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RESEARCH ARTICLE

Ultrastructural and Circadian Morphometric Characteristics of Rat Pinealocytes Under Physiological and Toxic Conditions

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Abstract: Carbon tetrachloride (CCl_4) exhibits pronounced neurotoxic effects following acute or chronic exposure. Recent studies indicate that the pineal gland responds to CCl_4 intoxication by altering its secretory function, although detailed ultrastructural changes in pinealocytes remain inadequately investigated. This research addresses the urgent need to elucidate the mechanisms of CCl_4 toxicity on the pineal gland, which could contribute to developing strategies for mitigating disorders induced by chlorinated hydrocarbons. The study aims to examine CCl_4-induced alterations in rat pinealocyte ultrastructure, melatonin secretion rhythms, and micromorphometric parameters of pineal organoids. Eighty male Wistar rats, allocated into two equal groups, were included in the study. Group I was under the fixed lighting regime (light: dark / 10:14). Group II was under the same conditions with additional injection of CCl_4 oil solution. All animals were kept in the experiment for 21 days, with removal on the 22nd day, and the pineal gland was taken, and blood collection was performed. Electron microscopic study of the pineal gland, micromorphometric analysis of some parameters of mitochondria, Golgi Complex (GC), and lipid droplets were performed. Quantitative determination of melatonin concentration was performed. Graphs of 24-hour dynamics of the analyzed parameters were plotted and statistically processed. Circadian rhythm amplitude and acrophase were calculated using cosinor analysis. The conducted study allowed for the revelation of significant changes in the morphology and function of the rat pineal gland under the influence of carbon tetrachloride, accompanied by impaired melatonin synthesis and ultrastructural restructuring of pinealocytes.

Keywords: Pineal Gland, Pinealocyte, Mitochondrion, Carbon tetrachloride, Circadian Rhythm

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Introduction

Through the production of melatonin and other bioactive molecules, the pineal gland (epiphysis cerebri) plays a key role in the regulation of circadian rhythms, the endocrine system, and the neuroimmune system [1, 2]. Pinealocytes, the main secretory cells of the pineal gland, are highly sensitive to the effects of toxic substances, which can lead to disruption of their functional activity and structural organization [3-5].

As the principal site of melatonin synthesis, the pineal gland performs an essential physiological role. Damage to this organ can lead to suppression of melatonin production, which may affect the functioning of other systems. However, information on the effects of various toxicants on the pineal gland is extremely limited [6, 7]. Evidence in the literature indicates that heavy metal salts influence both the pineal gland and the structure of circadian rhythms [8, 9]. Mirisola's studies have revealed a correlation between elevated fluoride levels and calcification of the pineal gland [10]. Glyphosate binds to aluminum ions through its metabolite p-cresol, and also impairs the synthetic activity of the pineal gland [11].

Carbon tetrachloride (tetrachloromethane, CCl_4) - a chlorinated hydrocarbon, widely used in industry- is known for its hepatotoxic effect, but its influence on neuroendocrine structures, including the pineal gland, is poorly studied [12, 13]. Metabolism of CCl_4 generates reactive radical species that cause lipid peroxidation, destruction to cell membranes, and disorganization of ultrastructural components of the cell [14].

CCl_4 toxicity involves multiple adverse outcomes of tetrachloromethane, contingent upon the administration route, dose, and exposure time in mammals.

In studies, tetrachloromethane is administered to experimental animals most commonly by intraperitoneal injection, by inhalation, and by administration through an intragastric tube. Most researchers prefer to use intraperitoneal injections because this method is simple, safe, and well reproducible [15-17].

The pathological effects of CCl_4 are based on its biotransformation and the resulting formation of toxic metabolites.

Tetrachloromethane is also highly neurotoxic and can adversely impact the nervous system under both acute and chronic exposure conditions. Owing to its lipophilic nature, CCl_4 easily penetrates the Blood-Brain Barrier (BBB). Within neurons, it undergoes metabolism by cytochrome P450, producing potent free radicals (CCl_4, Cl) that trigger lipid peroxidation of neuronal membranes. Tetrachloromethane depresses mitochondrial function by reducing ATP synthesis, leading to axonal degeneration and neuronal death. There is some evidence of the effects of CCl_4 on the GABAergic and glutamatergic systems [18, 19].

Recent evidence demonstrates that exposure to toxic doses of CCl_4 results in pronounced changes in organs beyond its classical targets, including the kidneys, lungs, and endocrine glands [20]. In particular, the pineal gland has been shown to respond to CCl4 intoxication with changes in secretory activity, but detailed ultrastructural rearrangements of pinealocytes remain poorly studied [21]. CCl_4-induced toxicity undoubtedly causes ultrastructural alterations in the organelles of target cells. This disruption may decrease melatonin production, thereby compromising the organism's adaptive capacity and promoting the development of pathological states [3, 22-24].

