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Experimental Determination of Permeability of Liver Mitochondrial Pores in Toxic Hepatitis

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KEYWORDS

toxic hepatitis, mitochondria, membrane, permeabilization, megachannel.

Abstract

Most of the factors leading to the permeabilization of the mitochondrial high permeability pore are known. However, the effects and substances that cause a decrease in permeability are much less. As a result of blocking the formation of bile fluid synthesized in the liver and functional disturbances are observed in the mitochondria of hepatocytes. One such disturbance can be through a change in the conformation of the high permeability pore of mitochondria. Experiments were conducted on 60 rats in order to identify pathophysiological disturbances at the level of the ion transport system, and their results were reported.

Introduction

At present, most of the factors leading to the open conformational state of the mitochondrial permeability transition pore (mRTP-mitochondrial permeability transition pore) are known: the change in matrix pH, $\Delta\psi_m$ decreasing, High concentration of Ca^{2+} ions, prooxidants, induction of LPO, oxidation of thiol groups in mPTP complex, inorganic phosphates, adenine nucleotide translocase (adenine nucleotide translocase -ANT), inhibitors atractylate and carboxyatractylate, acetyl-SoA and free fatty acids, meso, lysophospholipids, heavy metals, thyroid hormones, etc [Halestrap., 2009; Faustin et al., 2004]. However, the effects and substances that cause the closing state of the mPTP, i.e., the reduction of permeability, are much less: in the presence of adenine nucleotides ATF and ADF, Mg^{2+} ions, high $\Delta\psi_m$, a decrease in pH, antioxidants, bongkrekic acid and cyclosporine A (CsA), the mPTP switches to the closed state [Peixoto et al., 2010]. *MPTP* participates in the homeostasis of Ca2+ ions in the cytosol in the low-conductance state, and in cell death in the high-conductance state [Ichas., 1998]. This mitochondrial megachannel becomes hyperconductive in various pathologies, including diabetes, toxic hepatitis, and many other diseases [He et al., 2005; Halestrap et al., 2000]. Pathophysiological processes such as overload of calcium ions in mitochondria, formation of free radicals from the respiratory chain, increase of prooxidant factors, acceleration of formation of malondialdehyde as a result of peroxidation of membrane lipids are ideal conditions for the opening of mRTP. As a result of blocking the formation of bile fluid synthesized in the liver, functional disturbances are observed in the mitochondria of hepatocytes. One such disturbance can be through a change in the conformation of the high permeability pore of mitochondria. The following experiments were conducted in order to identify pathophysiological disturbances at the level of such an ion transport system.

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Materials and Methods

Experiments were carried out on purebred white male rats weighing 180-200 g. Laboratory animals were fed under standard rational conditions in vivarium conditions. The experiments were carried out under in vivo conditions in the following steps (Table 1):

Table 1
Research plan

Steps	Conducting research
Step I	The livers of healthy animals were ligated from the midline ventricular section of the bile duct using a ligature, and after 1 hour, the functional state of liver mitochondria and membrane-dependent pathophysiological processes were determined.
Step II	The functional state of liver mitochondria was studied 3 hours after ligation of the common bile duct.
Step III	The functional status of liver mitochondria was studied 8 hours after ligation of the common bile duct.

Mitochondria were isolated from liver tissue by differential centrifugation at all stages of the study, i.e. 1, 3 and 8 hours after ligation of the common bile duct. Hepatic mitochondria isolated from healthy rats without common bile duct ligation were used as controls.

Nembutal anesthesia at a dose of 40 mg/kg was administered subcutaneously to the animals for general anesthesia. Each experimental group consisted of 3 rats. Mortality of rats was also observed in these model groups. Experiments were repeated 4 times.

