RRPG94020126 (31 .P)

計畫編號：**DOH-94-TD- F-113-028**

行政院衛生署九十四年度科技研究計畫

建立保健食品改善血液黏度之評估方法

研 究 報 告

執行機構：台北醫學大學生物醫學材料所

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執行期間： 94 年 1 月 1 日至 94 年 12 月 31 日

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Improving Effects of Epigallocatechin-3-gallate on Hemorheological abnormality in Aging Guinea Pigs

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Submitted to : J. Nutrition. Nonember 2005

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Abstract

In our previous studies, epigallocatechin-3-gallate (EGCG), the most abundant catechin in green tea, has been shown to have anti-oxidative activity in animal models. In this present study, 50mg/kg/day of EGCG were given orally to aging guinea pigs for 28 days and studied the effects of EGCG on hemorheological parameters of aging guinea pigs. The results of blood biochemical parameters showed that after 28 days there were no significant difference whether in erythrocyte counts, hematocrit levels, hemoglobin, fibrinogen level, albumin or cholesterol concentration in the aging guinea pigs. In the viscosity experiment, the results demonstrated that after taking EGCG orally for 28 days, the whole blood viscosity was significantly reduced at different shear rates, by 0.09 ± 0.11cp (γ=500 s^{-1}) , 0.23 ± 0.347 cp (γ=250 s^{-1}) and 0.07 ± 1.17 cp (γ=5 s^{-1}). Viscoelasticity was significantly reduced in aging guinea pigs by 0.127 ± 0.164 cp (0.1Hz). In the erythrocyte property, after taking EGCG orally for 28 days, the level of erythrocyte malondialdehyde (MDA) was reduced by 14 % and the flow resistance (β) was reduced by 10 %; however, the deformability of erythrocyte was increased by 10 %. Furthermore, after taking EGCG orally for 28 days of the aging guinea pigs, we also found that oxygen transport efficiency (T_E) of whole blood was increased from 16.86 ± 0.40 to 17.47 ± 0.63 (γ=500 S^{-1}) .

To sum up our works, after taking EGCG orally for 28 days of the aging guinea pigs, EGCG acted as an effective antioxidant was exhibited very encouraging results for improving

the hemorheological abnormalities, such as blood viscosity, blood viscoelasicity, erythrocyte aggreability, erythrocyte deformability and oxygen transport capacity of blood. These findings seem to shed a light for the elderly health care and preventive medicine in the future.

Keywords: epigallocatechin-3-gallate, aging, blood viscosity, blood viscoelasticity, erythrocyte deformability.

Nomenclature and Abbreviation

AI	aggregate index of erythrocyte
β	flow resistance of erythrocytes suspension
DI	deformaibility index of erythrocyte
EGCG	epigallocatechin-3-gallate
Hct:	hematocrit (%)
Hgb:	hemoglobin
MCV:	mean corpuscular cell volume of erythrocytes
MDA	malondialdehyde
γ :	shear rate of steadily flow
T_E:	oxygen transport efficiency (or oxygen delivery index) of blood
T_K:	erythrocyte rigidity (or internal viscosity of erythrocyte)

Introduction

Hemorheology is the study of the properties of blood flow through the blood vessels in relation to corresponding biophysical parameters such as blood viscosity, erythrocyte deformability, erythrocyte aggregation, platelet aggregation, etc. In the past three decades, numerous studies have indicated a strong correlation between hemorheological abnormalities and a number of diseases including cardiovascular diseases [1-4], cerebrovascular accident [5-7], and hypertension [8,9] have been proven in direct coincidence with abnormal hemorheological parameters (high blood viscosity, high plasma viscosity, high fibrinogen level, high erythrocyte aggregation and impaired erythrocyte deformability). Recent evidences have revealed the possible link between hemorheological abnormalities with neuropathies such as Alzheimer's disease [10,11], glaucoma [12-14] and hearing loss [15]. Therefore, we should pay less attention to the causation between hemorheological abnormalities and diseases. Nonetheless, it is certain that, physiologically hemorheological abnormalities do have the adverse effects.