Mitochondria represent a major target of oxidative stress induced by CCl_4.

In pinealocytes, impairment of mitochondrial function may lead to diminished melatonin synthesis, as the production of melatonin depends on the activity of mitochondrial enzymes responsible for the conversion of tryptophan to serotonin [25-27]. Furthermore, the accumulation of reactive oxygen species (ROS) within mitochondria initiates a cascade of events that culminates in either necrotic or apoptotic cell death [24-32].

Electron microscopic studies show that exposure to CCl_4 in cells results in changes of ultrastructural components [33-38].

The present research is justified by the necessity to characterize the mechanisms of CCl_4-induced toxicity on the pineal gland, which may inform the development of corrective approaches for disorders resulting from exposure to chlorinated hydrocarbons. The objective of this study was therefore to evaluate the impact of CCl_4 on the ultrastructure of rat pinealocytes, the circadian rhythm of melatonin secretion, and specific micromorphometric parameters of pinealocyte organelles.

Materials and Methods

Experimental Subject

This study involved 80 male Wistar outbred rats (6 months old, weighing 350±15 g) with no visible pathologies. The animals were supplied by the Stolbovaya nursery, an affiliate of the Scientific Center for Biomedical Technologies under the FMBA. They were initially kept under natural light and were housed in plastic cages under controlled environmental conditions. The rats had free access to drinking water and standard pelleted feed. All procedures involving animal care and

experimentation were performed in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, March 18, 1986).

Study Design

Before the research began, animals meeting the inclusion criteria were divided into groups. Animals with the absence of visible abnormalities in behavior and appearance (condition of fur, eyes, and limbs) were selected and randomly allocated to groups, with 40 animals per group.

The control group (n = 40) was maintained under a fixed light regime (light: dark = 10:14 h, lights on at 8:00 a.m. and off at 6:00 p.m.). Animals of group II (n = 40) were kept under the same regime as the control group, but these rats received an intraperitoneal injection of CCl_4 in olive oil (0.2 mL per 100 g body weight) once every three days. Each animal received a total of seven injections [39].

The experiment lasted 3 weeks. Rats were euthanized using a ZOONLAB GmbH Model L CO_2 box (Germany) at four time points (9:00, 15:00, 21:00, and 3:00), with 10 rats per time point. Blood was collected for hematological and biochemical studies, and the pineal gland was excised.

Methods of Electron Microscopy

Fixation and preparation of pineal gland samples (2 mm^3) for electron microscopy were performed according to the standard protocol [40].

Following sectioning on a UC Enuity ultramicrotome (Leica Microsystems CMS GmbH, Germany), ultrathin sections were counterstained with Reynolds' lead citrate and subsequently examined and imaged with a HIMERA EM50X field emission scanning electron microscope (CIQUTEK, China).

Micromorphometric analysis of the pinealocyte mitochondrial apparatus was performed on each preparation by examining 20 non-overlapping fields at ×20,000 and 10 fields at ×50,000.

Stereometric studies were performed using the dissector method [41]. Cross-sections of mitochondria in light pinealocytes were used for all measurements. Pinealocytes with preserved membrane organoids and nucleus were subjected to micromorphometry.

The QuPath program was used to determine: mitochondrial numerical density; cross-sectional area, perimeter, cristae number, area-to-perimeter ratio, and roundness coefficient (circularity index) for mitochondria; along with Golgi complex cross-sectional area, Golgi vesicle count, and cytoplasmic lipid droplet count of 1 pinealocyte.

Enzyme Immunoassay Method

Blood melatonin concentrations were quantified by enzyme-linked immunosorbent assay using a StatFax 4200 analyzer (USA) and a Cloud-Clone Corp. Melatonin ELISA Kit (China).

Statistical Analysis

Statistical analysis and data visualization were carried out with GraphPad Prism (version 8.41, USA). Normality of distribution was assessed using the D'Agostino-Pearson test. Normally distributed data were analyzed using Student's t-test for pairwise comparisons and Tukey's test for comparisons involving three or more groups.

Differences between each experimental group and the control group were assessed using Dunnett's test. For data that did not follow a normal distribution, pairwise comparisons were performed using the Mann-Whitney test, and comparisons among three or more groups were carried out using Dunn's test. Differences were considered statistically significant when the significance level (α), or the probability of erroneously rejecting the null hypothesis, was less than 5% ($p < 0.05$). The arithmetic mean and standard deviation were used for graphical presentation. The strength of differences was indicated as follows: "*" corresponds to $p < 0.05$; '**' - $p < 0.005$; '***' - $p < 0.0005$.

