0.592

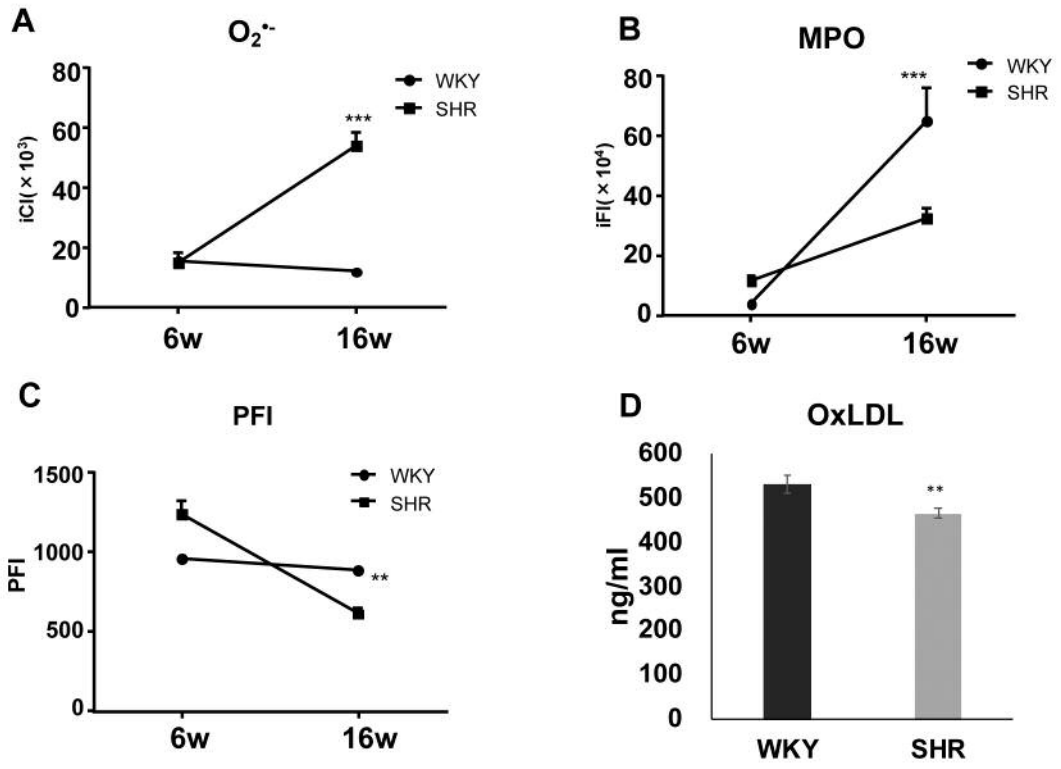

Figure 3. *Changes in $O_2^{\bullet-}$, MPO, PFI, and OxLDL. O_2^- changes at 6 weeks old and 16 weeks old rats (A), MPO changes in at 6 weeks old and 16 weeks old rats (B), PFI changes at 6 weeks old and 16 weeks old rats (C). Black circle: WKY group; black square: SHR group. OxLDL was measured at 16 weeks old rats (D). Black column: WKY group; gray column: SHR group. The data are presented as the means±SEM and were obtained from 3 rats per group. **p<0.01, ***p<0.001 compared to the WKY group.*

to be formed, and oxidative stress is developed (19). Also, the decrease in the phagocytic activity in SHR rats of 16 weeks of age was due to deterioration in the ability to eliminate *xenobiotics*. There is no difference in the amount of OxLDL in rats with hypertension and rats that are normal, but OxLDL is not accumulated in the early stages of hypertension. As the duration of hypertension increases, there is a possibility that OxLDL gets accumulated in SHR rats. The periodical changes in OxLDL in animals raised over a long duration of time should be confirmed in the future.

When the usefulness of this evaluation system was verified in the past using arteriosclerosis model mice, the generation amount of $O_2^{\bullet-}$ generation, MPO activity, and OxLDL increased significantly in the arteriosclerosis model mice compared to the regular mice, while the phagocytic activity showed a slightly decreasing trend (13). The 4 indices indicated changes that were different from the hypertensive model. This is because, the indices are considered to indicate different dynamics according to the condition of each disease.

On the other hand, items of each of the four indices related to the general biochemical markers were identified to be different. For example, T-BIL did not show any correlation with $O_2^{\bullet-}$ generation, but indicated a negative correlation with MPO activity. Further, the four indices were also observed to have a relationship with ALT, but not with AST. Each of these four indices can be considered as independent evaluation factors, and measuring each index of this evaluation system is meaningful.

If the health condition of an individual (state of oxidative stress, xenobiotic exclusion ability, and xenobiotic accumulation status) is evaluated using this system, then the indices can be expected to become new indices for the treatment policy of patients with various diseases such as cancer. This is a convenient system that is minimally invasive with fewer ethical problems, and causes less burden on the patients.

Conflicts of Interest

The Authors have no financial conflicts of interest.

Acknowledgements

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