Aging is an inevitable process that all living creatures go through. Many studies, either in animal models or human models, have extensively reported the influence of aging on the hemorheological alternations including the rise in fibrinogen [16,17], blood viscosity [18,19], plasma viscosity [20], erythrocyte rigidity [21], and decreased erythrocyte

deformability [22,23]. These aging-induced abnormal hemorheological parameters are generally believed to reflect the cumulative impacts of oxidative stress [24]. Cell aging with loss of antioxidant defense and free radicals from outside may result in a massive damage to cell activity. There may be a significantly decreased auto-antioxidant defense activity of erythrocyte as aging continues. Indeed, erythrocytes are susceptible to oxidative stress which increases the membrane rigidity leading to decrease erythrocyte deformability [25]. Consequently, not only impaired microcirculation but shortened the life span of erythrocyte were noted.

Green tea is a rich source of flavonoids, a class of polyphenols. The major flavonoids present in green tea are (-)-epicatechin, (-)-epicatechin-3-gallate, (-)-epigallocatechin and (-)-epigallocatechin-3-gallate (EGCG). Of these, EGCG is the most abundant in green tea and accounts for approximately 80% of the flavonoids present in green tea [26]. Previous studies have demonstrated that the molecular mechanisms of anti-tumor promotion activity of green tea polyphenol-EGCG involved in blocking EGF binding to its receptor [27] and inhibition of AP-1 activity induced by EGF or TPA [28]. The anti-tumor growth effect of EGCG was also associated with the inhibition of several key G1 regulatory proteins such as Cdk2 and Cdk4, and induction of Cdk inhibitors of p21 and p27 proteins in human breast carcinoma cells [29]. Various tea extracts and tea polyphenols were evaluated the antioxidant activity by ORAC

(oxygen-radical absorbance capacity) assay and shown that EGCG was the most radical scavenger [30]. These results suggested that green tea polyphenols, especially EGCG, indeed have the activities of anti-tumor, anti-inflammation, and antioxidation and may useful for prevention of cancer and cardiovascular disease. However, little is known the effect of EGCG on the haemorheological parameters. In this study, the main work of this study is to estimate the improvement of EGCG on the hemorheological abnormalities in aging guinea pigs by orally given EGCG. Measurements of plasma viscosity and blood viscosity under high, medium, low shear rate, and blood viscoelasticity under oscillatory flow, respectively, were carried out. In addition, results of erythrocyte deformability, lipid peroxidation of erythrocyte membrane, erythrocyte internal viscosity, erythrocyte aggregation and oxygen transport efficiency were further described and elucidated in detail.

Methods

Animals

After 1 week of acclimation, a group of 17 guinea pigs, 24 months-old, were fed diets containing EGCG (50mg/kg/day) for 28 days and sacrificed after 28 days to collect samples of blood from hearts for hemorheological measurements.

Haematological measurements

Fresh blood samples were collected from patients by venipuncture into plastic test tubes that contained EDTA (1.5 mg/ml) as an anticoagulant. Blood cell counts and other haematological data such as mean cell volume (MCV), haematocrit (Hct), etc. were determined by an automatic cell counter (SYSMEX NE-800, TOA Medical Electronic Co., Kobe, Japan). Plasma was separated from blood by centrifugation at 1500g for 10 min. Plasma fibrinogen was determined by the thrombin clot technique [31].

