

Effects of ultrasound waves on rat liver mitochondria

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Abstract. Succinate dehydrogenase has a wide range of properties in plant and animal cells and is distributed throughout the inner membrane of mitochondria, therefore it is the enzyme without which energy metabolism cannot occur. The goal of our research was to investigate the effect of ultrasonic waves on the intensity of lipid peroxidation, activity of succinate dehydrogenase and cytochrome c oxidase in rat liver mitochondria, as well as antioxidant effect of mulberry leaf extract and biosep oil extract. Study involved white lab rats weighing 180-200 grams. The ultrasound device used was Mindrey DP-50 Vet. The work was based on the following methods: Schneider differential centrifugation, spectrophotometry, chromatography, pH-metry, photometry. The results of the study explain the elucidation of the mechanisms of damage to liver tissue by ultrasonic waves and the correction of the mitochondrial dysfunction caused by it with the extract of mulberry leaves and the oil extract of biosep. It was revealed that changes in lipid peroxidation led to disruption of the functional activity of membrane-bound cytochrome c oxidase and succinate dehydrogenase.

1 Introduction

Ultrasound waves are extensively utilized in medical diagnostics today. Although ultrasound methods are recognized as effective in biological and medical studies, some sources indicate that ultrasound can produce a variety of biological impacts, such as mechanical, thermal, and physicochemical effects [1, 2].

A considerable body of information regarding the biological impacts of ultrasound has been amassed to date, yet much of this data stems from observations of ultrasound used for therapeutic applications [3].

Given this context, investigating the generation of free radicals due to external factors and the corresponding antioxidant system is highly relevant.

It is established that detoxification processes occur in all organs, particularly the liver. Liver impairment influences metabolism. The onset and progression of various pathological

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conditions in humans and animals are often linked to the activation of lipid peroxidation (LPO) in cells [4].

Under normal conditions, LPO in membranes is regulated by the antioxidant defense system. Living organisms produce oxidized products, namely "free radicals" and peroxide compounds of various organic and inorganic substances, as a result of metabolism. Adverse factors accelerate this process significantly. Free radicals cause cellular damage and disrupt immune system functions, leading to a range of diseases, including infectious, degenerative, cancerous, and cardiovascular conditions. Known protein free radicals in the body include peroxides, hydroxides, various lipid peroxides, hydrochloride radicals, etc. Stress-induced reactive oxygen species enhance LPO in the inner and outer mitochondrial membranes, causing damage to tissues and organs [4].

LPO products in the body impact several processes; they inhibit membrane-dependent enzyme activities, increase membrane permeability, and ultimately lead to its degradation [5].

Thus, this study aims to explore the impact of ultrasound waves on rat hepatocytes at the molecular level, focusing primarily on the effects of ultrasound on LPO, membrane-bound enzymes, and certain activities of the antioxidant system in liver mitochondria.

Cytochrome c oxidase (cytochromoxidase), also known as cytochrome-c-oxidoreductase, cytochrome aa3, and terminal oxidase, is crucial in the aerobic respiratory chain complex IV for electron transport, facilitating the transfer of electrons from cytochrome-c to oxygen and water formation. This enzyme, present in the inner mitochondrial membrane of all eukaryotes (commonly referred to as complex IV) and in many aerobic bacterial cell membranes, is vital for electron transport from complex III to complex IV in the mitochondrial membrane [5]. Various external factors alter the activity of the cytochrome-c-oxidase enzyme, leading to reduced mitochondrial membrane potential and ATP synthesis [6].

Succinate dehydrogenase (SDG), widespread in plant and animal cells and located in the inner mitochondrial membrane, is a key enzyme in energy metabolism [7]. SDG catalyzes the conversion of succinate to fumarate in the Krebs cycle, with the resultant electrons being transferred to complex III of respiration to reduce oxygen and produce water [8].

However, the effect of ultrasound waves on the activities of cytochrome-c-oxidase and SDG enzymes in rat liver mitochondria remains understudied [9].

The study's objective is to examine the influence of ultrasound waves on the activities of LPO, succinate dehydrogenase, and cytochrome-c-oxidase in rat liver mitochondria, as well as the corrective effects of mulberry leaf extract and bioseep oil extract.

