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HISTOBIOCHEMICAL SIGNS OF APOPTOSIS IN GROUND SQUIRREL TISSUES DURING AWAKENING AFTER HIBERNATION = ГИСТОБИОХИМИЧЕСКИЕ ПРИЗНАКИ АПОПТОЗА В ТКАНЯХ СУСЛИКОВ ПРИ ПРОБУЖДЕНИИ ПОСЛЕ СПЯЧКИ

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Исследованы гистологические препараты тканей печени, сердца и мозга сусликов в середине баута спячки (температура тела 1–4 °C), при самосогревании (температура тела 6–29 °C) и полном пробуждении (температура тела 37–38 °C). В тканях сусликов в состоянии глубокой спячки признаков апоптоза не обнаружено. Наблюдается нарушение микроциркуляции, выраженное в виде нарушения реологии крови: резкое полнокровие капилляров мозга и миокарда, центральных вен и синусоидов печени и слабовыраженный отек тканей, агрегация эритроцитов в виде монетных столбиков в сосудах микроциркулярного русла. При самосогревании обнаружены признаки начальной стадии апоптоза: в цитоплазме кардиомиоцитов – слабо выраженная зернистость как проявление белковой дистрофии, а в гепатоцитах – слабо выраженные признаки вакуолизации цитоплазмы клеток. Морфологические признаки этих процессов выявляются уже при полном пробуждении зверьков. Отмечено сморщивание единичных гепатоцитов, появляются клетки с пикнотичными ядрами, что можно объяснить конденсацией хроматина и внутриклеточных органелл, а также обнаруживаются клеточные фрагменты с явлениями кариорексиса, напоминающие апоптозные тельца. Таким образом, признаков апоптоза в тканях сусликов в состоянии глубокой спячки не установлено, при самосогревании выявлены признаки начальной стадии апоптоза и лишь при полном пробуждении отмечены единичные гепатоциты и нейроны, сходные с апоптозными тельцами. Отсутствие воспалительной реакции в исследуемых тканях при полном пробуждении также позволяет предположить, что наблюдаются стадии апоптоза. Пробуждение сусликов сопровождается значительным повышением активности маркеров состояния лизосомальных мембран, катепсина Д и кислой фосфатазы.

Ключевые слова: *гибернация, суслики, апоптоз, лизосомы, катепсин Д.*

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Introduction. Varying natural conditions have led to the emergence of organisms that are genetically adapted to different ranges of temperature fluctuations and therefore represent convenient natural models for studying the adaptability of cells. Animal hibernation is an evolutionarily established process of adaptation to adverse environmental conditions. In the cells of heterothermal mammals, metabolic equilibrium is maintained regardless of body temperature: both at 37 °C (active in summer and awake in winter) and at low body temperature in a state of hibernation. The state of torpor allows animals to significantly save the body's energy resources.

Exiting the state of hypometabolism involves the activation of genes whose products contribute to the normalization of the body's metabolism. Throughout the period of hibernation, intracellular mechanisms may fail, so upon exiting hibernation, after the state of the intracellular systems has been checked, such cells enter apoptosis. However, the initiation of the apoptotic programme at body temperature restoration in hibernators has not been covered in available literature.

The purpose of this study was to identify the morphological features of ground squirrel tissue cells at the stages of body temperature normalization after awakening. The results of our previous studies on lysosomal marker enzymes – cathepsin D and acid phosphatase – are also analysed and discussed in this regard [1].

Materials and methods. The study used ground squirrels (*Citellus pygmaeus* Pall.) caught in the lowlands of Dagestan.

The animals were divided into 4 groups:

1) control animals in the summer at the body temperature of 38 °C;

2) dormant animals at the body temperature of 1–4 °C;

3) animals awakening from hibernation at the body temperature of 6–29 °C;

4) animals awakening from hibernation at the body temperature of 30–37 °C.