Circadian rhythm (CR) parameters, specifically amplitude and acrophase, were statistically calculated using Cosinor analysis, an internationally accepted method for the unified study of biological rhythms, with the aid of the CosinorEllipse2006-1.1 program.

Cosinor analysis is specifically designed for studying wave processes and analyzing chronobiological data. This method fits experimental data to a sinusoidal curve using least squares approximation, enabling the identification of a reliable circadian rhythm along with its acrophase and amplitude. The main output parameters of cosinor analysis are as follows: the mesor, which represents the average level of the sinusoid and corresponds to the parameter's mean daily value; the amplitude (A); and the acrophase (Phi), defined as the time at which the function reaches its peak, indicating the moment of maximum rhythmic variability over a 24-hour period. Acrophase is expressed in hours, while amplitude is reported in the same units as the original measurements.

Then the construction of the error ellipse is carried out, with the help of which the reliability of the existence of rhythms at the accepted level of confidence (0.95) is determined. The circadian rhythm is considered reliable if two conditions are met: the averaged approximating chronogram sinusoid (indicated by a cross) must fit within the ellipse, and the ellipse itself must not pass through the center of the coordinates (since in this case the acrophase would be observed throughout the entire 24-hour period) [42].

The standard designation of chronobiological time was used in the study to denote time points, where 3 hours is 3 a.m., 9 hours is 9 a.m., 15 hours is 3 p.m., and 21 hours is 9 p.m.

Results of the Study

Immunoassay Results

The study showed that in intact animals, the average daily blood melatonin content is 37.34±3.64 pg/mL. Exposure to CCl_4 significantly decreased hormone levels to 12.17±3.19 pg/mL (Fig. 1).

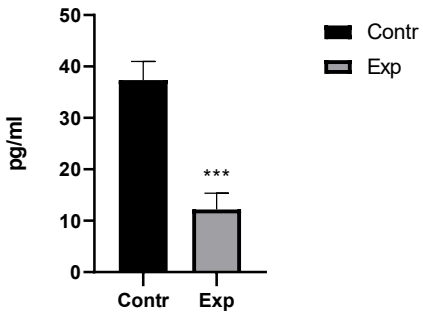

Fig.1: The average daily concentration of melatonin in rat blood

Note: In all subsequent figures/tables, statistical significance relative to the control group is denoted as follows: *p ≤ 0.05; **p ≤ 0.005; ***p ≤ 0.0005.

Ultrastructural Condition of the Pineal Gland of Rats

Pineal glands of animals of the control group have lobular parenchyma separated by thin layers of connective tissue. The lobules are represented by secretory active pinealocytes, among which the larger ones are light pinealocytes, and the smaller ones are dark pinealocytes. The cytoplasm of light pinealocytes contains a large number of secretory vesicles and cisternae, large mitochondria with a small number of cristae, and an electron-transparent matrix. Light pinealocytes are characterized by moderate development of rough EPR and pronounced development of the Golgi complex (Fig. 2, A).

Dark pinealocytes, diffusely located between light ones, are characterized by smaller linear dimensions, electron-dense cytoplasm containing a large number of granules, developed smooth and rough EPR, containing a large number of small condensed mitochondria with densely packed cristae, and small secretory vesicles. Dark pinealocytes are characterized by a higher content of lipid droplets in the cytoplasm. These cells have long outgrowths in contact with numerous blood vessels (Fig. 2, B-D). Cells of both types contain large euchromatic nuclei of irregular shape. At the ultrastructural level, the pineal gland of the "Control" group is consistent with the normal state.