A method for isolating mitochondria from rat liver

Rat liver mitochondria were isolated by differential centrifugation [Schneider W.C., 1948]. Composition of separation medium: 250 mM sucrose, 10 mM tris-chloride, 1 mM EDTA, pH 7.4. The rat was anesthetized under general anesthesia, and the liver was excised 1, 3, and 8 hours after ligation of the common bile duct. The dissected liver was placed in a pre-prepared dissection medium and weighed on a scale. To grind the tissue, it was passed through a micropress with a hole size of 1 mm. The minced liver tissue was placed in a special homogenizer, a separation medium was added in a ratio of 1:6 (6 ml of separation medium per 1 g of liver) and homogenized using a Teflon pestle. Homogenization was carried out using a pestle mounted on an electric motor at a speed of 600-800 revolutions per minute. The resulting homogenate was poured into a centrifuge tube. A SLR-1 centrifuge was used to separate mitochondria from the homogenate. The environment in the centrifuge was 0-2°C, and the centrifugation process was carried out in 2 stages. In the first stage, centrifugation at a speed of 1500 rpm (relative centrifugal acceleration of 600 g) lasted 7-8 minutes. At this time, the heavy aggregates of the cell settle down. The sediment supernatant was collected in another clean test tube and placed in the centrifuge rotor. In the second step, centrifugation was performed at a speed of 6000 rpm (relative centrifugal acceleration of 6000 g) for 15 minutes. Then the test tube was removed from the rotor and the mitochondria that had settled to the bottom were separated from the liquid part. Liquid residues and oil particles were cleaned from the walls of the test tube using filter paper. Mitochondria purified from the separation medium were taken into a special container using an autopipette. For experiments, mitochondria were washed in a 1:5 volume of EDTA-free separation medium and stored in an ice-cold container in the freezer.

Determination of high-conductance pore and ATF-dependent potassium channel activity in mitochondria

Kinetics of mitochondrial hyperpermeability (swelling) (0.3-0.4 mg/ml) was determined by the change in optical density at 540 nm in an open cell (volume 3 ml) of a mitochondrial suspension at 26°C with constant stirring. The following incubation medium was used to determine mitochondrial PTP

Journal of Chemical Health Risks
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permeability: 200 mM sucrose, 20 μM EGTA, 5 mM succinate, 2 μM rotenone, 1 μg/ml oligomycin, 20 mM Tris, 20 mM HEPES, and 1 mM KH_2PO_4, pH 7.4 [He L., 2003].

MitoK_{ATF}-channel conductance (0.3-0.4 mg/ml) was determined by changes in optical density at a wavelength of 540 nm in 3 ml cells. IM as follows: 125 mM KCl, 10 mM Hepes, 5 mM succinate, 1 mM $MgCl_2$, 2.5 mM K_2HPO_4, 2.5 mM KH_2PO_4, 0.005 mM rotenone, and 0.001 mM oligomycin, pH 7.4 [Вадзюк О.Б., 2008].

Statistical processing of the obtained results

Statistical processing of the obtained results and drawing of pictures were carried out using the computer program OriginPro 7.5 (Microsoft, USA). The difference between the values obtained from control, experiment and experiment+study material was calculated by t-test. In this, $R<0.05$; $R<0.01$; and $R<0.001$ values represent statistical reliability.

In *in vivo* studies, ligation of the common bile duct in rats causes functional impairment of liver cells. Ligation of the common bile duct caused a sharp change in the amount of total bilirubin in the blood plasma. In this setting, ligation of the common bile duct has been found to induce liver mitochondrial dysfunction. It was found that the content of arachidonic acid, palmitic acid and stearic acid in the phospholipids of liver mitochondria decreased. However, as a result of ligation of the common bile duct, a change in the conformational state of the high permeability pore, i.e. mPTP, in liver mitochondria was not detected. For this purpose, in our experiment mPTP permeability was studied after liver mitochondria were isolated by differential centrifugation after ligation of common bile duct of rat liver for 1, 3 and 8 hours. The outer and inner membranes of mitochondria contain various ion channels and transporters, one of which is the mRTR channel, which play a key role in the activity of human and animal cells, in the control of metabolism, and in the development of various pathological conditions [Brenner et al., 2006; Kwong et al., 2015].

MRTR formation takes place in the conditions of a marginal increase in the concentration of Ca^{2+} ions in the mitochondria, including oxidative stress, an increase in the concentration of inorganic phosphate (Pi) and a decrease in the concentration of adenine nucleotides. [Halestrap et al., 2004; Halestrap., 2009; Javadov et al., 2009]. Mitochondrial megapore shows sensitivity to redox-potential value, concentration of Ca^{2+} ions, $\Delta\psi_m$, adenine nucleotides, Pi and pH value. As an inhibitor of this pore, adenine nucleotides (ADF, ATF) and a decrease in the value of pH are shown [Morin et al., 2009]. In conditions of oxidative stress of the cell, the permeability value of the high permeability pore of mitochondria is observed to increase sharply. How the time-dependent binding of the common bile duct of the liver changes its conductance value is currently unknown.