Haemorheological parameter measurements

Plasma and blood viscosity

Plasma and blood viscosity were measured using a Rheostress 1 double cone viscometer (HAAKE Mess-Technik, Karlsruhe, Germany), with a cone angle of 1° at 310 K. The serial blood viscosities at different shear rates were determined via a computer controlled testing programs. Shear rates of 500, 250, and 5 s^{-1}, reflecting high, medium, and low shear rates were given. In terms of the viscoelastic properties of blood, the viscoelasticity of blood was tested in an oscillatory mode. The oscillatory shear strain was set at a constant 5% at the

frequencies ranged from 0.3 to 0.1 Hz [25]

Erythrocyte membrane MDA analysis

To measure the oxidative stress of erythrocyte membranes, the level of malondialdehyde (MDA), a product of lipid peroxidation which reacts with thiobarbituric acid (TBA), was examined by determining the quantities of the MDA-TBA complex at 532 nm with a spectrophotometer (Hitachi U2000, Hitachi Corp. Japan) [32]; the detailed preparation procedures for measuring the MDA-TBA complex are described elsewhere [33]. Quantities of MDA presented in the results were based on 10^{10} erythrocytes [33]. Both biochemical analyses were determined in a blinded manner.

Flow resistance of erythrocytes

For the flow resistance of erythrocytes, we used constant flow rate filtration methods to preparation the erythrocyte suspensions [34]. After being separated from plasma by centrifuging the sample at 1500 g for 10 min, erythrocytes were washed 3 times in PBS. Then, erythrocyte suspensions at 5% haematocrit, in which leukocyte concentrations were usually less than 100 cells mm^{-3}, were filtered through a 5-μm pore size Nuclepore® membrane with a disc diameter of 13 mm and an effective area of 0.8 cm^2 at a constant flow rate of 1.5 ml min^{-1}.The pressure-time data were measured with a pressure transducer (Model DP45,

Validyne Engineering Corp, Northridge, USA) connected to a Validyne digital transducer indicator (Model CD-23). The continuous output data of the indicator were digitized and recorded on a computer. Recorded data were played back off-line, and Po values for ringer solutions and Pi values for erythrocyte suspensions were determined as reported [36]. β values were calculated using the data of Pi/Po and were indexed as the flow resistance of erythrocytes when flowing through the pores [35].

The deformability and aggregability of erythrocytes

Erythrocytes deformability and aggregability were measured by laser diffractometer. The measurement of erythrocytes deformability was based on a laser diffraction method in which the laser beam traversed the diluted blood suspension (0.8ml) and was diffracted by RBC. The diffraction pattern was projected on a screen monitored by the photoelectric sensors, linked to a frame grabber integrated in the computer. The best fitting ellipse will be found to represent the deformed RBC. The long and short axes, A and B, respectively, of this ellipse would be used to calculate the elongation index: EI=(A-B)/(A+B). Before the measurement, the erythrocyte suspensions should be suspended in phosphate-buffered saline solutions with 5.5% polyvinylpyrrolidone (PVP). More detail information regarding the method is described elsewhere [36]. For erythrocytes aggregation test, 0.8 ml EDTA-blood sample was filled into the plate where the diffraction of the laser radiation on the undiluted

blood samples had been used for the aggregation degree estimation. The signal obtained from the intensity of the back-scattered light measured by the photoelectric sensors was further processed by the computer to estimate the aggregation degree. In our model, the optional disaggregation shear rate and its duration prior to stopping the motor were generally set on $600sec^{-1}$ and 15 sec, respectively. Using syllectogram analysis for measuring erythrocytes aggregation, we can obtain aggregation index (AI) of erythrocytes. The relevant description for the Syllectogram in detail could be found in other literatures [37].

Erythrocyte rigidity and oxygen transport efficiency of the blood

Erythrocyte rigidity (T_K) at a shear rate of $500 \cdot 250s^{-1}$ was calculated by the equation of Dintenfass [38]. Oxygen transport efficiency (T_E) of the blood was calculated as the ratio of the Hct to blood viscosity at a fixed shear rate [39].

Statistical Analysis

All data are shown as the mean ± standard deviation (n) and paired *t* test (α= 0.05) was used for the comparison of means. Linear regressions with higher than a 95% confidence level were also calculated. All calculations were analyzed by SigmaStat® Statistical Software (Jandel Scientific, San Rafael, CA, USA).

