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THE POSSIBLE ROLE OF LIPOPEROXIDE IN AGING

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ABSTRACT

According to the free radical theory by Harman, free radicals formed under various conditiors can cause the changes in DNA, which may result in chromosomal aberrations, and can initiate lipid peroxidation in subcellular and cellular membrane systems.

The accumulation of lipofuscin with age also supports the significance of lipid peroxidation on aging.

In this paper two lines of research were carried out.

In experiment 1, peroxidized methyl linoleate was administered to the rats with or without supplementation of antioxidant (α-tocopherol felurate) in order to observe the toxic effects and the possibility of absorption from gastrointestinal tract. The changes of total nitrogen, total lipid, phospholipid, and total tocopherol and TBA reactive substances, which were estimated by two different ways, were observed in the livers of rats. The changes in appearance and the decrease of body weight were recognized in peroxidized oil administered groups. Total lipid and total tocopherol decreased in the liver of peroxidized oil administered groups, whereas there was no significant difference in the ratio of total tocopherol per total lipid. TBA reactive substances by non-shaking method increased in peroxidized oil administered groups. Considering the above findings, serious toxic effects due to the oral administration of peroxidized methyl linoleate could not be protected by supplementation of antioxidant, and the peroxidized methyl linoleate or the destroyed products of it might be absorbed directly from the gastrointestinal tract.

In experiment II, age trends of the same substances as estimated in experiment I and of water soluble antioxidants by DPPH method were investigated in the liver of rats and human subjects. In both, content of water-soluble antioxidants decreased significantly with age and in contrast, total tocophérol increased in mature adult and mature old. In the rat liver the increase of TBA reactive substances was associated with age. In the human liver TBA reactive substances by shaking method increased from the young to the mature adult and decreased again in the old senescense. Assuming from the results of TBA reactive substances, the capacity of lipoperoxide formation reaches to maximum in mature adult and the amount of lipoperoxide formation may increase with age.

INTRODUCTION

Up to date, numerous theories have been offered to explain the causes and biological changes of aging, but the principal mechanism of aging has not been

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elucidated because of difficulties in obtaining experimental evidence and related complicated factors.

First is the theory which postulates the accumulation of deletorious products of metabolism as a cause of aging. Second is the theory which has been proposed as the "wear and tear" theory by Comfort[1] in 1956. According to this theory, cells and tissues wear out as a result of accumulation of mental and physical stresses, finally developing into senescense. The third is the mutation theory[2]. The fact that radiation accelerates aging experimentally and deleterious genes accumulate in the somatic cells by mutation supports this theory. The fourth is the autoimmune theory[3] that immunological reactions due to somatic mutation and so on, result in biophysical and biochemical damage in cells and tissues. Cross-linking theory is the fifth, considered as an important cause of aging by Bjorksten, *et al.*[4], suggests that aging begins with the formation of cross-linkages in proteins as well as in nucleic acids.

The last is the free radical theory by Harman[5], who strongly insisted on the possibility that aging starts with functional and structural damage in cells and tissues which resulted from the side effects of free radicals normally formed in the course of biochemical reactions. According to the report by Medvedev[6]. free radicals are one of the factors which cause mutation changes in DNA molecules, and also, in the cross-linking theory, Bjorksten regards free radicals as one of the cross-linking agents[4].

By the chain reaction of free radicals which occurred from irradiation of ultraviolet rays, X-rays, and cosmic rays, and catalyzed by Co, Mn, Fe, and so on, lipoperoxides easily produced *in vivo* which denature the structural proteins in the membrane system[7] and inactivate enzymes, especially SH enzymes, which participate in the oxidation-reduction system[8]. The destruction of the membrane system result in the liberation of proteolytic enzymes from lysosome[9] and thus the cell is destroyed.

Although peroxide has such deleterious effects on the organism, there are numerous substances which prevent the reaction of peroxide formation and protect the subject from its toxicity. Namely, Vitamin C, cysteine, and glutathione among the water-soluble antioxidants and Vitamins A and E, carotene, and coenzyme Q among the fat-soluble antioxidants, provide against the obstruction of chain reactions by reacting with free radicals and electrons, and thereby protect cells and tissues from serious toxic effects.

However, under some conditions where these antioxidants are much less or the amount of peroxides increase as a result of abnormal metabolism of fats and fatty acids, especially of unsaturated fatty acids, the formation of lipoperoxide and its chain reaction could be increased and finally irreversible changes in cells and tissues might result with age.

In the tissues of muscle, brain, liver, and nerve the deposition of a pigment

called lipofuscin was found to increase with age[10], which led to some speculation about its relationship to the aging process[11]. Experimentally, substances which resembled this pigment were found in the adipose tissue of animals fed on a Vitamin E dificient diet. These have been called ceroids and regarded as a precursor of lipofuscin[12]. Strehler, *et al.*[11] postulated the process of lipofuscin formation whereby polymerized unsaturated fatty acids combined with protein in lysosomes of cell and thus denatured insoluble substances were produced.

Considering aging from a morphological aspect, the amount of lipofuscin in tissues is adequate as an index of aging and also for investigation of its mechanism. On the other hand, from a biochemical aspect, it is also available for the elucidation of the aging process to investigate the trend of the presence of lipoperoxides and antioxidants in animal tissue with age. In the present paper the possible role of lipoperoxide in aging is discussed on the basis of results from experimentation. The estimation of antioxidants and TBA reactive substances in the liver of rats and humans was carried out under the following conditions:

1) oral administration of peroxidized methyl linoleate and antioxidant to rats.

2) different aged livers of rats and humans

ESTIMATION METHOD

Total nitrogen

200 mg of liver, wet weight, was homogenized in 3.8 ml of distilled water, and the homogenate was diluted to 500 volumes by adding distilled water. After adding 1 ml of 50% H_2SO_4 to 0.5 ml of the diluted homogenate, the mixture was ashed on a KOH bath.

For the colorimetric determination at 513 mμ, 1 ml of Nessler reagent was added to the ashed content, and the volume adjusted to 10 ml with distilled water[13].

Total lipid

From 100 mg to 200 mg wet weight of liver tissue was homogenized in several ml of ethanol bloor, and the homogenate was diluted with ethanol bloor to 25 ml and heated in a water bath at 60°C for 60 minutes. 2 ml of the filtrate was evaporated completely and determined according to the method of Bradgon[14]. The concentration of total lipid was expressed as mg per g of wet tissue.