First, the common bile ducts of experimental rats were ligated for 1 hour, and then a high-throughput pore of their isolated mitochondria was studied. A classic inducer of the mitochondrial mPTP is Ca^{2+} ions, and the addition of these ions to the incubation medium results in increased conductance. In our experiment, a concentration of 20 μM of the mPTP inducer $CaCl_2$ was used.

According to the obtained results, mPTP permeability did not occur without the presence of $CaCl_2$ ions in the incubation medium of liver mitochondria of healthy rats (Fig. 1).

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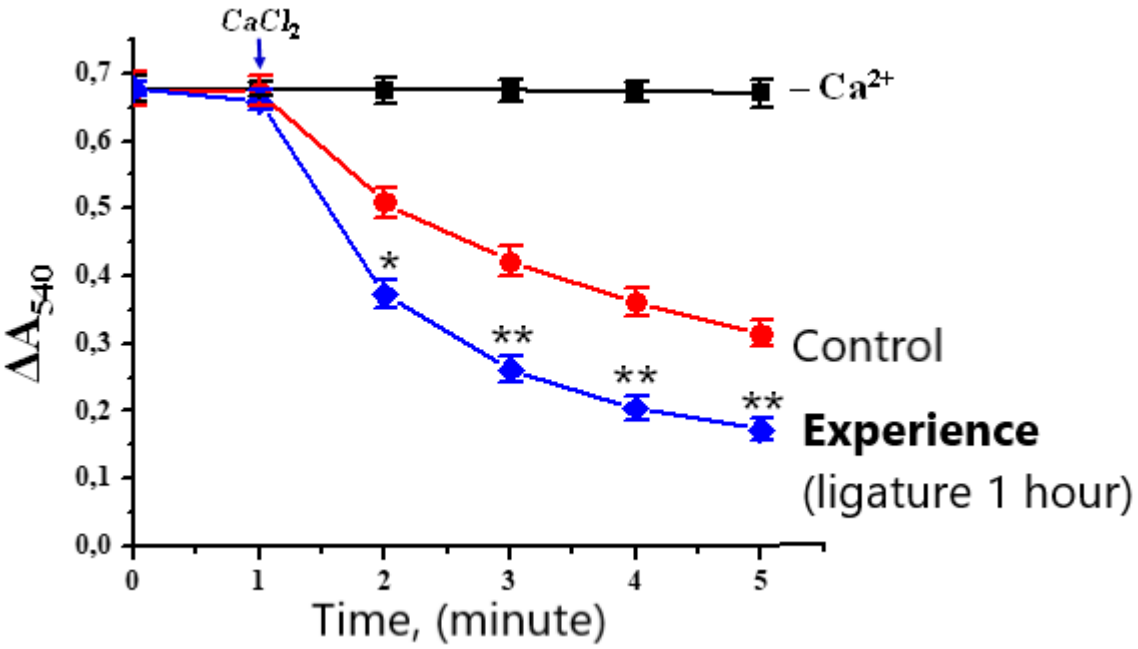

Figure 1. Changes in the permeability value of the high permeability pore (mPTP) of rat liver mitochondria following 1 hour ligation of the common bile duct (*R<0.05; **R<0.01; n=4).

However, it was found that the permeability value of the high permeability pore increased in the presence of 20 μM concentration of CaCl2 in the incubation medium of the liver mitochondria of the control group. In our next experiment, liver mitochondria were isolated after ligation of rat liver common bile duct for 1 h. It was found that mPTP permeability of common bile duct ligated rat liver mitochondria was increased by 39.5% compared to control (Figure 1). This indicates that the mPTP of the liver has changed to an open conformational state. In the open state of the mPTP, solutions with water begin to enter the mitochondrial matrix, and in the presence of inorganic phosphates in the mitochondria, the accumulation of Sa2+ ions causes the mitochondria to swell (swell), as a result, the outer membrane of the mitochondria ruptures [Bernardi P., 2007]. The increase in the permeability of the mitochondrial membrane associated with the connection of the bile duct is manifested by the increase in bile acids in the content of bile, which increases the intensity of the processes of lipid peroxidation of the liver cell and mitochondrial membranes.