2 Materials and Methods

The research was conducted on white lab rats, each weighing between 180-200 grams (permission No. 6/14-1697 dated September 27, 2022 from the Bioethics Committee of the Ministry of Health of the Republic of Uzbekistan). The Mindrey DP-50 Vet (Mindray, China), an ultrasound device specifically designed for animals, was employed in the study. The rats were subjected to ultrasound waves at a frequency of 7.5 MHz for 5 minutes.

For the experiment, the rats were categorized into distinct groups to analyze the effects of ultrasound waves and their subsequent mitigation:

Group I: Healthy control (n=5)

Group II: Subjected to 5 minutes of ultrasound exposure (n=5-6)

Group III: Ultrasound exposure followed by mulberry extract treatment (n=5-6)

Group IV: Ultrasound exposure followed by bioseep treatment (n=5-6)

Post-ultrasound exposure, rats in Group III were administered 1 ml/kg of mulberry leaf extract daily for 5 days using a specialized probe, while rats in Group IV received 1 ml of bioseep oil extract orally for the same duration.

The activities of LPO, succinate dehydrogenase, and cytochrome-c-oxidase in liver mitochondria were assessed on days 1, 3, 5, 10, and 15 following the administration of mulberry leaf and biosep oil extracts to the ultrasound-exposed rats.

Liver mitochondria from rats were isolated using W.C. Schneider's differential centrifugation (Polikom, Moscow) method. A 0.25 M sucrose - TKM buffer solution was utilized for mitochondrial isolation from liver tissue. Tissue homogenate (1:10 ratio) was prepared and centrifuged at 1000 rpm for 10 minutes. The supernatant was then centrifuged at 12000 rpm for another 10 minutes. Lipid peroxidation detection was based on the reaction of malondialdehyde (MDA) with thiobarbituric acids (TBK), forming a colored trimethine complex at high temperature and acidic pH [10], measured using a UV/VIS spectrophotometer (Metash Instruments, China) at 532 nm wavelength.

Cytochrome-c-oxidase enzyme activity was quantified spectrophotometrically by measuring the oxidation rate of cytochrome-c regenerated by dithionite [11]. The enzyme's activity was determined at a 550 nm wavelength in a spectrophotometer. The assay involved a 3 ml cuvette containing 2.2 ml of 0.2 M potassium phosphate buffer (pH 7.0) and 0.2 ml of 2×10^{-5} M reduced cytochrome c. The reaction commenced with the addition of mitochondria suspended in 0.25 M sucrose in Tris-HCl buffer, and enzyme activity was expressed in $\mu\text{mol}/\text{min}/\text{mg}$ of protein.

SDG activity was measured in a UV/VIS spectrophotometer at a 540 nm wavelength. This enzyme's activity determination is based on the reduction of tetrazole salts, where N^+ ions are transferred from the substrate through FAD^+ to tetrazole salts. Tetrazole's unique property is its easy reduction, forming brightly colored, water-soluble formazins, insoluble in acetone. The incubation medium required for this assay included 0.2 ml of 0.2 M magnesium chloride solution, 0.2 ml of 33 mM ATP solution, and 0.4 ml of 0.2 M phosphate buffer (pH 8.0). To this, 0.2 ml of mitochondrial suspension was added to 0.8 ml of incubation medium and incubated at 37°C for 10 minutes. The reaction was extended by adding 0.1 ml of sodium succinate solution, followed by 0.4 ml of 0.1% nitroretazol blue, and incubated at 37°C for 30 minutes.

The reaction was halted by adding 3.5 ml of acetone, and the precipitate was removed via centrifugation at 3000 rpm for 10 minutes. The optical density of the solution was measured at a 540 nm wavelength. Enzyme activity is expressed in $\text{nmol}/\text{min}/\text{mg}$ of protein [10].

Protein content in the mitochondria was determined using Lowry's method. Differences between the results from control, experimental, experimental+mulberry, and experimental+biosep groups were analyzed using the t-test.

3 Results and Discussion

The study's findings revealed that when rats' livers were subjected to 7.5 MHz ultrasound waves for 5 minutes using the Mindrey DP-50 Vet ultrasound device, there was a significant increase in the level of the LPO product MDA in the liver mitochondria membranes of these rats. Specifically, compared to the control group (group I), the increase was noted as $72.73 \pm 0.7\%$, $47.02 \pm 0.4\%$, $21.39 \pm 0.2\%$, $19.76 \pm 0.2\%$, and $7.91 \pm 0.1\%$ on the days 1, 3, 5, 10, and 15, respectively.