Phospholipid

2 ml of the fiitrate obtained from the same procedure for the estimation of total lipid described above was evaporated to 0.5 ml. Colorimetric determination was perfomed according to the method of Fiske-Subbarow[15]. The

concentration of the phospholipid was expressed as mg per g of wet tissue.

Water-soluble antioxidants

For water-soluble antioxidants determination the DPPH method was applied, which was reported by Glavind, *et al.*[16]. The homogenate of 500 mg wet weight of liver tissue was brought to a volume of 200 ml with distilled water, and 1 ml of this was mixed with 1 ml of 9 mg/dl DPPH ethanol solution and allowed to stand for 5 minutes. Then, 4 ml of xylen was added to the mixture, which was shaken and adequately mixed and then separated by brief centrifuging. The upper xylene layer was transferred to a cuvette and a colorimetric determination was carried out.

Total tocopherol

For the determination of total tocopherol in liver, a partly modified method reported by the Vitamin E Panel of the Analytical Committee in 1958 was employed[17].

About 500 mg of liver was homogenized with 5 ml of distilled water. After addition of 5 ml of 2.5% pyrogaroll solution and 1 ml of 50% KOH solution to the homogenate, it was heated in a water bath at 98°C for 20 minutes for saponification. 20 minutes later, the contents were cooled rapidly and extracted with 15 ml of diethyl ether three times. The ether extract was dissolved in ethanol solution. Again the ethanol solution was evaporated and the residue was dissolved in 4 ml of benzene. 2 ml of the benzene solution was passed through a Floridin XSS column and thereafter eluated with 25 ml of benzene for 45 minutes or more.

After evaporation of the benzene eluate, the residue was dissolved in 3 ml of ethanol and 1 ml of 0.12% Fe_2Cl_3 and 0.3% of α, α'-dipyridyl ethanol solution were added for the colorimetric determination at 520 mμ.

TBA reactive substances[18]

One gram of liver was homogenized with 3 ml of phosphate buffer, pH 7.4, at 0°C. The homogenate was shaken in a water bath at 37°C for 60 minutes. After adding 2.5 ml of 20% trichloroacetic acid to the homogenate, the contents were allollwed to stand for 15 minutes. Three ml of the supernatant obtained was heated at 100°C for 30 minutes with 1.25 ml of 0.65% thiobarbituric acid. To remove the turbidity from the pink colored supernatant, the contents were centrifuged at low-speed. The absorbancy at 535 mμ was determined with a spectrophotometer.

This method is referred to as the shaking method.

Another method was performed for the determination of TBA reactive substances. In this method the homogenate was not shaken, only allowed to settle in a water bath during incubation.

Except for this step the procedure through the estimation was identical to

the method described above. This method is referred to as the non-shaking method.

Experiment I. TBA Reactive Substances and Antioxidants in the Rat Liver after Oral Administration of Peroxidized Methyl Linoleate and Antioxidant

Design of experiment

For this experiment Wistar strain rats weighing about 100 g were used. After feeding with a standard diet for a week, they were divided into four groups, the average body weights which were similar. They were housed individually in metal cages in an air-conditioned room and maintained on different diets for 8 to 10 weeks. The groups were divided as follows and fed with different diets as shown in the Table 1.

TABLE 1. Administration Schedule of Different Diets

	Mon.	Tues.	Wed.	Thu.	Fri.	Sat.	Sun.
N group			Standard diet.				
AO group	AO	SD	AO	SD	AO	SD	SD
AO+PO group	PO	AO	PO	AO	PO	AO	AO
PO group	PO	SD	PO	SD	PO	SD	SD

AO: Standard diet supplemented with antioxidant.
PO: Standard diet added peroxidized methyl linoleate.
SD: Standard commercial stock diet.

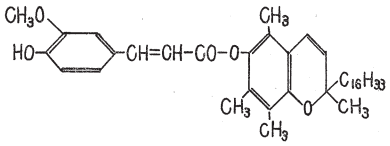
Normal diet group (expressed as N group).
Antioxidant diet group (expressed as AO group).
Antioxidant and peroxidized oil diet group (expressed as AO+PO group).
Peroxidized oil diet group (expressed as PO group).

Standard diet; 30 g of commercial standard stock diet was given per rat per day. The composition and vitamin content is shown in the Table 2.

TABLE 2. Components of the Standard Stock Diet

		Vitamin A	1000 I.U.
Water	7.0 g	D	200 I.U.
		E	1.66 mg
Protein	24.2 g	B_1	0.9 mg
		B_2	0.8 mg
Fat	5.5 g	B_6	0.8 mg
		Niacin	4.0 mg
Minerals	6.2 g	Pantothenic acid	1.9 mg
		Folic acid	0.03 mg
Carbohydrate	57.1 g	Inositol	60 mg
		Choline	100 mg

Antioxidant; α-tocopheryl felurate, the chemical structure of which is shown in Fig. 1 was given to a rat 4 mg a day mixed with the standard diet.



Feluric acid α-Tocopherol Molecular weight: 606.85
FIG. 1. The structure of α-Tocopheryl felurate.

Peroxidized methyl linoleate; Under radiation of ultraviolet rays, methyl linoleate was aerated with oxygen gas at 37°C for 20 to 24 hours until the peroxide value of the oil reached to approximately 1200 m equiv./kg by the measurement of Wheeler's method[19]. The oil prepared in this way was stocked in the refrigerator until used. 4 ml of the oil was mixed with the standard diet and given to a rat as a daily dose.

After 8 to 10 weeks feeding, animals were sacrificed by decapitation and bled with the head held downward. Their livers were removed immediately for analysis.

Results

General appearance and body weight

All animals were inspected daily and weighed every 2 weeks. The body weight of the AO group increased steadily and reached the maximum among the four groups, whereas, as shown in Fig. 2, the body weights of both groups administered peroxidized methyl linoleatte began to decrease gradually after 6 weeks feeding. The mean value of the bodyweight of N group was 315 g, of AO group was 338 g, of AO+PO group was 276 g and of PO group was

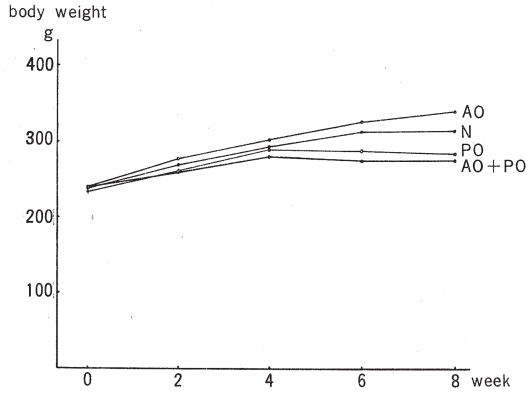


FIG. 2. The body weight change of each group.