Liver, heart and brain, extracted in the cold, were fixed in formalin; 5–7 μm thick paraffin sections were stained with haematoxylin and eosin. To prepare the sections, we took cerebral hemispheres, cardiac tissue from the left ventricle, and tissue from different parts of the liver. In each group, at least 4 animals were examined. For this, the method of simple light microscopy was used. Microphotographs of histological sections were obtained using a Motic microscope at 20×40 and 20×60 magnifications.

Results. The results of our histological examination of ground squirrel tissues are presented in Figures 1–4.

In liver, myocardium, and brain tissues, microcirculatory disturbances were observed in the middle of the hibernation bout (body temperature of 1–4 °C), compared with the control (*Fig. 1*).

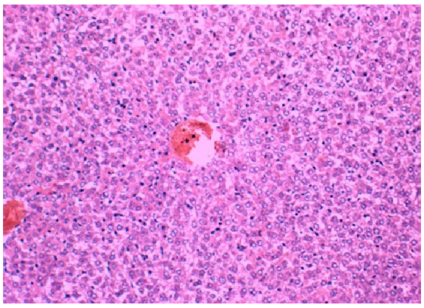

Fig. 1. Hyperaemia in the central and sinusoidal veins of ground squirrel liver (20×40 magnification)

Microcirculatory dysfunction was manifested in abnormal blood rheology: acute hyperaemia in brain and myocardial capillaries, central veins and sinusoids of liver, as well as mild tissue oedema, blood stasis, and aggregations of red blood cells (rouleaux) in cerebral microvasculature (*Fig. 2*).

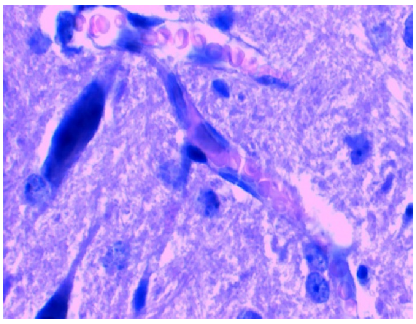

Fig. 2. Blood stasis in the microvasculature; rouleaux in the lumen of the blood vessels in ground squirrels (20×60 magnification)

However, no increase in capillary permeability of vessel walls, plasma leakage, haemorrhage or tissue hypoxia were observed. Abnormal blood rheology reflects adaptive tissue restructuring and metabolic characteristics associated with changes in the biological rhythm.

Self-heating animals (body temperature of 6–29 °C) showed more pronounced morphological disturbances. For instance, in cardiomyocyte cytoplasm, slight granularity as a sign of protein depletion was detected, while in hepatocytes, mild cytoplasmic vacuolization was observed. Granularity in cardiomyocytes is a reversible process and is most likely associated with metabolism (*Fig. 3*).

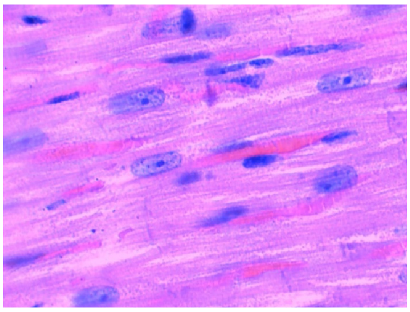

Fig. 3. Protein dystrophy of cardiomyocytes in ground squirrels (20×60 magnification)

During ground squirrels' self-heating to the body temperature of 30–37 °C, morphological signs similar to apoptotic bodies were identified in single hepatocytes at 20×60 magnification. Shrinkage of individual hepatocytes and emergence of cells with pyknotic nuclei were observed; cell fragments with karyorrhectic phenomena resembling apoptotic bodies were also found (formation of oddly shaped bumps, fragmentation of the nucleus) (*Fig. 4*). Moreover, hyperchromia and nuclear compaction were observed in the brain neurons, which, theoretically, can be attributed to apoptosis.