Results

Table 1 presented the comparison of biochemical parameters of 24 months-old aging guinea pigs before and after 28 days oral administration of EGCG. The results showed that there was no significant difference in erythrocyte counts (P=0.48), hemoglobin (P=0.48), MCV (P=0.11), hematocrit levels (P=0.5), cholesterol (P=0.3), albumin (P=0.22) and fibrinogen level (P=0.07) of aging guinea pigs before and after oral administration of EGCG. Hemorheological parameters listed in **Table 2** showed that after oral administration of EGCG, on the steadily flow, the blood viscosity were significantly reduced whether at high (γ=500 s^{-1}), medium (γ=250 s^{-1}), and low (γ=5 s^{-1}) shear rate. In addition, results from oscillatory flow field measurements showed that blood viscosity (P<0.01) and viscoelasticity (P<0.01) were significantly reduced. The results of plasma viscosity showed that there were no significant changes in the aging guinea pigs after oral administration of EGCG (P=0.57). Furthermore, there was a reduction in erythrocyte aggregability (P<0.01) after 28 days oral feeding of EGCG.

<< Please insert Table 1 、2 and 3 here. >>

Regarding erythrocyte properties, despite the results showed no significant change was found in microviscosity of erythrocytes (or erythrocyte rigidity) ($P=0.33$), the lipid peroxidation (MDA) of erythrocyte membrane exhibited a significant decrease ($P<0.01$) after 28 days oral feeding of EGCG **(Table 3)**. Therefore, the deformability of erythrocyte was significantly increased whether at high ($P<0.01$) and medium ($P<0.01$) shear rates and the flow resistance of erythrocytes was significantly decreased ($P<0.05$) on the steadily flow. Furthermore, Oxygen transport efficiency of whole blood was found to significantly increase after 28 days feeding whether at high ($P<0.01$), medium ($P<0.01$) and low ($P<0.05$) shear rates.

Fig. 1 and **Fig. 2** illustrated the correlations among MDA of erythrocyte membrane, erythrocyte deformability, and flow resistance of erythrocytes. The results showed good correlations with each other, that is, as MDA was increased, erythrocyte deformability was lowered which means that also increased flow resistance of erythrocytes.

<< Please insert Fig. 1. and 2 here. >>

Discussion

Patients with hemorheological abnormalities, physiologically, are at high risk of developing cardiovascular diseases, cerebrovascular accidents, and hypertensions. Such diseases are susceptible to the aging process, a rise in blood viscosity, and hypoperfusion which results in impaired microcirculation. From the point of view in preventive medicine, it is important to obtain appropriate hemorheological properties and blood flow models.

For blood biochemical parameters, Yamarat et al.[40] reported that adults had higher fibrinogen concentration, blood viscosity and lowered MCV as compared to newborns. Coppola et al.[19] further indicated that the elderly not only have higher fibrinogen levels but also lower erythrocyte counts. Therefore, the changes in blood biochemical parameters of 24 months-old aging guinea pigs after 28 days oral administration of EGCG was interested to investigate. The observation illustrated that orally given EGCG slightly increased erythrocyte counts and hemoglobin concentration erythrocyte MCV and hematocrit levels in contrast to a decreasing tendency in erythrocyte MCV, hematocrit levels and fibrinogen levels despite of no statistically significant differences.

For the relationship between blood viscosity and aging, previous studies extensively reported that blood viscosity rises with aging. The results of 60- 150- 320- and 710 days-old