286 g after 8 weeks feeding. Accompanied with a decrease of body weight, it was observed that some rats of PO and AO+PO groups had lusterless furs and suffered from eczematous skin lesions in their ears and tails and looked low spirited, nervous, and irritated.

Total nitrogen

In Table 3 are shown the mean values and standard deviations of total nitrogen concentration in the liver, expressed as mg per g of wet tissue. There were no significant differences in the total nitrogen concentration in the livers among the four groups. The mean value of the N group, however, was slightly higher and of the PO group was lower than the others.

TABLE 3. Contents of Total Nitrogen, Total Lipid, and Phospholipid in the Rat Liver of Four Groups Fed on Different Diets

	Total nitrogen (mg/g)	Total lipid (mg/g)	Phospholipid (mg/g)	PL/TL
N (6)	4.20±0.59	58.8±7.2	35.5±2.1	61.2± 7.1
AO (7)	4.18±0.35	62.7±7.1	38.6±2.5	62.1± 5.7
AO+PO (7)	3.95±0.69	*53.9±6.6	37.6±1.9	69.8±10.1
PO (8)	3.73±0.68	54.9±6.8	37.7±2.9	70.7± 8.9

Mean value±SD
* Significant difference from AO group value.
The figures in parenthesis indicate the number in each group.

Total lipid, phospholipid, and the ratio of phospholipid to total lipid

In Table 3 the mean values and standard deviations of total lipid and phospholipid content in the livers of the groups were expressed as mg per g of wet tissue. The content of total lipid showed a similar trend to their body weights. The content of total lipid was higher in the livers of AO group and lower in the livers of the AO+PO group than the others. Significant difference was recognized between the AO group and AO+PO group ($p<0.05$).

The change in phospholipid content in the liver of each group was not as apparent as seen in total lipid. Although there was no statistically significant difference, the content of phospholipid of the group was slightly higher and of N group was lower than the others.

The ratios of phospholipid to total lipid (PL/TL) in the liver of peroxidized oil administered groups were rather higher than the ratios of the N and AO groups. Significant difference between them could not be obtained.

Total tocopherol

As seen in Fig. 3, which shows the contents of total tocopherol in the livers of four groups, expressed as μg per g of wet tissue, the content of tocopherol was higher in the AO group and lower in the PO group. In comparison

with the content of the AO group, the content of the AO+PO group tended to be lower and of PO group was significantly lowered ($p<0.05$). Also, an apparent difference was observed between the N group and the PO group ($p<0.05$).

In Fig. 4 the content of total tocopherol per mg of total lipid (E/TL) in the liver of each group are demonstrated. In spite of the decrease of total lipid content in the liver of peroxidized oil administered groups, the mean values of these grpous were lower than the values of the N and AO groups.

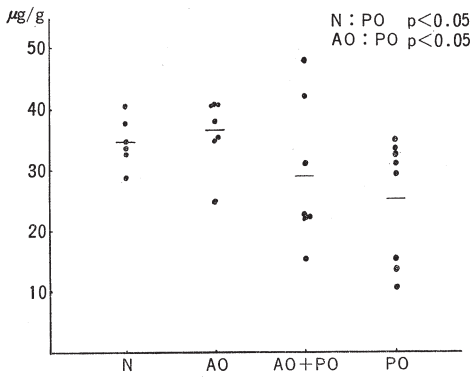


FIG. 3. Total tocopherol in the liver of each group.

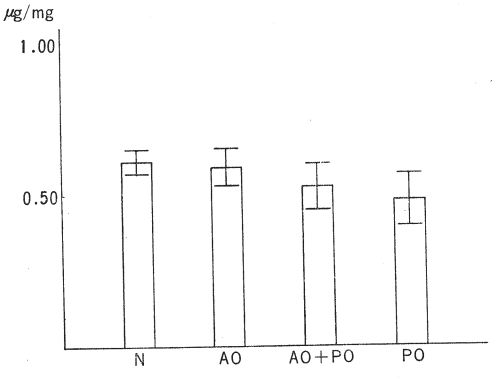


FIG. 4. Total tocopherol per total lipid.

TBA reactive substances

In this experiment, two kind of methods which were already described were employed for the estimation of TBA reactive substances in the livers of rats. Fig. 5 shows the mean values and standard deviations of TBA reactive substances in the livers of each group estimated by the shaking method. The value was expressed as extinction per g of wet tissue. In the N and PO groups the TBA values were almost the same and distributed over an extremely wide range. On the other hand, in the AO and AO+PO groups the values were significantly lower than the former and also distributed over a narrow range. Significant differences among these groups with or without supplementation of antioxidant existed ($p<0.05$).

Fig. 6 presents the TBA values of each group measured by the non-shaking method. It shows that the change of TBA values among the four groups was quite different from the change of TBA values estimsted by the shaking method. TBA values of the PO and AO+PO groups tended to be higher and distributed over a wide range, whereas, the TBA values of the N group and of the AO group were low and remained in a narrow range.

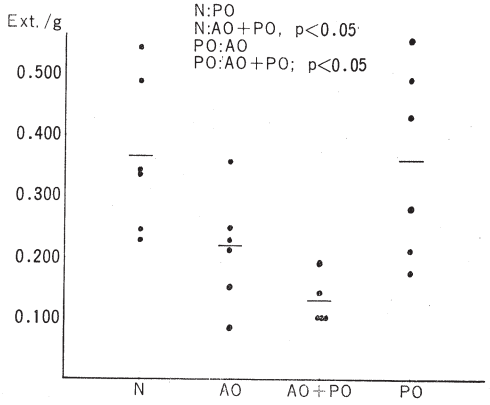


FIG. 5. TBA values of the livers of each group (by shaking method).