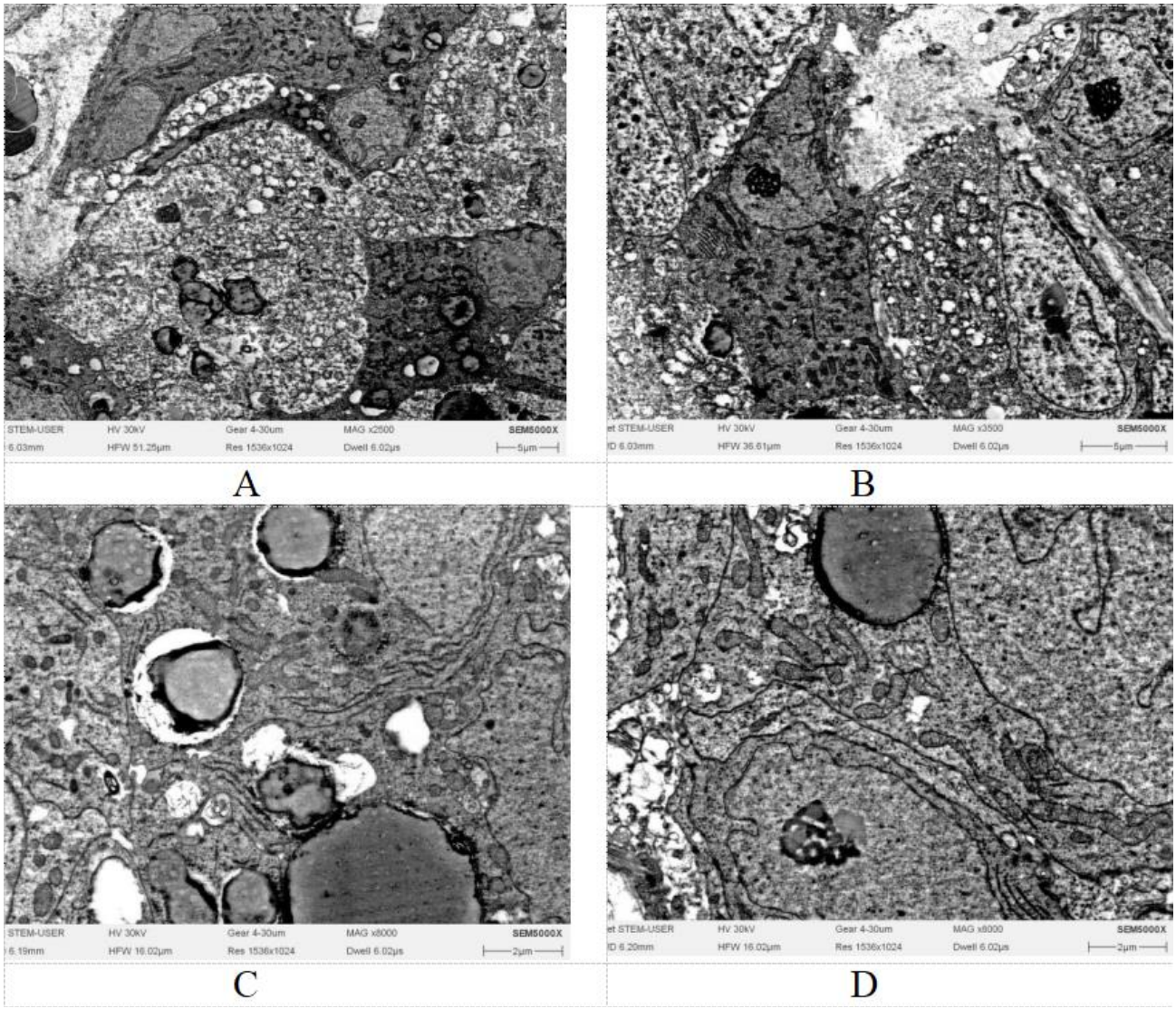

Fig. 2: Pineal gland of animals in the control group. Ultrastructure corresponds to norm. TEM, A – ×2500, B – ×3500, C, D – ×8000

The ultrastructure of the pineal gland under exposure to tetrachloromethane has a number of significant differences.

Thus, pineal vessels under the influence of carbon tetrachloride are characterized by hyperemia, and pronounced perivascular edema is noted (Fig. 3, A). The cytoplasm of both types of pinealocytes contains a great number of large confluent vacuoles (Fig. 3, B). Swelling of mitochondria of pinealocytes (edema of their matrix), destruction of cristae, and breakage of membranes are expressed (Fig. 3, C). This process is most pronounced in cells located perivascularly. In addition, a large number of lamellar myelin-like inclusions are observed in the cytoplasm, which are a product of the degradation of organoid membranes due to the process of lipid peroxidation of their constituent lipids. A similar process affects lipid inclusions in the cytoplasm of cells, leading to their destruction (Fig. 3, D). The number of lysosomes in the cytoplasm was also elevated. Numerous fragments of intracellular organelles are observed within autophagosomes in the cells. In the nuclei of pinealocytes, the formation of numerous invaginations of the nuclear membrane, a decrease in the size of nuclei, and clumpy condensation of chromatin are observed (Fig. 3, E). Additionally, we observed both individual and clustered necrotic pinealocytes in the pineal gland of animals of this group (Fig. 3, F).

