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Citation	日本医学放射線学会雑誌. 1966, 26(4), p. 345-350
Version Type	VoR
URL	https://hdl.handle.net/11094/16751
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Effects of X-ray irradiation on the lipid peroxidation and
swelling of mitochondria induced by ferrous ions

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X線全身照射によるラット肝ミトコンドリアの
脂質過酸化反応と膨潤に対する影響

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X線全身照射によるラット肝ミトコンドリアの脂質過酸化反応と膨潤に対する影響をみるために、酸化的磷酸化、 Fe^{2+} 添加後の脂質過酸化反応、 90° light scattering 及び $520\text{m}\mu$ に於ける吸光度の変化の測定を行い次の結果を得た。

1. Fe^{2+} によつて起るミトコンドリアの膨潤は一定濃度の Fe^{2+} 添加により一定の lag period のち形成される脂質過酸化物に原因して起る。

2. ミトコンドリアの酸化的磷酸化能と呼吸調節能はX線照射後6時間目のもので、80%に減少

し、12～17時間後のそれでは正常に戻る。

3. Fe^{2+} 添加後の脂質過酸化物形成までの lag period はX線照射後22時間目のミトコンドリアで著しく短縮がみられるが、48時間後のものでは正常に戻る。

4. X線全身照射後24時間目のラット肝より分離したミトコンドリアに於ける不飽和高级脂肪酸は、非照射肝よりのものに比べ著しい減少がみられる。

Previous studies showed that there is a definite acceleration with X-ray irradiation in the lipid peroxidation reaction *in vitro*⁷. According to the accepted mechanism, chain autoxidation is initiated by free radicals having a high content of chemical free energy, and may be terminated by formation of chemical low-energy free radicals³. The question whether X-ray induces autoxidation *in vivo* as well as *in vitro* remains unsolved but there are some evidences suggesting the lipid peroxidation reaction *in vivo*: with various methods of peroxide estimation, an increased lipid peroxide was found in X-ray irradiated mice³. Thus, it is clear that X-ray can initiate autoxidation *in vivo*, though the observed peroxide might be formed in some other way. In any case, from the idea that ionizing radiation produces lipid peroxide participating in the X-ray injury, it is important to reveal the mechanism of lipid peroxidation with X-ray irradiation. In many chemical systems ferrous ions catalyze to produce free radicals of lipid and induce the lipid peroxidation^{14, 15}. Recently, Hunter *et al*⁴ observed the lipid peroxidation of mitochondria induced by ferrous ions being accompanied with mitochondrial swelling. The mechanism of the Fe^{2+} -induced

peroxidation is obscure but a part of the reaction is associated with the electron transfer system of mitochondria, especially with cytochrome C⁹. Therefore, studies on the lipid peroxidation of mitochondria would give a new approach to clarify the mechanism of the lipid peroxidation reaction *in vivo*. The present paper describes about the findings that the X-ray irradiation shortened the lag period for the formation of lipid peroxide in mitochondria induced by ferrous ions and decreased the proportion of unsaturated fatty acids in mitochondria.

MATERIALS AND METHODS

Materials: Albino rats, weighing 150—200 g, fed on a semisynthetic diet (products of Oriental Co. Ltd.), were used for this investigation. Rats were irradiated in a petri dish under condition of maximum backscatter. The physical factors were as follows: 200 KVp, 25 mA, filter; 0.5 mm Cu+0.5 mm Al, H.V.L.; 1.37 mm Cu, dose rate; 116 R/min., whole body dose was 1000R. After irradiation in various periods they were killed by decapitation, the livers were immediately removed, weighed and transferred to ice cold sucrose solution (0.25 M). Mitochondria were isolated as previously described¹⁰.

Adenosine 5' diphosphate (ADP), adenosine triphosphate and antimycin A were obtained from Sigma Chemical Co. Other reagents were of analytical grade and were prepared by dissolving the deionized distilled water.