rats from Abe et al [41] confirmed that the impaired erythrocyte deformability of aging rats might result in an increase in blood viscosity. Kameneva et al [42] showed that aging led to a decrease in erythrocyte deformability, and an increase in both erythrocyte aggregation and blood viscosity. Ajmani and Rifkind [43] provided evidence that the aging process raised erythrocyte rigidity, plasma viscosity and blood viscosity. Oder et al. [44] clearly indicated that erythrocyte aggregation at low shear rate was increased by aging resulting in a rise in blood viscosity. In our study, we used two different flow fields of hemodynamics to simulate blood flow and measured the blood viscosity and viscoelasticity. In steady flow conditions, shear rates of 500 s^{-1}, 250 s^{-1} and 5 s^{-1} represented high, medium and low shear rates, respectively. The results demonstrated that 28 days oral feeding of EGCG obviously improved blood viscosity of aging guinea pigs at high, medium and low shear rates. It is generally known that decreased blood viscosity under high shear flow field was dominated by a rise in erythrocyte deformability, whereas under low shear rate flow field the blood viscosity was decreased by the reducing erythrocyte aggregation. The unsteady oscillatory flow, in fact, displays the closest simulation to human blood flow. In an oscillatory model shear stress force was dominated by frequency-dependent sinusoidal flow instead of continuous flow. Owing to low elasticity of blood, the measurement was performed with controlled strain and frequency. In this study, after oral feeding of EGCG for 28 days, it showed a significant reduction in blood viscosity and elasticity of 24 months-old aging

guinea pigs. We believe the main cause is probably due to flow resistance of erythrocyte.

Erythrocyte deformability has been thought to have detrimental influence on peripheral microcirculation or blood's oxygen transport efficiency. The previous studies of Kameneva et al.[42] and Terranova et al.[45] showed that the aging process significantly decreased erythrocyte deformability. Tillmann et al.[46] reported that the aging-induced impaired erythrocyte deformability was due to a increase in blood internal viscosity and erythrocyte rigidity. Therefore, to improve (or protect) the aging-induced impaired erythrocyte deformability was imperative. Since erythrocyte deformability depends on membrane mechanical property and erythrocyte internal viscosity (erythrocyte rigidity). In the studies, the erythrocyte rigidity of aging guinea pigs showed no significant changes after 28 days oral feeding of EGCG, despite of significant decrease in MDA of erythrocyte membrane. Malondialdehyde (MDA) is known as an end product of lipid peroxidation which is mainly formed under oxidative stress. The MDA levels of aging guinea pigs were decreased after 28 days oral feeding of EGCG, as we proposed, was due to EGCG which acted as an antioxidant by trapping free radicals and inhibiting lipid peroxidation. Therefore, it was plausible for us to believe that EGCG has the antioxidative effect which decreases the lipid peroxidation of erythrocyte and leading to an increase (or improvement) in erythrocyte deformability. Furthermore, an interesting finding (Fig.1 and Fig. 2) was the negative

correlations between erythrocyte membrane MDA and erythrocyte deformability, and erythrocyte deformability and flow resistance of erythrocytes. That means either before or after EGCG oral administration lipid peroxidation of erythrocyte membrane leads to decrease erythrocyte deformability. As erythrocyte deformability is impaired, flow resistance of erythrocytes is inevitably increased. Even more important is that we found EGCG oral administration for 28 days obviously improved lipid peroxidation of erythrocyte membrane, leading to improve erythrocyte deformability and decrease flow resistance of erythrocytes.

High erythrocyte aggregability not only increases the blood viscosity under low shear rate but also is a risky factor for the formation of blood clots. The mechanism of erythrocytes aggregation is extremely complicated that depends on the interaction energy (attractive force) between fibrinogen and erythrocyte which are correlated to erythrocyte surface charge distribution, surface geometry shape and fibrinogen levels. In this study, the fibrinogen levels showed no significant changes after oral feeding of EGCG for 28 days. That is probably owing to more flexible erythrocyte membrane (good deformability) decreasing interactions between erythrocyte membrane and fibrinogens which further decreases erythrocyte aggregation.

Hemorheological mechanism is actually complicated. The cause of decreasing blood

viscosity or blood viscoelasticity would be due to several possible factors including decreasing plasma viscosity, decreasing blood internal viscosity, decreasing erythrocyte aggregation, increasing erythrocyte deformability, etc. In this study, after 28 days oral administration of EGCG aging guinea pigs showed a reduction of erythrocyte membrane MDA leading to improve erythrocyte deformability. The improvement of erythrocyte deformability further facilitated a decrease in blood viscosity. In addition, our results confirmed that EGCG oral administration after 28 days reduced erythrocyte aggregation which also decreased blood viscosity, causing a decrease in the resistance of blood flow and an increase in oxygen-carrying capability of whole blood. We believed such physiological phenomenon would be positively helpful for health. To sum up our works, EGCG acted as an effective antioxidant was exhibited very encouraging results for improving the hemorheological abnormalities. These findings seem to shed a light for the elderly health care and preventive medicine in the future.