In our next experiment, the rat common bile duct was ligated for 3 hours. Ligation of the common bile duct of rats for 3 hours was observed to have a higher value of mPTP permeability of liver mitochondria. According to the obtained results, mPTP permeability increased 6.4 times in the incubation medium of liver mitochondria of control group of rats in the presence of 20 μM $CaCl_2$ ions compared to the medium without calcium. It was found that the high-permeability pore of mitochondria isolated from the liver of rats subjected to common bile duct ligation for 3 hours was 44.4% open (Fig. 2). This indicates that they have increased conductivity value compared to the control. Therefore, the long-term ligation of the common bile duct causes more profound pathophysiological changes in liver cells. Enzymatic processes in the liver, including the decrease in the activity of antioxidant enzymes (SOD, catalase, glutathione peroxide) and the increase in the amount of ALT in the plasma, affect mitochondria as stress. As a result, the generation of free radicals in the mitochondrial respiratory chain increases, the synthesis of ATF decreases, and ultimately causes a sharp increase in mPTP permeability.

Journal of Chemical Health Risks
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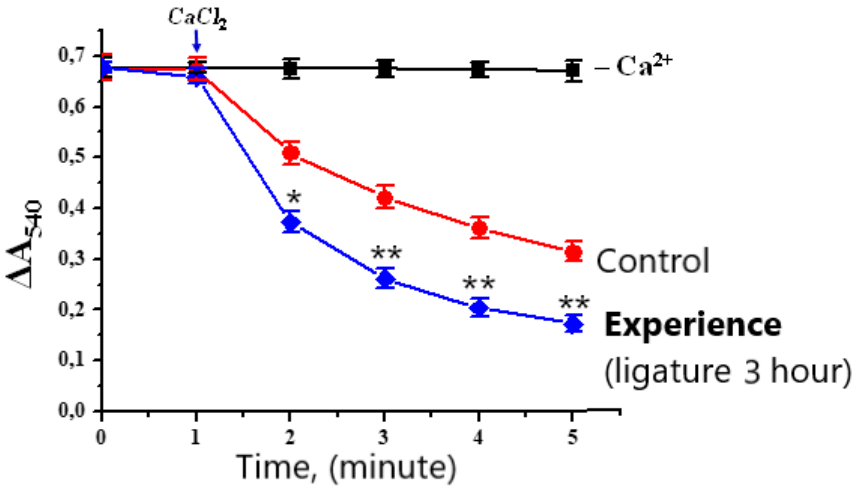

Figure 2. Changes in the permeability value of the high permeability pore (mPTP) of rat liver mitochondria following 3-hour ligation of the common bile duct (*R<0.05; **R<0.01; n=4).

In our next experiment, the rat common bile duct was ligated for 8 hours. Long-term binding of the common bile duct is manifested by a doubling of ALT, alkaline phosphatase, total lipid content in liver hepatocides, and a relative increase in the concentration of bilirubin in the blood serum. In this case, a decrease in the total content of P450 and β5 cytochromes was noted in the microsomal part of the liver of mice [Зверинский И.В., 2013]. Corresponding to these pathophysiological processes, total bile duct ligation of rats for 8 h resulted in dramatic changes in liver mitochondrial permeability. According to the obtained results, it was determined that the Ca^{2+} ion-dependent swelling of mitochondria isolated from the control group rats increased 6 times compared to the environment without calcium. It was found that the permeability value of mitochondria isolated from the liver of rats that were ligated for 8 hours by common bile duct increased by 62.0% compared to the control (Figure 3). This indicates that the mPTP has become open.