Measurements: The swelling of rat liver mitochondria was measured as the turbidity decrease of dilute suspension at 520 m μ (D_{520}) or 90° light scattering at 650 m μ as described in previous papers¹¹. The isolated mitochondria were incubated in amounts giving an initial OD_{520} m μ of approximately 0.500 and incubation was carried out at 25°C in air environment in the medium of 0.15 M KCl-0.02 M Tris-HCl buffer, pH 7.4, equivalent in tonicity. Readings were taken or aliquots were withdrawn at time intervals short enough to establish the character of the curves. There were 2 or 3 minutes in the early time periods in every cases.

The changes of 90° light scattering, fluorescence intensity of pyridine nucleotides and oxygen uptake were measured simultaneously by the apparatus constructed by the authors¹¹.

Lipid peroxide formation was followed by a modification of the thiobarbituric acid (TBA) method⁸. A 1-ml aliquot was withdrawn from the incubation mixture and pipetted into pyrex tube containing 40% trichloroacetic acid (0.25 ml) and 5N HCl (0.125 ml). After mixing, 0.25 ml of 2 per cent 2-thiobarbiturate was added promptly. The tube were topped with 18cm air-cooled condensers and placed in boiling water bath for 10 minutes, cooled, centrifuged at 2500 r.p.m. for 10 minutes, and the supernatant was volumed up to 4 ml with absolute ethanol. The color intensity was read at 532 m μ and the formation of lipid peroxide was graphed directly⁴.

Oxidative phosphorylation of isolated rat liver mitochondria was tested by rotating platinum electrode¹¹. Other experimental details of each experiment were given in the text and legends to figures.

Fatty acids of mitochondria were extracted by the method of Folch *et al*². The $CHCl_3$ - CH_3OH soluble fraction was saponified with 1 N KOH, extracted with ether and esterified with diazomethane solution in ice cooled ether. The methyl esters were analyzed by gas-liquid chromatography using Shimadzu Model GC-1B.

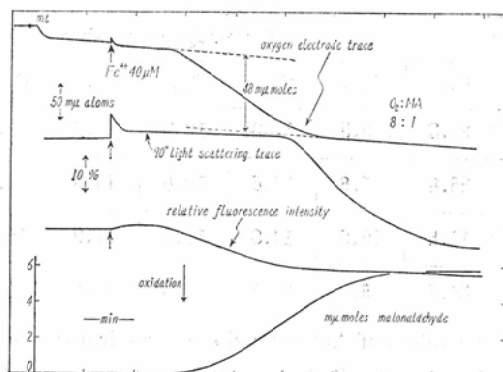
Columns 255 mm Cu-tube were packed with 10 per cent diethylen glycol succinate polyester on 60 to 80-mesh fire brick. Hydrogen was used as the carrier gas.

RESULTS

Effect of Fe^{++} on the oxygen uptake, oxidation-reduction state of pyridine nucleotides and volume of mitochondria:

The mitochondria isolated from non-irradiated rat liver show the oxygen uptake and oxidation of pyridine nucleotides accompanied with the increase of TBA color reaction at a certain lag period after addition of Fe^{++} ($40 \mu\text{M}$). The length of the lag period was lengthened by the increase of Fe^{++} in the medium of KCl-Tris buffer solution. At a certain period after causing the lipid peroxidation by Fe^{++} , it was followed by the mitochondrial swelling in a remarkable extent (so-called high amplitude swelling) and uncoupling of oxidative phosphorylation, as shown in Fig. 1. These phenomena induced by Fe^{++} were slightly inhibited by antimycin A ($2 \mu\text{g}/\text{ml}$) and KCN (1 mM) but not by uncoupler of oxidative phosphorylation and inhibitor of phosphorylating respiration.

Fig. 1. Effect of Fe^{++} on oxygen uptake, 90° light-scattering, oxidation-reduction of pyridine nucleotides and lipid peroxide formation of the mitochondria isolated from normal rat liver by the method described in a previous paper in the medium 0.15 M KCl - 0.02 M Tris-HCl buffer ($\text{pH} 7.4$). The mitochondria (0.56 mg protein) were incubated in 2 ml of the incubation mixture at 25° . The amount of lipid peroxide formed was estimated by the malonaldehyde per ml in the incubation mixture. Arrows indicate the addition of $40 \mu\text{M}$ of ferrous ammonium sulfate.