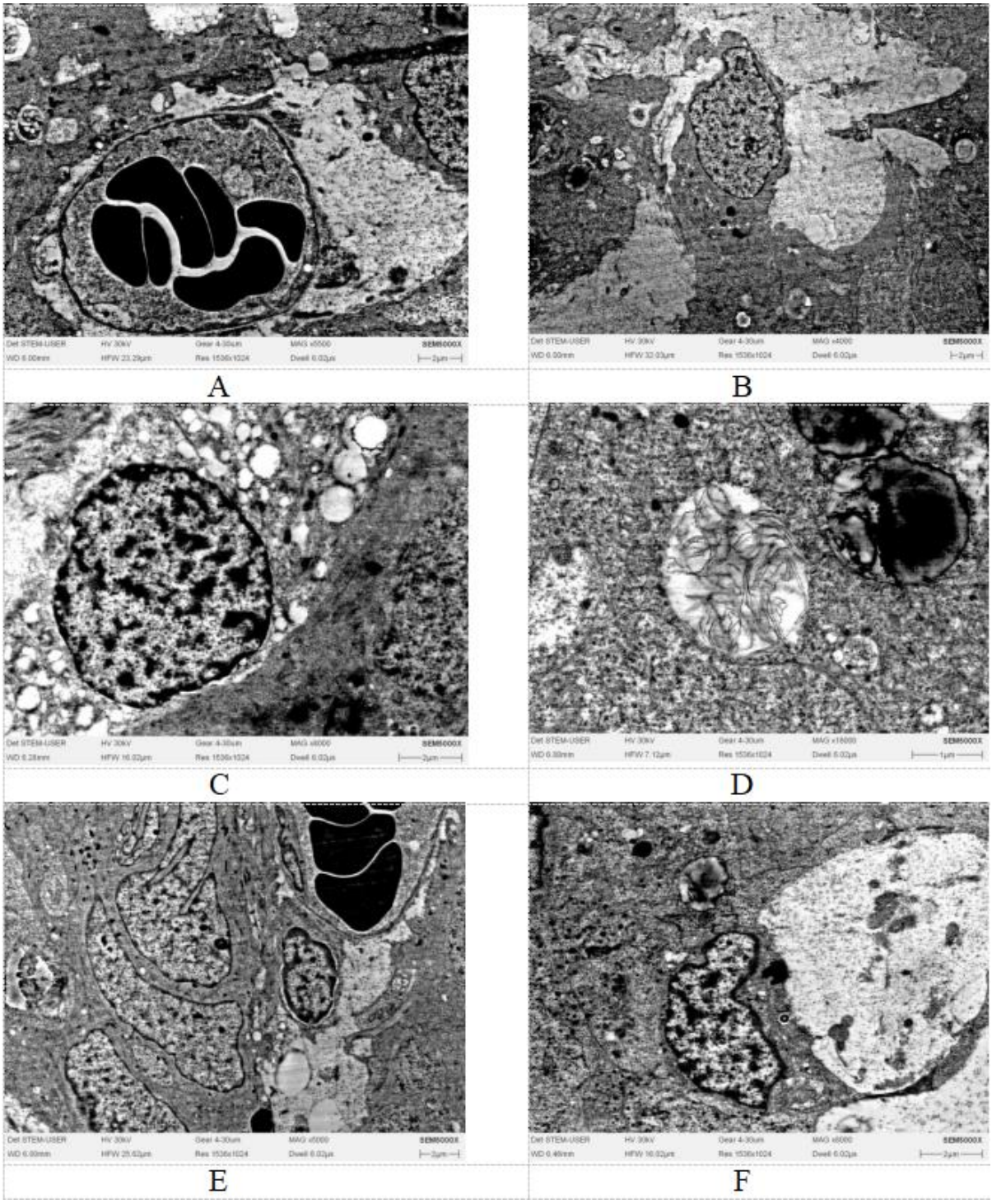

Fig. 3: Pineal gland of animals exposed to carbon tetrachloride. TEM, A − ×5500, B − ×4000, C, F − ×8000, D − ×18000, E − ×5000

Results of Micromorphometric Studies

CCl_4 administration significantly altered the micromorphometric parameters studied. Specifically, we observed a decreased numerical density of mitochondria, along with increases in both their mean cross-sectional area and perimeter. In contrast, the other mitochondrial parameters in pinealocytes of the experimental group decreased (Table 1).

Table 1. Value of investigated parameters of rat pinealocyte mitochondria

Group						
I	15.0±6.41	0.291±0.10	17.6±4.79	2.19±0.46	0.131±028	0.77±0.21
II	10.93±3.23 **	0.468±0.083 ***	15.47±2.89 ***	3.27±0.63 ***	0.146±0.029 ***	0.59±0.20 ***

Simultaneously, the Golgi complex showed an enlarged cross-sectional area from 3,54±0,79 μm2 in the control group to 4,41±2,01μm2 in pinealocytes of experimental rats, although the average number of vesicles of the complex does not change significantly, 1,89±0,73 in the control and 1,63±0,33 in the experimental group. The number of lipid droplets in pinealocytes in the control is 1,85±1,10 units, and in the experiment, it is reliably less - 1,35±0,80 units.