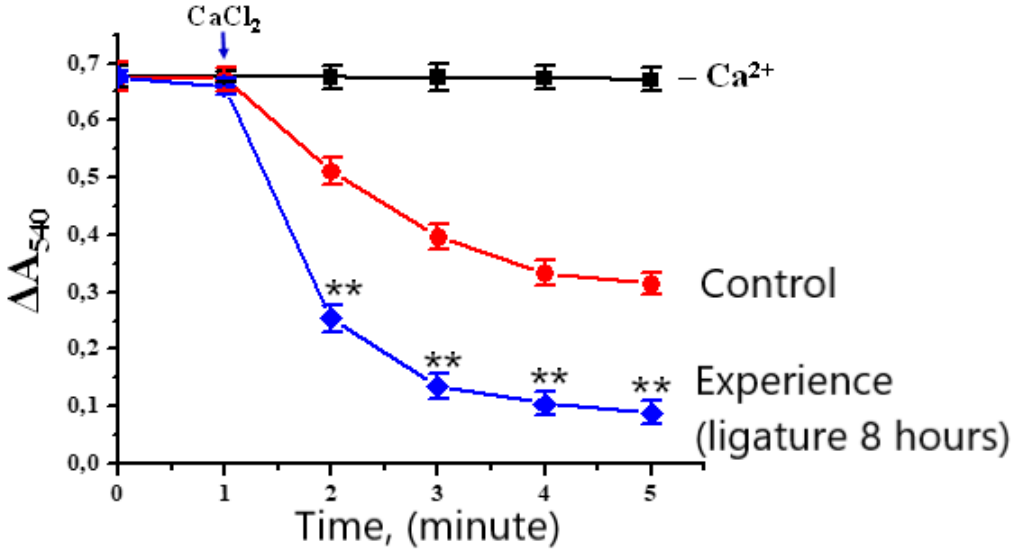

Figure 3. Changes in the permeability of the high permeability pore (mPTP) of rat liver mitochondria following 8-hour ligation of the common bile duct (**R<0.01; n=4).

Journal of Chemical Health Risks
www.jchr.org
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Therefore, failure of bile to enter the digestive system and their accumulation in the gall bladder leads to membrane damage of liver hepatocides and mitochondria. The manifestation of a highly permeable conformation of the mPTP in the liver leads to a disruption of matrix homeostasis, a decrease in membrane potential, a breakdown of metabolic processes, oxidative phosphorylation, and a decrease in ATF synthesis. Ligation of the hepatic common bile duct also causes release of cytochrome c from the matrix in the high permeability state of the mitochondrial pore. This can be explained by the decrease in the activity of respiratory chain enzymes and antioxidant enzymes in the inner membrane.

In our next experiment, studies were conducted with cyclosporin A, a classical inhibitor of this megachannel, in order to further confirm the increase in the permeability value of the high permeability pore of liver mitochondria as a result of ligation of the common bile duct for 1, 3 and 8 hours. According to the obtained results, the permeability of the high-permeable pore of the liver mitochondria of rats with common bile duct ligation for 1, 3 and 8 hours was 38.6%, respectively, compared to the control; It was found that it increased by 53.3% and 63.5%. Under these conditions, in the presence of Ca^{2+} ions in the incubation medium, the addition of 1 μM concentration of cyclosporin A to the cuvette inhibited the inhibition of liver mitochondria in all experiments (Fig. 4).

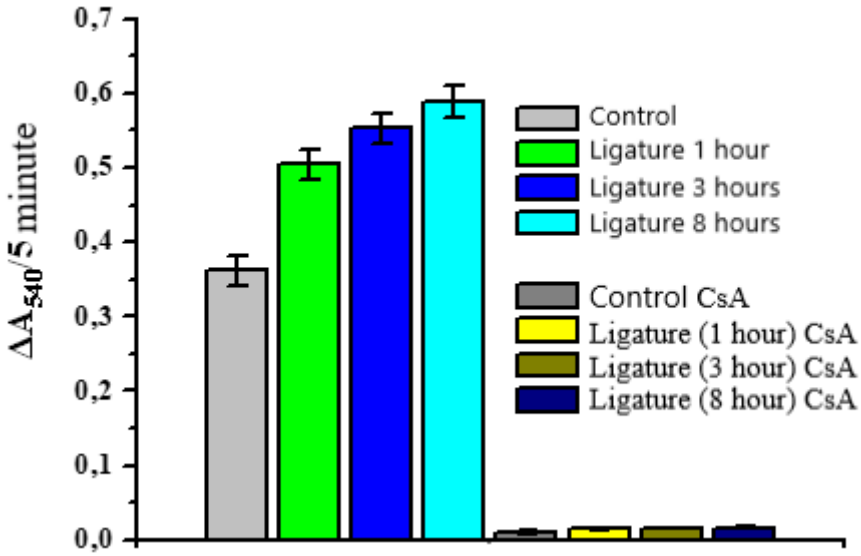

Figure 4. Changes in the permeability value of the high permeability pore (mPTP) of rat liver mitochondria after ligation of the common bile duct for 1, 3 and 8 hours and the effect of the classical inhibitor cyclosporin A (CsA 1 μM) on them (all cases $R<0.05$; $n=4$).