This data suggests an acceleration of the LPO process in the mitochondrial membranes on days 1, 3, 5, and 10 following the ultrasound wave exposure, as shown in Table 1. Such acceleration leads to the disruption of membrane structures and alterations in LPO within the rat liver mitochondria. The experiments also indicated that the LPO in the liver mitochondria of the rat groups, when treated with mulberry leaf extract and biosep oil extract, showed a degree of restoration.

In the study, the concentration of MDA in the liver mitochondria of rats from group III, treated with mulberry leaf extract, showed a notable decrease compared to group II. On the

first day, it was reduced to $44.85 \pm 0.9\%$, and on the third, fifth, tenth, and fifteenth days, the reductions were $27.38 \pm 0.3\%$, $25 \pm 0.2\%$, $10.18 \pm 0.2\%$, and $9.61 \pm 0.9\%$, respectively.

Table 1. MDA content in the liver mitochondria of rats

Groups	MDM content on different days after ultrasound exposure, nmol/min/mg of protein				
	1	3	5	10	15
Control	1.65±0.03	1.68±0.04	1.73±0.03	1.67±0.04	1.77±0.032
Ultrasound	2.85±0.02*	2.47±0.03*	2.1±0.02*	2.0±0.02*	1.91±0.01*
Ultrasound+ mulberry	2.11±0.01*	2.01±0.02*	1.94±0.02*	1.83±0.01*	1.74±0.02*
Ultrasound+ biosep	2.42±0.03*	2.24±0.02*	2.0±0.03*	1.92±0.03*	1.82±0.01*

Note: *p<0,05.

For rats in group IV, which received biosep oil extract, the MDA levels on days 1, 3, 5, 10, and 15 post-ultrasound exposure were reduced to $26.06 \pm 0.3\%$, $13.69 \pm 0.1\%$, $5.8 \pm 0.1\%$, $4.79 \pm 0.05\%$, and $5.09 \pm 0.3\%$ in comparison to group II, indicating a significant decrease.

In the groups treated with herbal antioxidants, namely mulberry extract and biosep oil (groups III and IV), lipid peroxidation in the liver mitochondria showed a substantial correlation effect compared to group IV. Specifically, in group III, the reductions in lipid peroxidation were 18.79% on day 1, 13.7% on day 3, 3.5% on day 5, 5.4% on day 10, and 4.5% on day 15.

The study also observed the activity of cytochrome-s-oxidase in hepatocyte mitochondrial membranes compared to the control group. The enzyme activity decreased by $44.5 \pm 0.3\%$, $40.7 \pm 0.2\%$, $35 \pm 0.2\%$, $31 \pm 1.8\%$, and $29.1 \pm 1.9\%$ on days 1, 3, 5, 10, and 15, respectively. This suggests the inactivation of the cytochrome-c-oxidase enzyme in mitochondria due to ultrasound exposure. Particularly on days 1 and 3 post-exposure, a sharp decline in cytochrome-c-oxidase activity was detected in the liver mitochondria of these rats.

Furthermore, a significant influence on the activity of the cytochrome c oxidase enzyme was observed in the liver mitochondria of rats from group III, treated with mulberry leaf extract (Fig. 1). Compared to group II, the enzyme activity on days 1, 3, 5, 10, and 15 was $5.1 \pm 0.6\%$, $5.4 \pm 0.9\%$, $5.7 \pm 3.9\%$, $8.8 \pm 0.6\%$, and $13.4 \pm 1.1\%$, respectively. Notably, a significant recuperation of enzyme activity in the hepatocyte mitochondria was recorded by day 15 (Table 2).

Table 2. Cytochrome-c-oxidase enzyme activity of rat liver mitochondria

Group	Cytochrome-c-oxidase enzyme activity on different days after ultrasound exposure, μmol/min/mg of protein				
	1	3	5	10	15
Control	22.49±0.77	25.72±0.90	25.42±0.76	25.64±0.77	23.93±0.65
Ultrasound	12.48±0.31*	15.25±0.42	16.52±0.43	17.89±0.50*	16.78±0.56*
Ultrasound+ mulberry	13.64±0.30	16.64±0.69*	17.98±0.99*	20.15±0.56	19.99±0.68
Ultrasound+ biosep	13.37±1.03*	16.28±0.39	17.59±0.49	19.05±0.53*	20.15±0.83*

Note: *p<0,05

In the case of Group IV rats treated with Biosep oil extract, an improvement in the activity of the cytochrome c-oxidase enzyme in their hepatocyte mitochondria was observed. The

enzyme activity increased to $3.9 \pm 1.8\%$, $4 \pm 1.2\%$, $4.2 \pm 3.8\%$, $4.2 \pm 3.8\%$, $4.5 \pm 0.4\%$, and $14.1 \pm 0.6\%$ compared to Group II.