Conclusion

EGCG of 50mg/kg/day were given orally to aging guinea pigs for 28 days. The oral administration of EGCG caused no significant difference in blood biochemical parameters and blood fibrinogen levels but actually improved abnormal hemorheological parameters of aging guinea pigs. The antioxidant activity of EGCG would reduce the impact of free radicals on erythrocyte membrane during aging process, resulting in a decrease of erythrocyte membrane MDA that further improved erythrocyte deformability and blood viscosity at high shear rates. In addition, the oral administration of EGCG obviously reduced erythrocyte aggregation, improving blood viscosity at low shear rates and viscoelasticity at oscillatory flow. Furthermore, the oxygen transport efficiency of blood in aging guinea pigs was therefore increased after 28 days of EGCG oral administration.

Acknowledgments

We would like to thank the Mackay Memorial Hospital, Taipei, Taiwan for financially supporting this work under grant no. **94MMH-TMU-08**

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Table 1

Haematogical parameters of 24 months-old guinea pigs before and after taking EGCG orally for 28 days supplementation.

Parameters	Before treatment mean ± SD (n=17)	After treatment mean ± SD (n=17)	Difference mean ± SD (n=17)	Paired t-test P value
MCV（fl）	81.6 ± 1.63	81.1 ± 1.10	-0.52 ±1.25	NS
Hgb (g/dl)	14.4 ± 1.93	14.3 ± 1.91	-0.08 ±0.2	NS
Hct（%）	43.6 ± 3.1	43.5 ± 3.0	-0.076 ± 0.5	NS
RBC (10^{12}/dl)	5.34 ± 0.36	5.36 ± 0.32	0.018 ± 0.1	NS
Fibrinogen (mg/dl)	338.6 ± 30.7	324.1 ± 21.7	-14.47 ± 30.16	NS
Albumin(g/l)	219.5 ±16.9	226.4 ±15.9	6.88 ± 22.42	NS
Cholesterol (mg/dl)	46.8 ± 8.7	44.6 ± 7.9	-1.88 ± 7.31	NS

*P<0.05 ** P<0.01

Table 2

Haemorrheological characteristics and oxygen transport efficiency of blood of 24 months-old guinea pigs before and after taking EGCG orally for 28 days supplementation

Parameter	Befor treatment mean ± SD (n=17)	After treatment mean ± SD (n=17)	Difference mean ± SD (n=17)	Paired t-test P value
η plasma (cp)	1.36 ± 0.12	1.34 ± 0.11	-0.014 ± 0.1	**
η blood (cp)[a] (γ =500 S^{-1})	2.59 ± 0.21	2.49 ± 0.20	-0.09 ± 0.11	**
η blood (cp)[a] (γ =250 S^{-1})	4.05 ± 0.82	3.82 ± 0.41	-0.23 ± 0.347	**
η blood (cp)[a] (γ =5 S^{-1})	12.1 ± 1.83	11.40 ± 1.84	-0.07 ± 1.17	*
η' blood (cp)[b]	10.52 ± 1.80	9.12 ± 1.98	-1.39 ± 1.182	**
η'' blood (cp)[b]	1.37 ± 0.14	1.25 ± 0.15	-0.127 ± 0.164	**
AI	2.57 ± 0.19	2.27 ± 0.35	-0.30 ± 0.2936	**
T_E (γ =500 S^{-1})	16.86 ± 0.40	17.47 ± 0.63	0.611 ± 0.626	**
T_E (γ =250 S^{-1})	10.76 ± 0.23	11.46 ± 1.06	0.715 ± 0.949	**
T_E (γ =5 S^{-1})	3.65 ± 0.30	3.88 ± 0.42	0.22 ± 0.36	*