Discussion. The analysis of the results allows us to assume that changes occur in the tissue cells of hibernating animals, which, upon their complete awakening, lead to programmed cell death. As is known, hepatocyte vacuolization is caused by dilatation of smooth endoplasmic reticulum. This phenomenon is characteristic of the early stage of apoptosis, i.e. the programming stage. At this stage, specialized proteins either respond to the apoptotic signal by launching the programme or block the potentially lethal signal. Two (not mutually exclusive) ways of implementing the programming stage are distinguished: 1) by direct activation of effector caspases and endonucleases (bypassing the cell genome) and 2) by genome-mediated signal transmission to effector caspases and endonucleases.

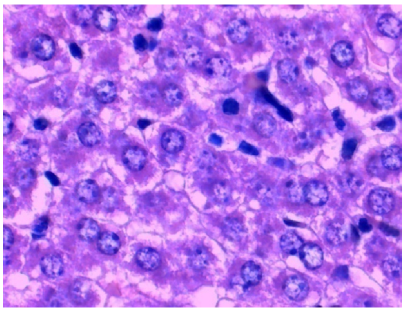

Fig. 4. Chromatin condensation and karyopyknosis, hepatocyte karyorrhexis in ground squirrels (20×60 magnification)

Physiologically, cytoplasmic vacuolization during apoptosis occurs due to the removal of fluid from the cytoplasm to compact it under the action of transglutaminase, which participates in the bonding of protein molecules, catalysing the formation of covalent bonds between free amino groups and γ-carboxamide groups of glutamine.

Covalent bonds formed by transglutaminases are resistant to proteolysis. The observed emergence of cells with pyknotic nuclei can be explained by the condensation of chromatin and intracellular organelles. Moreover, cell fragments with karyorrhectic phenomena resembling apoptotic bodies are found. Pyknosis and karyorrhexis are successive stages of the apoptotic process reflecting the dynamics of endonuclease activation. The absence of an inflammatory reaction in the tissues allows us to suggest that we are observing the stages of apoptosis.

The possibility of apoptosis at normalization of ground squirrel's body temperature after awakening is indirectly indicated by the result of our study into the activity of lysosomal enzymes [1]. Lysosomal enzymes are considered to be the most sensitive to the changes in the physiological status of the body, due to the characteristics of their membranes and the activation of membrane enzymes under various types of stress [2]. A limited release of cathepsins into the cytosol can lead to serious malfunction of intracellular structures and, subsequently, to apoptosis. A connection of lysosomal membrane permeabilization with cell damage and apoptosis has been established by numerous studies [3–7]. The detrimental effect of a number of cellular proteinases on lysosomal membranes has been demonstrated [3]. This ability is also possessed by cytosolic proteases (calpains and caspases). Moreover, phosphorylated p53 protein recruited by the LAPF adaptor protein to lysosomal membranes binds to them causing an early increase in their permeability and, possibly, forming pores in the membranes that are permeable to lysosomal enzymes [3]. In the case of lysosomal membrane permeabilization, the function of the endo/lysosomal compartment is disrupted and the contents of the lysosomes are, to varying degrees, released into the cytosol [4]. After destabilization of the target lysosomal membrane, cathepsins can be released into the cytosol and initiate the lysosomal apoptosis pathway through Bid cleavage and degradation of anti-apoptotic Bcl-2 homologues [8]. It has been found that microinjection of cathepsin D into the cytosol of human fibroblasts causes apoptosis, which is manifested in changes in the distribution of cytochrome *c*, cell shrinkage, activation of caspases, chromatin condensation, and the formation of pyknotic nuclei [5].

Ground squirrels' awakening is accompanied by a significant increase in the activity of lysosomal membrane markers cathepsin D and acid phosphatase [1]. The increase in the activity of cathepsin D in brain homogenates during complete awakening occurs at all incubation temperatures and is more significant compared to the data from the awakening bout. Such changes in enzyme activity in spring upon exiting hibernation can be explained by two reasons: firstly, depletion of the antioxidant membrane protection system and, secondly, cathepsin D activation. Lysosomal acidic aspartic proteases – cathepsins B and D – are involved as secondary messengers in the Fas-dependent apoptosis signalling pathway. In addition, cathepsins transmit apoptotic signals through the interaction with ceramide. Ceramide resulting from the breakdown of sphingomyelin upon activation of acidic sphingomyelinase and the Fas receptor specifically binds procathepsin D (52 kDa), causing its autocatalytic proteolysis with the formation of active cathepsin D (EC 3.4.23.5) in the form of two isoforms, p48 and p32 [9]. Furthermore, ceramide accumulation in the neutrophil membrane at the early stages of spontaneous apoptosis has been shown [10].

