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Dynamic changes in RNA integrity, gene expression, and tissue pathomorphology of experimental mice in the postmortem period

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ABSTRACT

Aim. To examine the pattern of morphological changes, RNA quality number, and gene expression in mouse tissues sampled at autopsy under controlled experimental conditions.

Materials and methods. Balb/c mice were euthanized and subsequently subjected to necropsy at 0, 3, 12, 24, 48, and 72 hours of the postmortem period. During the first three hours following euthanasia, the mice were maintained at room temperature, after which they were transferred to a refrigerator (4 °C). Total RNA was extracted from tissue samples taken from the kidney, liver, and brain; the integrity of the RNA samples was assessed by capillary electrophoresis, and the RNA quality number (RQN) was calculated. The expression levels of *Actb*, *Epas1*, and *Rps18* housekeeping genes were evaluated by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) with original primers and probes using the TaqMan assay. The histologic examination was performed according to standard techniques.

Results. Degradation of RNA extracted from mouse kidney tissues appeared to be greater than that of RNA taken from the liver. In the meantime, a negative linear correlation was observed between RQN and the duration of the postmortem interval for liver and kidney samples. In contrast, no significant changes in the RQN score were observed for brain RNA samples at any of the time points. The expression of the *Epas1* and *Rps18* genes was significantly decreased in mouse kidney and liver tissues. However, the level of *Epas1* and *Rps18* gene expression in the brain remained stable at all time points and did not exhibit a significant decrease at 72 hours after euthanasia. No obvious morphological changes were detected by the histologic examination, which does not exclude the presence of ultrastructural pathological changes.

Conclusion. RQN in autopsy tissues serves as a crucial predictor of sample quality for molecular biology studies, including gene expression analysis.

Keywords: postmortem interval, autopsy, RNA integrity, PCR, gene expression

Conflict of interest. The authors declare the absence of obvious or potential conflict of interest related to the publication of this article.

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Динамические изменения целостности РНК, экспрессии генов и патоморфология тканей экспериментальных мышей в посмертном периоде

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РЕЗЮМЕ

Цель. Изучение закономерности морфологических изменений, величины целостности РНК и паттернов экспрессии генов тканей мышей, отобранных при аутопсии в контролируемых условиях.

Материалы и методы. Мышей линии Balb/c подвергали эвтаназии с последующей некропсией через 0, 3, 12, 24, 48, 72 ч. Первые 3 ч после эвтаназии мыши находились при комнатной температуре, а затем были перемещены в холодильник (4 °C). Общую РНК выделяли из образцов тканей почек, печени, головного мозга, целостность образцов РНК измеряли при помощи капиллярного электрофореза с расчетом значений RQN (RNA Quality Number). Уровень экспрессии генов домашнего хозяйства Actb, Epas1, Rps18 оценивали при помощи обратно-транскриптазной количественной полимеразной цепной реакции в режиме реального времени (RT-qPCR) с оригинальными праймерами и зондами по технологии TaqMan. Гистологическое исследование выполнено по стандартной методике.

Результаты. Выделенная из тканей почек мышей РНК подвержена деградации в большей степени с увеличением посмертного интервала, чем РНК печени. При этом обнаружена отрицательная линейная зависимость между показателем RQN и длительностью посмертного интервала для образцов печени и почек животных. В то же время образцы РНК головного мозга не демонстрировали существенного изменения показателя RQN во всех временных точках. В тканях почек и печени мышей значительно снижается экспрессия генов *Epas1* и *Rps18*. Однако величина экспрессии генов *Epas1* и *Rps18* в головном мозге животных остается стабильной во всех временных точках и не демонстрирует значительного снижения через 72 ч после проведения эвтаназии. При гистологическом исследовании не обнаружено явных морфологических изменений, что не исключает наличия ультраструктурных патологических изменений.

Заключение. Величина целостности РНК (RQN) в аутопсийных тканях является важным предиктором качества образца для молекулярно-биологических исследований, включая анализ экспрессии генов.

Ключевые слова: посмертный интервал, аутопсия, целостность РНК, ПЦР, экспрессия генов

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INTRODUCTION

Postmortem tissue studies have fundamental importance for forensic medicine and biological research in the study of the etiology and pathogenesis of many diseases [1, 2], especially in oncology [3].