*P<0.05 ** P<0.01

a: the steadily flow model of blood

b: the oscillatory flow model of blood (0.1Hz)

η: the viscosity of steadily flow

η': the whole blood dynamic viscosity

η'' : the whole blood elasticity viscosity

γ: shear rate

AI_1: aggregate index of erythrocyte

T_E: oxygen transport efficiency

Table 3

The erythrocyte rigidity, deformaibility index of erythrocyte and MDA of erythrocyte membranes for the 24 months-old guinea pigs before and after taking EGCG orally for 28 days supplementation.

Parameters	Before treatment mean ± SD (n=17)	After treatment mean ± SD (n=17)	Difference mean ± SD (n=17)	Paired t-test P value
Tk(γ =500 s^{-1})	0.52 ± 0.06	0.50 ± 0.06	-0.018 ± 0.073	NS
Tk(γ =250 s^{-1})	0.82 ± 0.06	0.78 ± 0.09	-0.03 ± 0.089	NS
DI(γ =500 s^{-1})	0.33 ±0.02	0.36 ± 0.04	0.032 ± 0.032	**
DI(γ =250 s^{-1})	0.28 ±0.07	0.32 ± 0.03	0.025 ± 0.033	**
MDA	5.67 ±0.46	4.93 ± 0.62	-0.736 ± 0.595	**
β	9.8 ±1.20	8.9 ± 1.30	-0.906 ±1.389	*

*P<0.05 ** P<0.01

β: flow resistance of erythrocytes suspension

DI: deformaibility index of erythrocyte

γ: shear rate of steadily flow model

T_K: erythrocyte rigidity (or internal viscosity of erythrocyte)