So, indeed, it was found that as a result of the ligation of the common bile duct of the liver, the permeability of the liver mitochondria was completely blocked by the action of the classical inhibitor.

Ligation of the common bile duct may not only increase hepatic mPTP permeability but also affect mitoK_{ATF}-channel activity. The liver mitoK_{ATF}-channel plays an important role in the cycling of potassium ions between the mitochondria and the cell. There are a lot of potassium channels in mitochondria, but among them, mitoK_{ATF}-channel is of high physiological importance, it is a uniporter channel that actively participates in the maintenance of matrix volume and formation of membrane potentials. When the physiological concentration of ATF in the cell decreases, this channel can be activated or inhibited. In order to clarify this, changes in the activity of the mitoK_{ATF}-channel in the inner membrane of mitochondria were studied

Journal of Chemical Health Risks
www.jchr.org
JCHR (2023) 13(4), 2325-2332 | ISSN: 2251-6727

against the background of binding of the common bile duct of this channel. According to the obtained results, mitoK$_{ATF}$-channel activity of mitochondria isolated from their liver as a result of ligation of common bile duct of rat liver for 1, 3 and 8 hours was 15.7%, respectively, compared to the control; 24.5% and 42.5% were found to be activated.

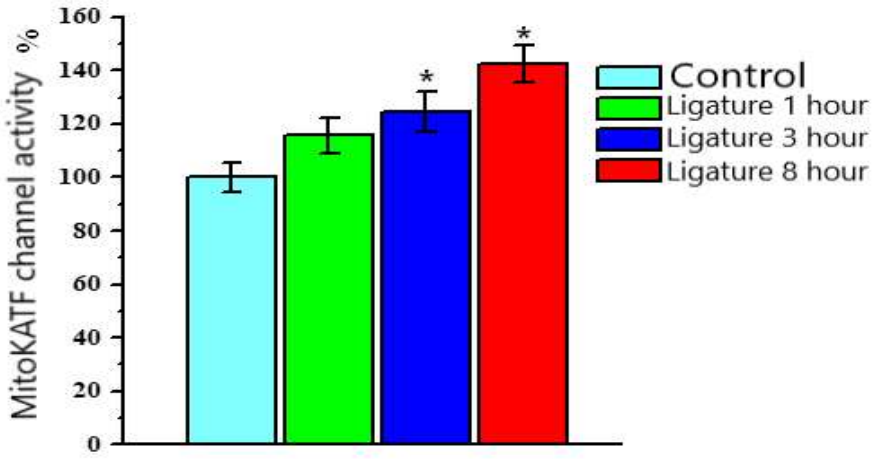

Figure 5. Changes in mitoK$_{ATF}$-channel activity of rat liver mitochondria following ligation of rat common bile duct for 1.3 and 8 hours (*R<0.05; n=4).

Therefore, it can be explained by the emergence of adaptation mechanisms and reduction of ATF synthesis in liver mitochondria of rats as a result of connecting the common bile duct of the liver with the help of a ligature for different periods of time.

In the development of cholestasis, the connection of the common bile duct plays a leading role in the damage of common cell membranes, activation of hydrolases and necrosis of hepatocytes due to the detergent effect of bile acids. Bile acids inhibit the regeneration of hepatocytes, activate fibrogenesis, in addition, they contribute to the activation of free radical formation processes [Monte M.J., et al., 2009; Sedlaczek N., et al., 2001]. The following conclusions can be drawn based on the obtained results.

Ligation of the rat common bile duct for 1, 3, and 8 hours increases their hepatic mPTP permeability. An increase in the binding time of the common bile duct resulted in a marked unfolding of the hepatic mPTP conformation.

Inhibition of the mPTP in the presence of a classical inhibitor of the mitochondrial high permeability pore indicates that this megachannel can indeed open.

Ligation of the common bile duct of the liver for 1, 3 and 8 hours caused a decrease in the synthesis of ATF in mitochondria, which was explained by an increase in the permeability of the mitoK$_{ATF}$-channel.

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