Effect of X-ray irradiation on the lipid peroxidation of mitochondria induced by Fe^{++} : The lag period of lipid peroxidation of mitochondria by Fe^{++} was shortened by Fe^{++} . A similar phenomenon was observed in the mitochondria isolated from the liver of X-ray irradiated rat. Even the concentration of Fe^{++} was the same for the lipid peroxidation of mitochondria, the lag period being shortened gradually according to the time after X-irradiation, and about 12 to 22 hours after X-ray irradiation the lag period disappeared almost completely. Then the shortening action of X-ray irradiation gradually disappeared from 48 hours after X-ray irradiation as shown in Table I.

Oxidative phosphorylation and respiratory control index of mitochondria: Oxidative phosphorylation and respiratory control index of rat liver mitochondria in the medium containing succinate, were traced by rotating platinum electrode following the method of B. Chance and R.C. Williams¹. As the result it was found that both of them were decreased in the mitochondria isolated from the liver of

Table 1. Effect of X-ray irradiation on the lag period of Fe^{++} induced swelling and lipid peroxidation in rat liver mitochondria. Incubation mixture is consisted of 0.15M KCl-0.02 M Tris-HCl buffer (pH 7.4) and the concentration of added ions is 160 μM Fe^{++} +40 μM Fe^{++} . Details for the measurement of mitochondrial swelling and of lipid peroxidation are as described under "methods".

Time after irradiation (hours)	Lag period	
	Swelling (min)	Lipid peroxidation (min)
Initial	7~9	6-7.8
4.5	7.3	5
12.0	4.5	3
22.0	1.0	0.5
48.0	7.2	5

Table 2. Oxidative phosphorylation and respiratory control index of rat liver mitochondria after whole body irradiation. ADP/O ratio and respiratory control index are measured by polarographic method in the medium of 0.05 M sucrose, 0.02 M KCl, 0.01 M Tris-HCl buffer pH 7.4, 1 mM MgCl_2 , 100 μM EDTA, 3 mM sodium succinate and 3 mM potassium phosphate buffer (pH 7.4). Incubation is carried out at 25°C.

Animal groups: post radiation (hours)	ADP/O ratio	Respiratory control index
Initial	2.0	3.6
4.5	1.6	2.5
6.0	1.6	2.6
17.0	1.8	3.0
22.0	2.0	3.5
52.0	2.0	2.8

Table 3. Effect of X-ray irradiation on the composition of total fatty acids of mouse and rat liver mitochondria. Mitochondria are isolated from non-irradiated and irradiated mouse and rat liver: 19 hours and 24 hours after irradiation for mouse and rat respectively.

Group	Percentage composition							
	$\text{C}_{16:0}$	$\text{C}_{16:1}$	$\text{C}_{18:0}$	$\text{C}_{18:1}$	$\text{C}_{18:2}$	$\text{C}_{18:2}'$	$\text{C}_{20:0}$	$\text{C}_{20:\text{poly}}$
Mouse control	28.3	9.9	17.9	12.3	19.2	5.7	3.6	3.3
Irradiated	36.8	5.6	24.6	10.6	15.9	2.0	0.65	2.4
Rat Control	19.5	10.6	14.8	15.2	17.0	10.3	6.5	6.4
Irradiated	34.8	6.8	28.5	10.3	10.2	2.2	2.8	2.0

rats irradiated with X-ray. The values of ADP/O ratio and respiratory control index reached to their minimum at 3 to 6 hours after X-ray irradiation but these values returned gradually to normal level at 12 to 17 hours after irradiation, as shown in Table 2.