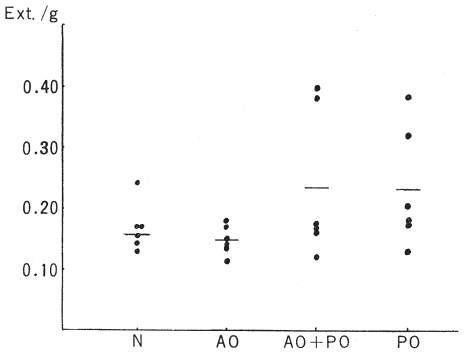


FIG. 6. TBA values of the livers of each group (by non-shaking).

Experiment II. TBA Reactive Substances and Antioxidants in the Liver of Differently Aged Rats and Humans

A) Rat liver

Design of experiment

In this experiment, the estimations of total nitrogen, total lipid, water-soluble antoxidants, total tocopherol, and TBA reactive substances were carried out as previously described on the livers of differently aged rat groups of the Wistar strain, which consisted of rats of 3 months, 12 months, and 24 months of age. Rats were fed on the same standard diet used in the previous experiment. After starvation for 24 hours they were sacrificed by decapitation, bled with the head held downward, and the livers were removed immediately for analysis.

Results

Total nitrogen

Table 4 shows the mean values and standard deviations of total nitrogen in the livers of each age group. The mean value of total nitrogen increased slightly with age, but there was no significant difference among them.

TABLE 4. Contents of Total Nitrogen, Total Lipid, and Phospholipid in the Liver of Different Aged Rats

	Total nitrogen (mg/g)	Total lipid (mg/g)	Phospholipid (mg/g)	PL/TL
3 months	4.56±0.38 (10)	55.5± 6.5 (8)	43.6±1.9 (8)	0.798±0.104
12 months	4.64±0.34 (7)	62.5± 6.8 (6)	46.0±5.2 (6)	0.740±0.096
24 months	4.80±0.29 (8)	57.3±11.3 (8)	43.9±3.3 (8)	0.775±0.106

Mean value±SD

The figures in parenthesis indicate the number of samples in each group.

Total lipid, phospholipid, and the ratio of phospholipid to total lipid

Table 4 shows the mean values and standard deviations of total lipid, phospholipid, and the ratio of phospholipid to total lipid in the livers of each age group. The total lipid of the 12 months age group tended to be higher than the other two groups, and of the 24 months age group decreased again.

Concerning the phospholipid content, a similar trend as observed for the total lipid was shown by the mean values. However, the differences between them were less than in the case of total lipid and not statistically significant.

The ratio of phospholipid to total lipid was slightly lower in the 12 months age group than the others.

Water-soluble antioxidants

In Fig. 7 the amounts of water-soluble antioxidants measured by the DPPH method were exhibited. It can been seen that the amount of water-soluble antioxidants was higher in the liver of the 3 months age group than of the older groups. A significant difference was observed statistically between the 3 months age group and 24 months age group ($p<0.05$).

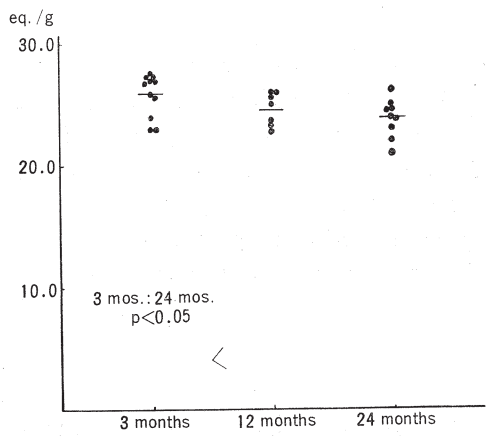


FIG. 7. Water-soluble antioxidants of the rat livers of each age group.

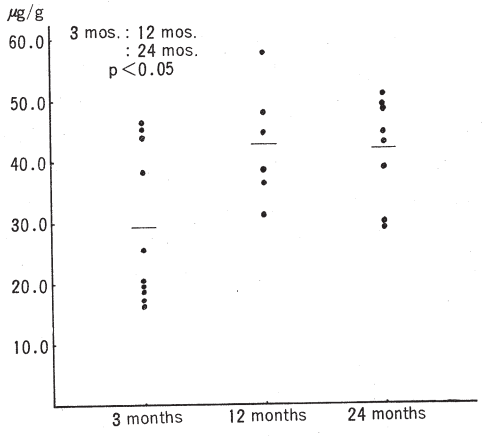


FIG. 8. Total tocopherol levels in the rat livers of each age group.

Total tocopherol

Fig. 8 shows the tocopherol level in the livers of each group. In spite of the wide range of levels, there were significant differences statistically between the 3 months age group and the other two older groups ($p<0.05$). No difference could not be obtained between the tocopherol levels of the 12 months and 24 months age groups.

The ratio of total tocopherol to total lipid.

As shown in Fig. 9 E/TL tended to increase slightly with age, but significant difference could not be recognized statistically between the three age groups.

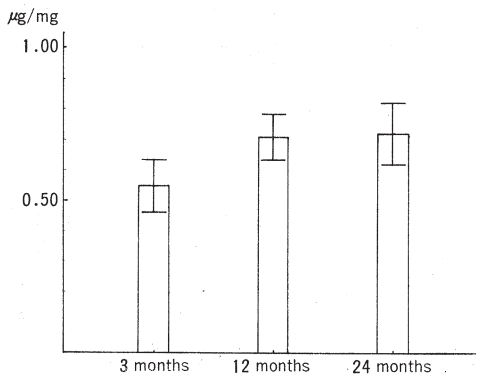


FIG. 9. Total tocopherol per total lipid in the rat livers of each age group.

TBA reactive substances

For the estimation of TBA reactive substances in the livers of each group, both of the methods previously mentioned were employed.

In Fig. 10 the TBA values, which were estimated by the shaking method on 0.5 g of wet liver tissue, are presented. In the colorimetric determination the pink colored supernatant was diluted to 4 volumes with distilled water. The values obtained in this method were distributed over a markedly wide range and showed no certain trend with age.