At the same time, there is evidence of periodic updates of the lysosomal apparatus and activation of lysosomal enzymes in autophagosomes during hibernation with recurring bouts of torpor and awakening. In hippocampal neurons, the onset of torpor is accompanied by a pronounced reduction and transformation of the structure of endoplasmic reticulum (ER) and Golgi apparatus (GA) concurrently with an increase in the number of autophagosomes containing fragments of membrane structures and ribosomes. In the middle of the torpor bout, signs of the formation of a new ER and multilamellar structures are observed, while GA cisternae reappear only after the animal has warmed up. The structure of both ER and GA in awakened ground squirrels is completely normalized within 2 or 3 hours [11].

The activation of acid phosphatase and cathepsin D during hibernation and awakening is a process necessary for the renewal of subcellular structures. Acid phosphatase is a marker of stability of the lysosomal membrane, and its rising activity indicates an increase in the membrane's permeability. Considering that lysosomal membranes are attacked by calpains and caspases, it can be assumed that at periodic awakenings during hibernation, some of the brain cells undergo apoptosis. Cathepsin D, in particular, being a secondary messenger of the apoptotic signal, itself becomes a direct participant in apoptosis by degrading cellular and subcellular proteins. Morphological signs of these processes are detected upon complete awakening.

Conclusions. No signs of apoptosis were found in ground squirrel tissues during deep hibernation. At self-heating, signs of the initial stage of apoptosis were detected, and only in the state of complete awakening were single hepatocytes and neurons similar to apoptotic bodies identified.

The connection of the results of morphological changes in ground squirrel tissues with the state of stability of subcellular structures, lysosomes in particular, may serve as an evidence of apoptosis. However, at the tissue level and with the given magnification it is only detected after complete awakening and only in hepatocytes and neurons.

Conflict of interest. The authors declare no conflict of interest.

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HISTOBIOCHEMICAL SIGNS OF APOPTOSIS IN GROUND SQUIRREL TISSUES DURING AWAKENING AFTER HIBERNATION

Histological preparations of ground squirrel liver, heart and brain tissues were studied in the middle of the hibernation bout (body temperature of 1–4 °C), at self-heating (body temperature of 6–29 °C) and upon complete awakening (body temperature of 37–38 °C). In the state of deep hibernation, no signs of apoptosis were identified in ground squirrel tissues. We observed microcirculatory dysfunction manifested in abnormal blood rheology: acute hyperaemia of brain and myocardial capillaries, central veins and sinusoids of the liver, as well as mild tissue oedema and aggregations of red blood cells (rouleaux) in microvasculature. At self-heating, signs of the initial stage of apoptosis were detected: mild granularity in cardiomyocyte cytoplasm as a sign of protein dystrophy, and mild cytoplasmic vacuolization in hepatocytes. Morphological signs of these processes were revealed upon the animal's complete awakening. Shrinkage of individual hepatocytes was observed; cells with pyknotic nuclei appeared, which can be explained by the condensation of chromatin and intracellular organelles; cell fragments with karyorrhectic phenomena resembling apoptotic bodies were identified. Thus, no signs of apoptosis were found in ground squirrel tissues during deep hibernation. At self-heating, signs of the initial stage of apoptosis were detected, and only in the state of complete awakening were single hepatocytes and neurons similar to apoptotic bodies identified. In addition, the absence of an inflammatory reaction in the tissues under study allows us to suggest that we are observing the stages of apoptosis. Noteworthy, ground squirrel awakening was accompanied by a significant increase in the activity of lysosomal membrane markers cathepsin D and acid phosphatase.

Keywords: *hibernation, ground squirrels, apoptosis, lysosomes, cathepsin D.*

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