Proportion of fatty acids in mitochondria: Analysis of fatty acids of total compound lipids proved the reduction of unsaturated fatty acids in quantity in the mitochondria obtained from X-ray irradiated rats comparing to that of non-irradiated one. The kind of fatty acids was quite similar as shown in Table 3 in both groups of X-ray irradiated and non-irradiated rats. But in the former, C_{16} monoenic, C_{18} monoenic, C_{18} trienoic, C_{20} and C polyenoic acids were decreased in quantity and then C_{16} palmitic and C_{18} stearic acids were increased.

These various changes in mitochondrial function after X-ray irradiation were observed in the liver of mouse.

DISCUSSION

Mitochondrial swelling by Fe^{++} is characteristically associated with lipid peroxidation of mito-

chondria in KCl medium^{12,5}. This swelling is more extensive than electron transport chain supported swelling⁶. As shown in this paper the mitochondrial swelling is followed to the transient increase of oxygen consumption and of pyridine nucleotides oxidation which is closely correlated to the increase of malon-aldehyde formation. Namely, Fe^{++} -induced mitochondrial swelling is brought about by the formation of lipid peroxide, even the question whether initiation of lipid peroxidation by Fe^{++} involves specific sites or is a generalized non-specific effect in mitochondria is a very complicated one. At any rate, the exact role of Fe^{++} in the mechanism of such reaction is still uncertain.

Under certain conditions as described in this paper this lipid peroxidation occurs at a certain lag period, which is largely obliterated by addition of Fe^{+++} with Fe^{++4} . Namely this function is coupled with the oxidation-reduction of Fe ion. Although for explanation of the mechanism leading to the autoxidation of lipid by X-ray irradiation is quite difficult, the most acceptable hypothesis is that the production of free radical by X-ray irradiation induces the autoxidation of lipid. From this fact, there arises a question, whether the shortened lag period of Fe^{++} induced peroxidation with X-ray irradiation is the result of increased autoxidizable lipid or increase of lipid free radicals in mitochondria by Fe^{++} . Gas chromatographic analysis of fatty acids shows the decrease of the substrate high unsaturated fatty acid to the formation of malon-aldehyde. High unsaturated fatty acids readily form free radicals by the formation of conjugated double bonds by the treatment of some chemical catalizer. These results suggest that the possibility of the increase of free radical or initiation of lipid peroxidation may be dismissed. Against this, a shortening of the lag period for the lipid peroxidation by Fe^{++} is also observed on the aged mitochondria⁵. Therefore, it is possible to assume that the shortening of the lag period observed in the mitochondria isolated from X-ray-irradiated rat liver may be due to the mitochondrial disorder. But the ratio and the respiratory control index are decreased at 6—12 hours after X-ray irradiation inspite of the extensively shortened lag period being at 24 hours.

Then the shortened lag period for the lipid peroxidation might have occurred by the complicated mechanism and the mechanism of this shortened lag period remains obscure. But as demonstrated in this experiment, the contents of unsaturated fatty acid are remarkably decreased in the mitochondria which have shortened lag period for the Fe^{++} -induced lipid peroxidation. This results may mean an increase of easily oxidizable unsaturated fatty acid content in mitochondria with X-ray irradiation.

SUMMARY

Effects of X-ray irradiation on the lipid peroxidation and swelling of the mitochondria of whole body irradiated rat liver were studied by measuring the oxidative phosphorylation, lipid peroxidation, 90° lightscattering or absorption at 520 $\text{m}\mu$ after addition of Fe^{++} and obtained the following results.

1. The mitochondrial swelling induced by Fe^{++} is caused by the formation of lipid peroxide which is formed at a certain lag period after addition of certain concentration of Fe^{++} .
2. The activity of oxidative phosphorylation and respiratory control of mitochondria was reduced by 80 per cent after X-ray irradiation for 6 hours, which returned to normal after 12—17 hours.
3. The lag period for the formation of lipid peroxide after addition of Fe^{++} was shortened remarkably with X-ray irradiation for 22 hours, which returned to normal after 48 hours.
4. In the mitochondria isolated from rat liver after X-ray irradiation for 24 hours, a remarkable decrease was observed in the content of unsaturated high fatty acids in comparison with that of non-irradiated liver.

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