Diurnal Dynamics of the Investigated Parameters

The circadian profile of blood melatonin concentration in control animals was characterized by a pronounced maximum at 3:00 and a minimum at 15:00. In the experimental group, although the maximum occurred at 3:00, the minimum was shifted to 21:00 (Fig. 4).

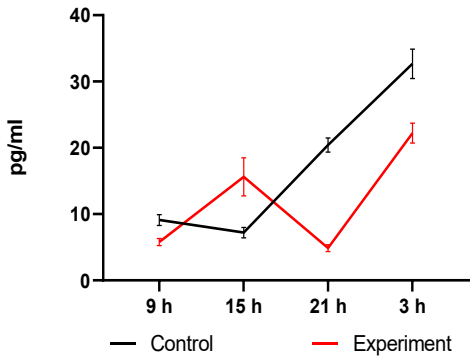

Fig.4: Diurnal dynamics of melatonin concentration in the blood of rats

Table 2: Outcomes of cosinor analysis applied to the daily rhythm of the investigated parameters

Parameter	Control		Experiment	
	Acrophase	Amplitude	Acrophase	Amplitude
Melatonin, pg/ml	1:24	13,94	1:04	10,06
Numerical density of mitochondria, units	4:42	6,77	23:42	3,86
Area of mitochondria, μm^2	8:18	0,132	11:12	0,095
Number of cristae, units	2:38	6,14	13:36	1,03
Perimeter of mitochondria, μm	9:06	0,52	1:53	0,01
Circularity index	14:26	0,59	4:19	0,19
S/P ratio	7:24	0,031	7:04	0,022
GC area	21:52	0,93	0:29	2,43
Number of Golgi complex vesicles	21:48	0,23	1:06	0,81
Number of lipid droplets in pinealocyte	1:30	1,49	5,15	0,1

In accordance with cosinor analysis results, the significant CR of melatonin concentration in the control is characterized by an acrophase at 1:24 and an amplitude of 13.94 pg/ml. In rats of the experimental group, with an acrophase at 1:04, the amplitude of the rhythm was 10.06 pg/mL (Table 2).

TAnalysis of the daily dynamics of micromorphometric parameters revealed that mitochondrial numerical density was lowest in the morning and peaked at night in both control pinealocytes and those from the experimental group (Fig. 5, A). Nevertheless, cosinor analysis demonstrated that while the acrophase of the rhythm was observed at 4:42 in the control

group, it shifted to 23:42 in the experimental animals, with a concomitant and significant reduction in rhythm amplitude (Table 2).

In both groups, the largest mitochondrial cross-sectional area was observed in the morning, with a decrease to a minimum by 21 hours (Fig. 5, B). Herewith, the acrophase of the rhythm in the experimental group is shifted relative to the control, and its amplitude decreases. In the control animals, the mitochondrial perimeter reached its minimum at 3:00. In the experimental group, while the maximum occurred at 9:00, as in controls, the minimum was observed at 21:00 (Fig. 5, C). The acrophase of the rhythm of this parameter in the control falls on the morning hours; in the experiment, it shifts to the night hours with a significant decrease in amplitude.

Over the 24-hour period, cristae number in the control group follows a pattern defined by a minimum at 15 hours and an increase to a maximum at night hours. At the same time, in mitochondria of pinealocytes of animals of the experimental group, the maximum number of cristae was registered at 9 hours, and the minimum at 3 hours (Fig. 5, D).