fig.1a

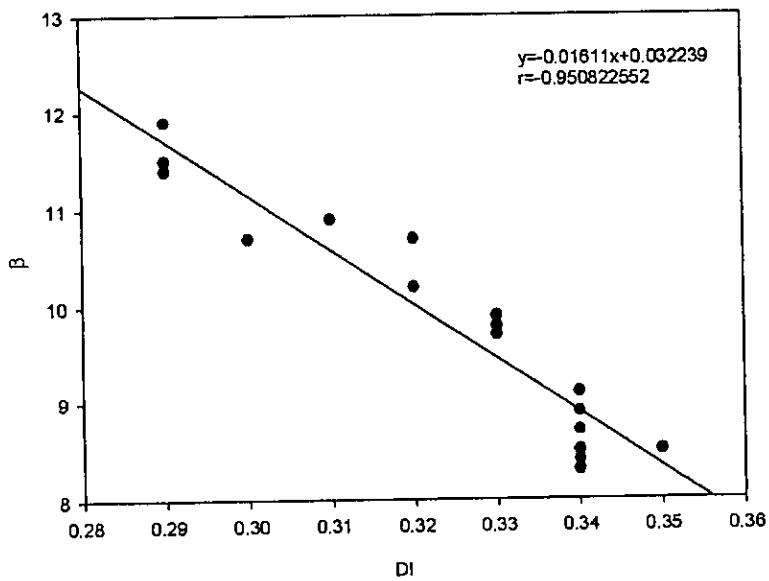

fig.1 b

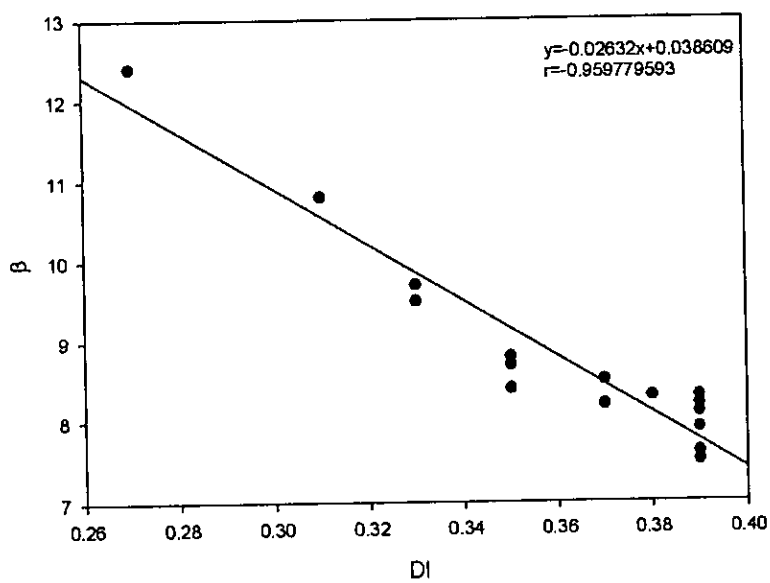

Figure 1:

Linear correlations between the erythrocyte flow resistance index (β) and deformability of erythrocyte in aging guinea pigs before (a) and after (b) taking EGCG orally for 28 days.

fig.2a

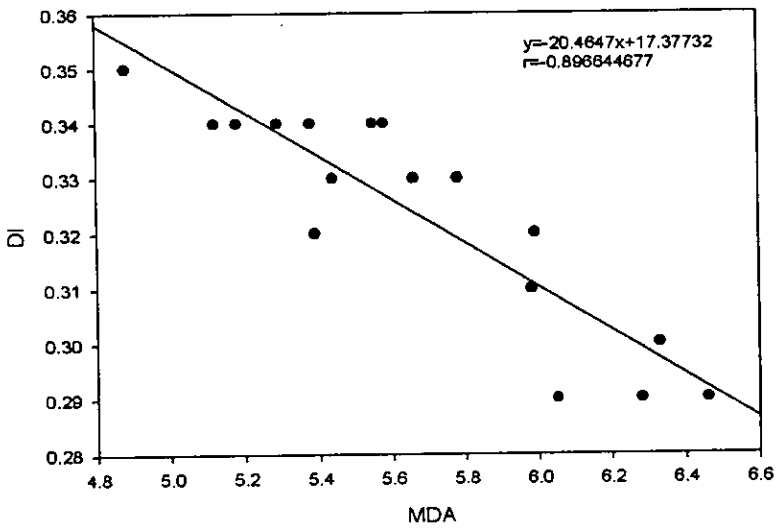

fig.2b

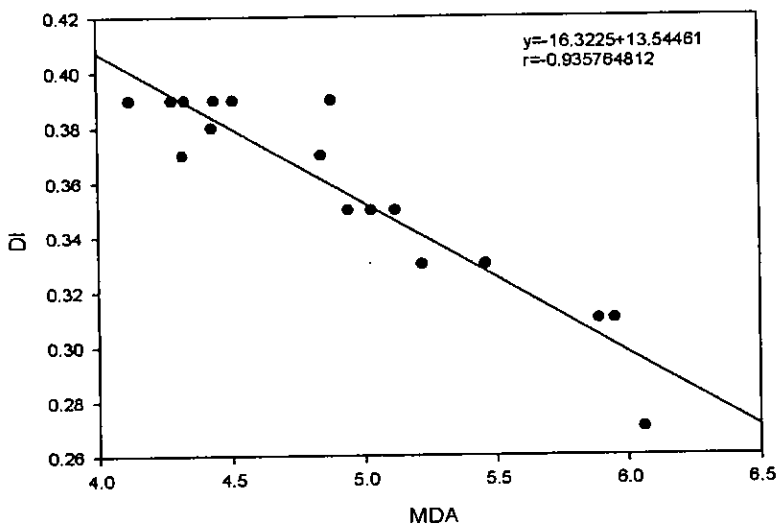

Figure 2:

Linear correlations between the deformability of erythrocyte(DI) and MDA of erythrocyte membranes in aging guinea pigs before (a) and after (b) taking EGCG orally for 28 days.