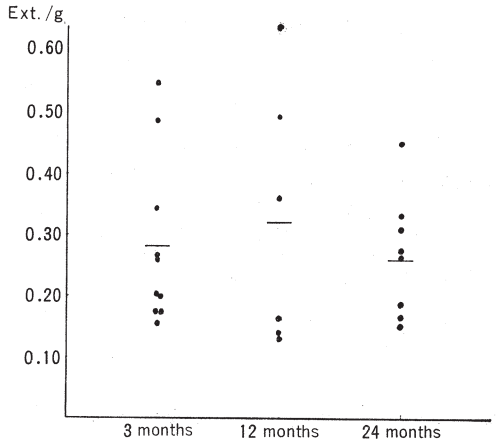


FIG. 10. TBA values of the rat liver of each age group (by shaking method).

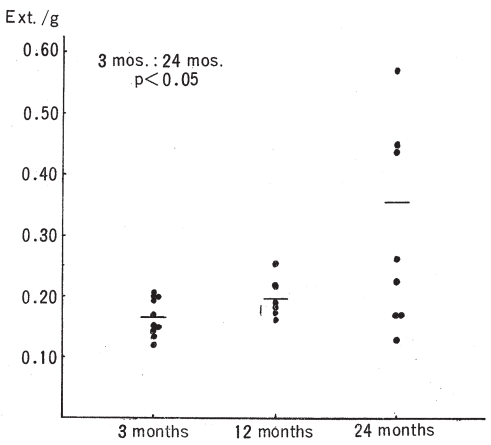


FIG. 11. TBA values of the rat livers of each age group (non-shaking method).

Fig. 11 shows TBA values, estimated by non-shaking method. Compared to the value of the 3 months age group, TBA value showed a tendency of

elevation in the 12 months age group ($p<0.05$). It is also apparent that the TBA values are distributed over a rather wide range in the 24 months age group.

B) Rat liver mitochondria

Design of experiment

A considerable amount of tocopherol has been found in the membrane system of cells, especially in mitochondria, in which tocopherol plays an important role in the electron transport system and also may participate in oxidative phosphorylation. In order to see the age trend of total tocopherol content in mitochondria, the following experiment was carried out. Wistar strain rats were used for the estimation and divided into three groups according to age. Three age groups consisted of 4 months old rats, 22 months old rats and 34 months old rats. They were raised on a standard stock diet, the contents of which were already described in the previous experiment. After the animals were sacrificed by decapitation and bled with the head held downward, the livers were removed immediately and fractionated by a method described elsewhere[20].

Results

Table 5 shows the contents of total nitrogen and total tocopherol in the mitochondria fractionated from 3 g of liver in each age group. It seems likely that there was some difference due to age in tocoperol content of mitochondria, when their contents were compared with the value originally obtained from 3 g of wet tissue. When the contents were evaluated on a nitrogen basis, no difference was found between each age group.

TABLE 5. Contents of Total Nitrogen, and Total Tocopherol in the Mitochondria Fractionated from 3 g of Rat Liver

	Total nitrogen (mg)	Total tocopherol (mg)	Tocopherol/nitrogen
4 months (6)	16.9±1.3	28.4±11.9	1.68±0.68
22 months (4)	17.4±1.6	39.5± 8.0	2.27±0.44
34 months (5)	15.8±2.5	34.5± 5.9	2.23±0.44

Mean value±SD

The figures in parenthesis indicate the number of samples in each group.

C) Human liver

Design of experiment

The estimation of the same substances as in the rat liver were carried out in human liver by the same estimation methods for the purpose of observing

their trend with age. The analyzed materials were obtained from the subjects at the upper abdominal surgical operation, who were in good general condition and had no significant change as determined by physical examination, and no liver dysfunction according to biochemical examination. The subjects included in this experiment comprise 46 males and 15 females, and their age varied from 13 to 72 years. The materials were kept in dry ice until analysis.

Results

Table 6 shows the mean values and standard deviations of total nitrogen, total lipid, phosholipid, and the ratio of phospholipid to total lipid in the human livers divided into five groups by age.

TABLE 6. Contents of Total Nitrogen, Total Lipid, and Phospholipid in the Human Liver of Different Age Groups

	Total nitrogen (mg/g)	Total lipid (mg/g)	Phospholipid (mg/g)	PL/TL
10, 20 yrs.	39.7±8.1 (4)	*39.5±6.5 (7)	30.6±4.0 (6)	0.74±0.09 (6)
30 yrs.	32.7±3.2 (7)	46.2±7.3 (12)	32.7±3.4 (10)	0.78±0.07 (9)
40 yrs.	32.5±2.5 (6)	44.9±7.8 (6)	33.8±5.5 (7)	0.75±0.04 (6)
50 yrs.	32.6±8.1 (7)	41.1±6.5 (8)	30.4±5.2 (8)	0.75±0.14 (8)
60 yrs.	34.6±5.7 (8)	*40.4±5.0 (13)	30.8±4.2 (12)	0.78±0.10 (9)

Mean value±SD
The figures in parenthesis indicate the numbers of each group.
* Significant difference from the group of 30 years old group.

Total nitrogen

No certain age trend was found in the content of total nitrogen of the liver among each age group.

Total lipid, phospholipid, and the ratio of phospholipid to total lipid

Total lipid content was lower in the liver of the 1st and 2nd decade aged group than the others, and became higher in the 3rd decade aged group. A significant difference was observed between them ($p<0.05$). On the other hand, the content of total lipid in the liver of the 5th decade aged group tended to decrease to a certain extent, and, moreover, the 6th decade and higher aged group decreased significantly in comparison with the 3rd decade aged group. Comparing the trend of total lipid, the content of phospholipid of the human liver showed a somewhat different age trend, the content of phospholipid was higher in the 4th decade aged group than the others in mean value.

It can be seen in Table 6 that there was no certain tendency by age in the ratio of phospholipid to total lipid in the human liver.

Water-soluble antioxidants

Fig. 12 shows the mean values and standard diviations of water-soluble

antioxidants in 1 g of the human liver of each age group.

In this experiment the materials were insufficient to estimate a large number of determinations on the subjects. They were divided into three groups, *i.e.* young age group consisting of 1st, 2nd, and 3rd decade aged individuals, middle age group of 4th and 5th decade and old age group of 6th decade and above. The amount of water-soluble antioxidants tended to decrease with age, the value of the old age group being significantly lower than that of the young age group. There was an apparent difference between them ($p < 0.05$).