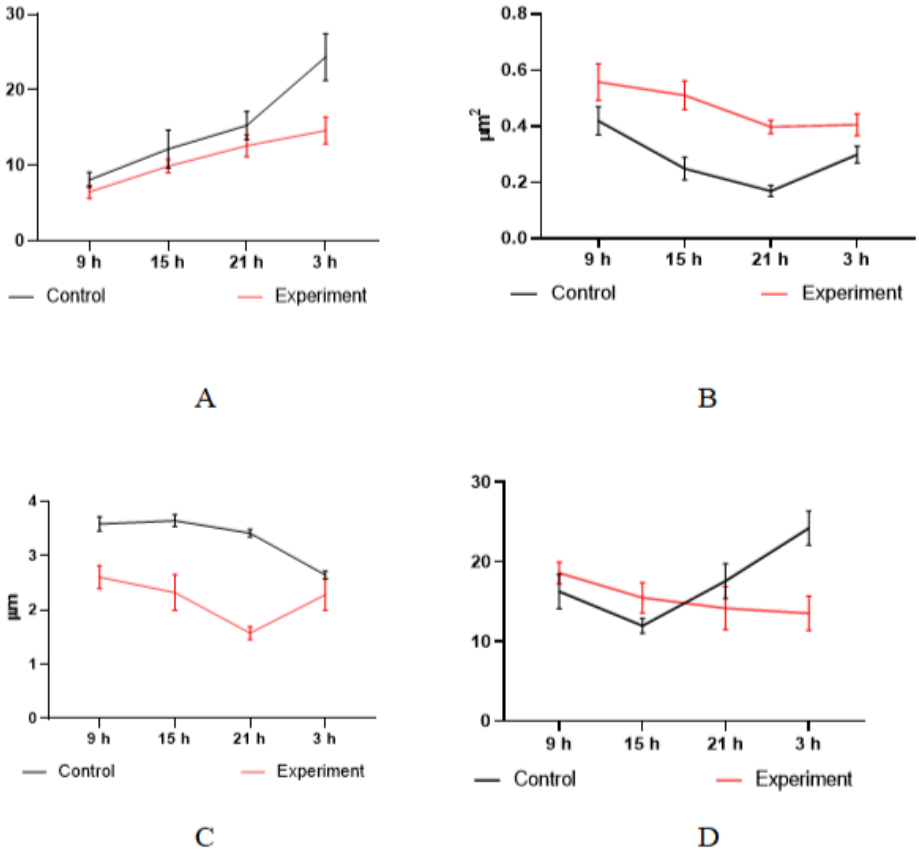

Fig. 5: Diurnal dynamics of studied parameters: A - numerical density of mitochondria, B - average cross-sectional area of mitochondria, C - perimeter of mitochondria, D - average number of cristae in a mitochondrion

In control animals, the mitochondrial Circularity Index (CI) reached its minimum at 15:00 and its maximum at 21:00. In contrast, the experimental group showed a maximum at 3:00 and a minimum at 21:00 (Fig. 6, A). Regarding the S/P ratio, controls exhibited a peak at 3:00 and a minimum at 15:00, whereas the experimental group displayed a maximum at 9:00 and a minimum at 3:00. (Fig. 6, B).

The diurnal fluctuations in Golgi complex area, observed in both control and experimental groups, exhibit a minimum in the morning hours and a maximum in the night hours (Fig. 6, C). The number of GC vesicles in the control was characterized by the same diurnal dynamics, while in the experiment, the diurnal dynamics were smoothed out (Fig. 6, D). In the control, the number of lipid droplets changes similarly, but in the experiment, there are 2 peaks of maximum of this parameter, at 15 and 3 hours, with minima at 9 and 21 hours (Fig. 6, E).

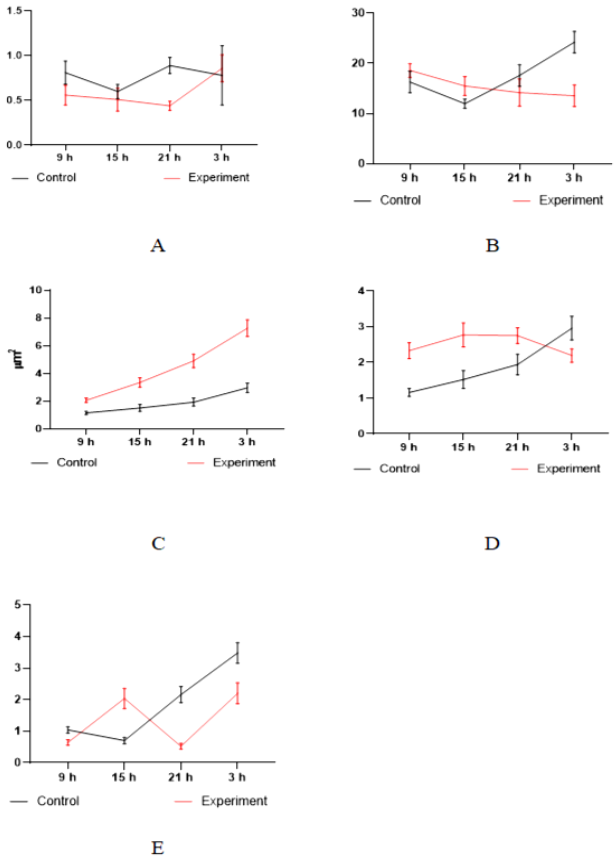

Fig. 6: Diurnal dynamics of studied parameters: A - circularity index of mitochondria, B - ratio of mitochondria area to their perimeter, C - area of Golgi complex, D - number of Golgi complex vesicles, E - number of lipid droplets in pinealocyte

Discussion

Tetrachloromethane exposure caused significant changes in the rat pineal gland, including disrupted melatonin synthesis and ultrastructural remodeling of pinealocytes.

The significant decrease in melatonin concentration in the experimental group reflects inhibition of pineal secretory activity. This finding is consistent with the established toxic profile of CCl_4, which is thought to act primarily through oxidative stress-mediated damage to cell membranes [12, 43, 44].