The study also found that the enzymatic activity of succinate dehydrogenase (SDH) in the liver mitochondria of rats decreased following exposure to ultrasound waves. The reductions were measured at $37.8 \pm 1.5\%$, $34.57 \pm 2.3\%$, $25.5 \pm 3.2\%$, $16.7 \pm 1.6\%$, and $8.6 \pm 1.1\%$ (see Table 3). Specifically, on the 1st and 3rd days, the SDH activity in this group was reduced by $37.8 \pm 1.5\%$ and $34.57 \pm 2.3\%$, respectively, compared to the control group.

A significant influence of mulberry leaf extract on the SDH activity in the liver mitochondria of rats from Group III was observed (see Table 1). On days 1, 3, 5, 10, and 15, the enzyme activity levels were $10 \pm 0.8\%$, $10.9 \pm 0.9\%$, $8.9 \pm 0.9\%$, $4.8 \pm 0.4\%$, and $4.7 \pm 1.1\%$, respectively. By the 10th and 15th days, there was a significant restoration of enzyme activities in the mitochondria of hepatocytes in this group.

Table 3. Succinate dehydrogenase enzyme activity of rat liver mitochondria

Groups	Succinate dehydrogenase enzyme activity on different days after ultrasound exposure, $\mu\text{mol}/\text{min}/\text{mg}$ of protein				
	1	3	5	10	15
Control	9.98 ± 0.06	10.04 ± 0.03	9.98 ± 0.02	9.9 ± 0.01	10.03 ± 0.01
ultrasound	$6.21 \pm 0.01^*$	$6.58 \pm 0.02^*$	$7.14 \pm 0.015^*$	$8.25 \pm 0.02^*$	$9.17 \pm 0.02^*$
ultrasound+ mulberry	$7.21 \pm 0.02^*$	$7.67 \pm 0.016^*$	$8.03 \pm 0.02^*$	$8.72 \pm 0.02^*$	$9.64 \pm 0.05^*$
ultrasound+ biosep	$6.83 \pm 0.03^*$	$7.35 \pm 0.01^*$	$7.93 \pm 0.03^*$	$8.49 \pm 0.04^*$	$9.46 \pm 0.03^*$

Note: $*p < 0.05$.

The activity of SDH in mitochondria of hepatocytes of group IV rats, corrected with Biosep oil extract, was $6.2 \pm 0.4\%$, $7.7 \pm 0.5\%$, $7.9 \pm 0.6\%$, $2.4 \pm 0.3\%$, $2.9 \pm 0.2\%$ compared with group II.

4 Conclusion

From the results obtained, it was concluded that the restorative effect of the mulberry extract is more pronounced than that of Biosep. Consequently, the effect of ultrasonic waves on rat liver mitochondria led to increased lipid peroxidation and a noticeable decrease in the activity of cytochrome c oxidase and succinate dehydrogenase. These changes in lipid peroxidation impaired the functional activity of membrane-bound cytochrome c oxidase and succinate dehydrogenase, which in turn affected the antioxidant defense system. The study showed that oil extracts of mulberry leaves and biosep have antioxidant properties, helping to partially restore their activity. Since the leaves of the medicinal mulberry contain substances with antioxidant properties such as vitamin C, flavonoids and general antioxidants, their extract can be used to correct the effects of ultrasound on liver cells. Changes in lipid peroxidation led to disruption of the functional activity of membrane-bound cytochrome c oxidase and succinate dehydrogenase. Such changes entailed changes in the antioxidant defense system. The results of the study explain the elucidation of the mechanisms of damage to liver tissue by ultrasonic waves and the correction of the mitochondrial dysfunction caused by it with the extract of mulberry leaves and the oil extract of biosep. The practical significance of the research results is that the extract of mulberry leaves and oil biosep can be used as a means of correcting mitochondrial dysfunction of the liver caused by ultrasonic waves. It was also found that mulberry leaf extract and biosep oil extract are biologically active compounds with antioxidant and antiradical properties.

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