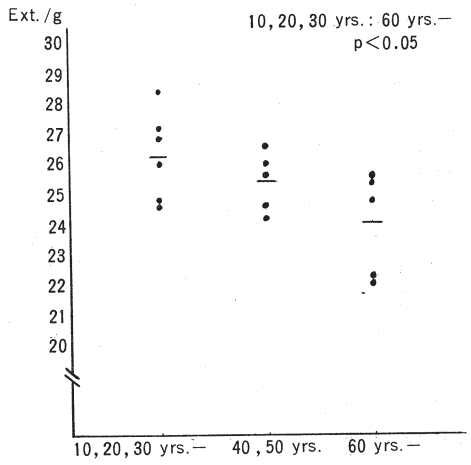


FIG. 12. Water-soluble antioxidants in human liver of different aged group.

Total tocopherol

In Fig. 13 total tocopherol contents in 1 g of wet tissue of human liver are shown. In contrast to the results of water-soluble antioxidants, the values

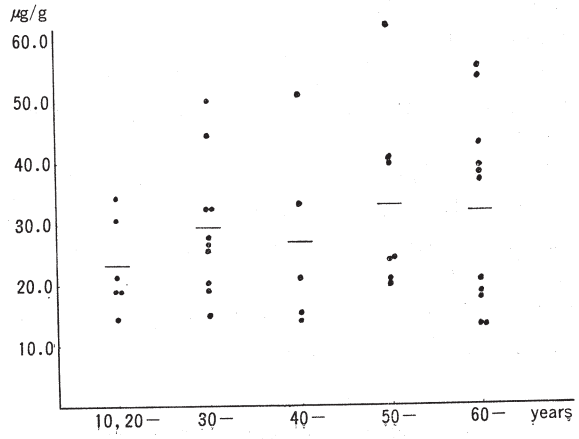


FIG. 13. Total tocopherol levels in the human livers of each age group.

for tocopherol were distributed over an extremely wide range, and the tendency was more prominent with age. Total tocopherol content of the 1st and 2nd decade aged group was slightly lower than the others, the 3rd decade aged group increased to a certain extent and the 5th decade aged group was higher, and then, the 6th decade and older group decreased again to some extent. There was no statistically significant difference.

The ratio of total tocopherol to total lipid

The age change in the ratio of total tocopherol to total lipid is shown in Fig. 14. A similar age trend as seen in the content of total tocopherol was observed among the ratios of these groups.

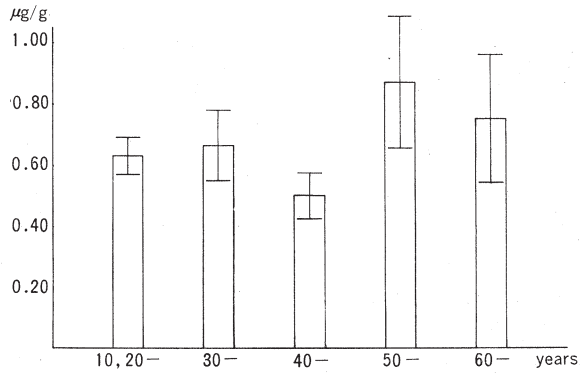


FIG. 14. Total tocopherol per Total lipid in the human liver of each age group.

TBA reactive substances

In Fig. 15 are demonstrated the TBA values of the human livers among the various age groups estimated by the shaking method.

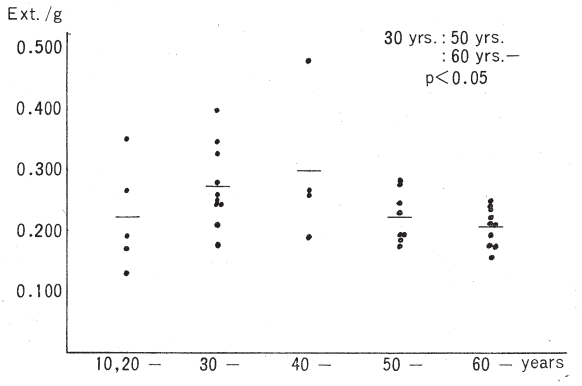


FIG. 15. TBA values of the human livers of each age group.

The mean value of the 4th decade aged group showed the highest among all groups, one of which was extremely high. The value of the 3rd decade

aged group was higher than that of the others except for the value of the 4th decade aged group.

Compared to the value of the 3rd decade aged group, a gradual decrease with age was observed in older age groups and there were significant differences between the 3rd decade aged group and both the 5th decade and 6th decade and older groups ($p<0.05$).

DISCUSSION

Peroxidized methyl linoleate administration.

The existence of lipoperoxide in the tissues of animals maintained on a Vitamin E deficient diet[21)22)], or exposed to high doses of x-rays[23)] and in atheroma of the human aorta have been reported[24)25)].

It is not clear whether the orally administered lipoperoxide can be absorbed from the gastrointestinal tract or not. Glavind and Tryding[26)] found no peroxide in the thoracic duct after feeding lipoperoxide to rats and considered that they were deperoxidized at the wall of the intestinal mucosa. Nishida and Kummerow[27)] observed a substance which has maximal absorption at 232 mμ in the thoracic lymph channel after the oral administration of relatively low concentration of methyl linoleate hydroperoxide and, therefore, speculated the possibility of lipoperoxide absorption from the intestine. According to Freeman[28)] a trace amount of [I-C^{14}] linoleic acid hydroperoxide given orally to rats was rapidly destroyed in the gastrointestinal tract, and the products were absorbed.

Andrews[29)] and Kaneda, *et al.*[30)] showed the strong toxicity of lipoperoxide to animals and also Nishida and Kummerow[31)] observed pathological findings similar to that of Vitamin E deficient animals in the animals which were administered methyl linoleate hydroperoxide per os.

In the present experiment the body weights of the rats of peroxidized methyl linoleate administered groups began to decrease gradually after 6 weeks feeding. A marked decrease in total lipid was found in the AO+PO group compared to the AO group, although considerable amounts of antioxidant were administered to them. It might be assumed that the serious toxic effects of lipoperoxide on the nutritional state could not be prevented by the administration of the antioxidant.