Under the action of CCl_4, perivascular edema develops in the pineal gland, which can impair the transport of synthesized melatonin into the blood [45]. The detected degenerative changes of mitochondria indicate a disturbance of energy metabolism [46, 47]. Mitochondrial damage inhibits a key step in melatonin production because the mitochondrial enzyme AANAT is responsible for catalyzing the conversion of serotonin (5-HT) to N-acetylserotonin (NAS) [48]. The increased numbers of vacuoles and myelin-like inclusions that were observed are linked to enhanced lipid peroxidation [49, 50].

Chromatin condensation in the nuclei of pinealocytes and reduction of nuclei indicate a decrease in the synthetic activity of cells; these changes are associated with suppression of transcription and protein synthesis [51, 52]. The observed necrosis of single pinealocytes confirms the cytotoxic effect of CCl_4 [53].

The decrease in numerical density of mitochondria with simultaneous increase in their area and perimeter confirms the presence of processes of their destruction and swelling [54-56].

A decrease in the number of mitochondrial cristae speaks in favor of impaired oxidative phosphorylation [57, 58]. This finding agrees with several previous reports that also described vacuolization and structural alterations of the mitochondrial apparatus [30, 33].

The increase in the area of the Golgi complex may be a compensatory response to the increased degradation of damaged organelles [59, 60]. The reduction in lipid droplet count is associated with the activation of lipid peroxidation (LPO) [61].

The presence of autophagosomes containing damaged cellular components in pinealocytes is indicative of autophagy as a potential survival mechanism. In contrast, the identification of necrotic cells suggests extensive cell death and an inability to effectively compensate for damage under stressful conditions [49].

The impact of CCl_4 on the circadian rhythm of melatonin secretion is reflected primarily in an altered amplitude, whereas the acrophase remains virtually unchanged. The observed modifications in the circadian oscillations of morphometric parameters of pinealocyte organelles point to a disturbance of intracellular rhythms, which may be a consequence of damage to the pineal oscillators [60, 62].

Conclusion

Thus, we can conclude that:

- CCl_4 induces a marked reduction in melatonin concentrations, a consequence of its direct toxic action on pinealocytes
- Ultrastructural changes (mitochondrial degeneration, LPO activation, cell necrosis) confirm the cytopathologic effect of CCl4 on pinealocytes
- Violation of the daily dynamics of melatonin content and morphofunctional parameters indicates disorganization of circadian rhythms

This study makes a significant contribution to our understanding of the morphological consequences of carbon tetrachloride intoxication on the pineal gland. The data obtained convincingly demonstrate that this key neuroendocrine organ is a target for toxic effects, which can impair its regulatory functions.

To further advance this knowledge and assess its potential practical significance, the following steps are logical and necessary:

Study the mechanisms of damage and protective pathways. Given the pivotal role of oxidative stress in the toxicokinetics of CCl_4, a natural progression of this research would involve evaluating the capacity of targeted antioxidant strategies, including the administration of melatonin, N-acetylcysteine, or mitochondria-targeted antioxidants, to prevent or ameliorate the structural damage to the pineal gland documented in this study. A comparative analysis of the hepatoprotective effect will allow us to identify general and organ-specific mechanisms of cytoprotection.

Assess the dynamics of recovery. It is critical to establish whether the described changes are reversible. This requires experiments that include an observation period after cessation of toxin administration, which will allow us to determine the timeframe and completeness of morphofunctional recovery of the pineal gland.

Determining the limits of the model's applicability. To extrapolate the findings, it is necessary to study how gender and age factors, as well as various intoxication regimens (dose, route of administration, chronic exposure), influence pineal gland sensitivity to CCl_4. This will allow us to more accurately define the range of conditions under which the identified toxic effect is observed.

Thus, this study not only describes a new aspect of carbon tetrachloride toxicity but also provides a clear basis for further study of xenobiotic-induced neuroendocrine pathology and the search for strategies for its correction. Further studies should be aimed at studying the protective effects of antioxidants and melatonin under conditions of CCl4 intoxication, as well as at clarifying the molecular mechanisms of pineal damage.

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Author's Contributions

Anna I. Anurkina: Investigation; Data curation; Formal analysis; Literature review; Writing - review & editing.
David A. Areshidze: Conceptualization; Methodology; Formal analysis; Literature review; Writing - original draft; Writing - review & editing.
Maria A. Kozlova: Investigation; Data curation; Formal analysis; Writing - review & editing.
Valery P. Chernikov: Investigation; Data curation; Writing - review & editing.

Ethics

Ethical approval for the study was obtained from the Bioethics Committee of the Avtsyn Research Institute of Human Morphology, protocol N 5 from 23.05.2025.

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