For the values of TBA reactive substances among the four groups, entirely different changes were observed, which resulted from slight differences in the estimation procedure. When the TBA values were estimated by the non-shaking method, a marked elevation of the values was observed in peroxidized oil administered groups, especially in the PO group. On the contrary, when the estimation of TBA reactive substances was carried out by the shaking method, TBA values of antioxidant supplemented groups were significantly

lower than for the others. In general, the TBA values estimated by the shaking method reached almost 4 times the values of the non-shaking method. These two different results indicate complicated factors related to it. Furthermore, the fact that TBA values of the rat livers were extraordinarily high compared to the human livers in experiment II may indicate that the ability to produce TBA reactive substances differs greatly from tissue to tissue in one animal, and also from species to species. Fig. 16 shows the relationship between TBA value and water-soluble antioxidants measured by the DPPH method in the following experiment. Estimation of TBA reactive substances and water-soluble antioxidants was carried out on 3 ml of the homogenate, to which 5 g of one rat liver was ground with 30 ml of distilled water, for each time during the incubation with shaking. As shown in Fig. 16, accompanying the decrease of water-soluble antioxidants, the TBA value began to elevate and reached a maximum value after 150 minutes.

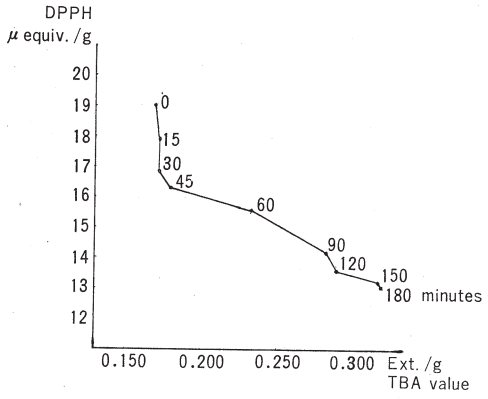


FIG. 16. TBA value and water-soluble antioxidants.

Although the TBA method used for the measurement of lipoperoxides in animal tissues has high sensitivity and simplicity, lipoperoxide themselves can not be measured directly by this method. However, the reacted products of TBA with malonyl aldehyde, which is one of the aldehydes formed from peroxidized unsaturated fatty acids, is estimated colorimetrically. When the TBA value of the tissue is estimated in the usual way, incubated aerobically with shaking, the value is influenced by the amount of unsaturated fatty acids, catalytic agents, and antioxidants in the tissue and based on the result of previous experiment, strongly affected by the amount of water-soluble antioxidants.

Althouth the TBA value estimated after aerobic incubation expresses the amount of lipoperoxide formation in tissue *in vitro*, not *in vivo*, it is a useful method for measuring the capacity of peroxide formation of the tissue under various conditions and reflects the possible capacity of peroxide formation *in vivo* to a certain extent.

The change of TBA values in the four groups, estimated by the shaking method, indicates depressed lipoperoxide formation *in vitro* due to the administration of antioxidant. Actually, the nutritional change in peroxidized oil administered animals observed in experiment I could not be protected by the supplementation of antioxidant and such a discrepancy in TBA value and the grade of tissue damage as seen in the AO+PO group was also reported by Desai, *et al.*[32] between TBA value and the grade of dystrophy in vitamin E deficient chicks. It would be necessary for the explanation of this discrepancy to know the effect of feluric acid, which is also a strong antioxidant, to TBA value and the change of the water-soluble antioxidants in each group.

On the other hand, the TBA value estimated by non-shaking method would represent the amount of TBA reactive substances formed at the initial stage of peroxide formation *in vitro*, and also may indicate the tendency of peroxide formation *in vivo*, because the incubation was carried out under relatively unaerobic conditions compared to the shaking method, it could be considered that the oral administration of peroxidized methyl linoleate produced the situation in which lipoperoxides were easily formed and also antioxidant supplementation could not suppress the lipoperoxide formation.

The reasonable explanations to the main causes of significant changes observed in the peroxidized methyl linoleate administered rats are as follows;

Decrease of total lipid:

1) Disturbance of fat synthesis in the liver.

2) Disturbance of fat absorption from the intestine.

Low concentration of total tocopherol:

1) Diminution in the concentration of absorbed tocopherol from the intestine.

2) Increased consumption of tocopherol for the decomposition of peroxide or for the destruction of additional free radicals.

Elevation of TBA value by non-shaking method:

1) The accumulation of the peroxidized methyl linoleate or the accumulation of the destroyed products of it, which were absorbed directly from the intestine.

2) The increased amount of lipoperoxides formed by autooxidation of lipids in the body.

Differently aged rats and humans.

In general, there is a gradual decrease in the content of various liver constituents to maximal values in the mature adult, which is followed by a gradual decrease to low values in the senescent adult[33]. Concerning the contents of total nitrogen in the liver, no certain age trend could be observed in both rat and human. It appears that the content of total lipid in the liver increased in the 12 month group and decreased slightly in the 24 month group. Although statistically significant difference between each age group could not be found,

the age change of total lipid agreed with the results by Rockstein and Hrachovec[33] that the content of total lipid in the rat liver was lower in young growing rats than in mature adult and decreased again to senescent animals. Likewise, in the human liver the content of total lipid was higher in the mature adult of the 3rd decade and decreased significantly in the 6th decade and older age group. It can be considered that the age trend of total lipid in the liver is similar for the human and rat.

The content of water-soluble antioxidants in the liver measured by the DPPH method decreased with age apparently in the human and rat. There have been no publications that reported this age change such as glutathione, cysteine, and so on, except for Vitamin C in the liver of animals. It is now reported that the reduction of glutathione, cysteine[34], and Vitamin C[35] in serum is accompanied with age. According to the report of Morehouse and Guerrant[36] a rapid decrease in total ascorbic acid concentration took place during the fourth week of life and this decrease was followed by further lowering of the concentration with age. A reduction with age in the ascorbic acid concentration of the human liver from childhood through adulthood to old age was observed by Girroud[37].

On the contrary to the age reduction of water-soluble antioxidants, the content of total tocopherol as a representative fat-soluble antioxidant tended to increase in the mature and aged. In the rat liver a significant increase of total tocopherol was found in the mature and old rats. In the human liver the number with a high tocopherol level tended to increase in middle and old age group. In regard to the ratio of total tocopherol to total lipid, significant difference with age could not be found in the liver of either rat or human, The hepatic Vitamin A levels of various age rat observed by Guerrant[38] reached a maximum value at the age of about 170 days and remained essentially unchanged and also the values observed by other investigators[9,40] are in agreement with the conclusions by Guerrant that a considerable increase in the liver vitamin A concentration occurs with age. According to the report of Mei Yu Dyu, *et al.*[41], total tocopherol in the liver of normal subjects appeared to reach maximal values during late childhood and adolescence and was considerably lower in the liver from the subjects who succumbed from various disease. Tocopherol absorbed from the intestine accompanied with fat reaches the tissue and is incorporated into cells as a natural component of lipid. It is considered that incorporated tocopherols function as intracellular antioxidants to stabilize lipids and their oxidation products in cells. Although there is a close relationship between tocopherol and lipid content in serum[42], it is not elucidated how tocopherol is metabolized and functions as an antioxidant in the cell.

There was no age trend of TBA value by the shaking method in the livers of three age groups because their values were distributed over a wide range

as seen in the values of the N and PO group in experiment II. On the other hand, according to the result estimated by the non-shaking method, the tendency of lipoperoxide formation in the rat liver increased with age apparetly in accordance with the diminution of water-soluble antioxidants, in spite of the decrease of total lipid and the increase of tocopherol content. A similar age trend of TBA reactive substances as observed in the present study was also found in rat brain by other investigators[43)44)].

TBA values in the human liver increased from younger subjects to mature adults, aged 3rd decade and 4th decade, then decreased again in senescent adults. The elevation of TBA values in mature adults may be explained on the basis that there was an increase in total lipid as a substrate of TBA reactive substances and decreasing tendency of water-soluble antioxidants in the liver of mature adults. On the contrary, in senescent adults, presumably a decrease of total lipid, metabolic or O_2 comsumption rate[45)], and increasing tendency of tocopherol are responsible for the significant decrease of TBA values in spite of the diminution of water-soluble antioxidants.

Although an increase in tocopherol associated with age was observed in crude tissue, age change of tocopherol concentration calculated on a nitrogen basis could not be found in the liver mitochondria of subcellular particles. Beside the participation of the electron transport system as a co-factor of cytochrome reductase in the biochemical process of oxidative phosphorylation, tocopherol acts an antioxidant to protect unsaturated fatty acids in the cell membrane system from peroxidation. Accordingly, it would be necessary to calculate on the basis of the ratio of tocopherol per total lipid or, if possible, unsaturated fatty acids for adequate evaluation of tocopherol content of mitochondria. However, there were no age associated changes in the fatty acid content[46)] of subcellular particles and coenzyme Q[47)] of mitochondria in rat liver.

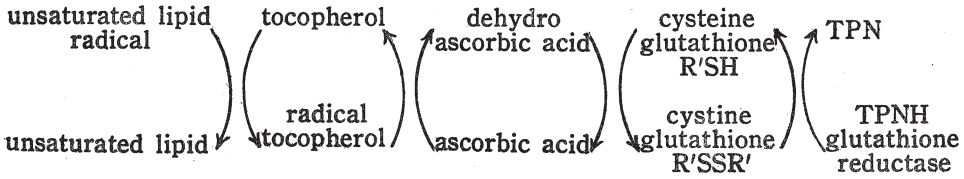
From the standpoint of morphology, a decrease in weight and size of many organs, diminution of parenchyme cell number[48)] and increased deposition of lipofuscin[11)] in a given area of tissue have been regarded as characteristics of the aging process. At the level of subcellular particles, in general, mitochondria in a cell tend to become larger in size, but less in number, and peculiar in shape in a liver cell of an old rat[49)].

The presence of lipofuscin, which increases linearly with age, indicates a close relationship between lipoperoxide formation and aging.

Concerning the results obtained in experiment I and II, it appears that the capacity of lipoperoxide formation in a tissue increases from young to mature and decreases again in old. For adequate quantitative speculation of the tendency of lipoperoxide formation *in vivo*, application of the non-shaking method is more appropriate. Based on the fact that TBA values by the non-shaking method increased significantly with age in the rat liver, it can be

thought that peroxide formation will take place more easily in the tissue of an old animal. In the present series of experiments estimation of TBA reactive substances by the non-shaking method could not be applied to the human liver, because a large amount of materials would be necessary to perform the estimation. However, if it could be determined, a similar age trend of TBA values might be observed in the human liver as seen in the rat liver.

The reduction of water-soluble antioxidants with age was another characteristic among biochemical changes associated with age. Sulfur aminoacids function as antioxidants by reacting with free radicals and decomposing lipid peroxide. Furthermore, cysteine, glutathione, and sulfhydryl proteins construct a large reactive system of redox compounds for reduction of vitamin E[49].



Assuming from the gradual decrease of water-soluble antioxidants and accumulation of disulfide groups in the course of aging in animals and humans[50], the reduction of tocopherol in the redox system, which increases the antioxygenic capacity of tocopherol, may be obstructed to some extent.

Based on the results that high doses of radiation reduced the level of water-soluble antioxidants and left no effect on the content of the fat-soluble antioxidants in the liver in spite of the occurence of peroxide formation in the adipose tissue of the rat, Glavind[51] concluded that contrary to the water-soluble antioxidants, the fat soluble antioxidants did not function as scavengers for the free radicals produced by radiation. Tocopherol will not be able to prevent lipoperoxide formation sufficiently, however, a considerable amount of tocopherol may exist in the membrane system of cells, if it can not function as a antioxidant in efficacy as the results of the disturbed redox system and because of chemically stable mobility in lipoprotein.

A number of theoretical hypothesis have been proposed to explain the mechanism of aging, many of which are based on the accumulation of chromosomal abnormalities[52][53]. According to the free radical theory by Harman[53], free radicals formed by various enviromental factors and lipid peroxides that occur with it's chain reaction cause DNA changes which result in chromosomal aberrations and destruction of subcellular and cellular membrane systems. If membrane damage of lysosomes takes place, a group of acid hydrolases which will hydrolyze tissue constituents will be released from them and magnify the lesion. Likewise in mitochondrial enzymes, probably all kinds of enzymes in mitochondria will be inactivated by free radical oxidation, furthermore, proteins and enzymatic proteins such as cytochrome c will be denatured by them.

Thus, the electrontransport system and oxidative phosphorylation reaction in mitochondria will be disturbed. In microsomes, as a result of lipid peroxidation, deletorious effects on protein synthesis will appear. The accumulation of such deleterious effects on the cell accelerates the dysfunction and the destruction of cells and tissues, and finally the organism falls into senescense.

Further investigations should be performed at subcellular and molecular levels from the morphological and physico-biochemical view in order to elucidate the possible role of lipoperoxide in the mechanism of aging.

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