Biological materials of cancer patients obtained at autopsy are of great interest for research since they provide an adequate assessment of the quality of clinical diagnosis and allow to develop optimized treatment strategies [4]. The main approach to identifying pathological changes occurring in the

body at the tissue level is morphological research, which is an interpretation of the tissue structure based on findings of the microscopy analysis, including the use of immunohistochemistry methods. In the meantime, implementation of advanced methods for assessing protein expression into clinical practice and translational multi-omics studies provide the most comprehensive understanding of cancer-related pathogenetic processes associated with changes in the molecular, metabolic, and genetic landscapes [5].

However, the use of postmortem tissues is invariably accompanied by a time delay, since samples cannot be immediately stored under conditions, which prevent biological molecules from degradation. Cell autolysis and tissue degradation threaten the reliability of gene expression data and multi-omics studies [2].

Therefore, the time interval between death and sample collection is an important factor in the accuracy and reliability of molecular biology research data, and we can consider postmortem RNA degradation as one of the markers of autopsy tissue integrity [2].

Traditionally, RNA integrity has been assessed qualitatively by comparing the intensities of 28S and 18S ribosomal RNA (rRNA) bands during agarose gel electrophoresis. More recently, automated electrophoresis systems employing microfluidic technologies have been developed that are capable of quantitatively assessing RNA quality based on the analysis of digital electropherograms [6].

Indeed, RNA molecules in cells are extremely vulnerable to degradation, and measuring the RNA Quality Number (RQN) is a standard method for assessing RNA degradation [7].

The literature data on the influence of RNA integrity on the reliability and validity of the results of molecular biology studies of autopsy tissues are contradictory [8–10]. Several studies have attempted to investigate the relationship between RNA integrity in postmortem human brain tissue and the results of gene expression analysis [11] or transcriptome profiling [7].

However, a significant limitation in the design of the presented studies is that they do not assess the influence of such factors as the duration of the postmortem interval and standardization of tissue sample preparation. Therefore, the study of the influence of these factors on dynamic changes in RNA integrity under strictly controlled experimental conditions has a great practical significance; these conditions can only be created using animal models.

The aim of this study was to investigate the patterns of morphological changes, RNA quality number, and gene expression patterns in mouse tissues, selected and sampled at autopsy under controlled experimental conditions.

MATERIALS AND METHODS

The experiments were carried out on 30 female Balb/c mice (aged 7 weeks at the beginning of the study) obtained from the specific pathogen-free facility of the Research Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (Novosibirsk).

The use of animals in this study was approved by the IACUC commission at Siberian State Medical University (Protocol No. 1 of 05.06.2023).

The animals of all groups were euthanized by cervical dislocation following Forane anesthesia (AESICA QUEENBOROUGH Limited, United Kingdom).

The animals of group 1 were subjected to necropsy immediately after euthanasia as a control (n = 5). The animals of the remaining experimental groups were subjected to necropsy at 3, 12, 24, 48, and 72 hours after euthanasia (n = 5 for all groups). In the meantime, in the first hours after euthanasia, the mice were kept at room temperature and then were placed in the refrigerator (+4 °C). This allowed to simulate conditions that were as close as possible to the routine autopsy protocol for patients who died in specialized medical facilities [4].

Total RNA was extracted from tissue samples using the HiPure Total RNA Kit (Guangzhou Magen Biotechnology, China) in accordance with the manufacturer's instructions. The concentration and quality of the isolated RNA were assessed by measuring the optical density at 260 and 280 nm using the Nanodrop 2000 UV-VIS spectrophotometer (Thermo Scientific, USA). The integrity of the RNA samples was assessed using capillary electrophoresis on the Bio-Fragment Analyzer (Bioptic Inc., China).

The isolated RNA was used to synthesize cDNA using the MMLV RT kit (Evrogen, Russia) in accordance with the manufacturer's instructions. Primers and probes (FAM-BHQ1) were designed using the Vector NTI Advance 11.5, Oligo 7,5 software and the NCBI Nucleotide Database (http://www.ncbi.nlm.nih.gov/nuccore). The expression level of *Epas1 (Endothelial PAS Domain Protein 1)* and *Rps18 (Ribosomal Protein S18)* genes was assessed using TaqMan RT-qPCR on the Rotor-Gene-6000 amplifier

(Corbett Research, Australia) (with original primers and probes (*ACTB: F* 5' TGGCAACGAGCGGTTC 3'; *R* 5' CATAGAGGTCTTTACGGATGTCA 3'; *Probe* FAM-5'-tggcaacgagcggttc-3'- BHQ1; amplicon of 134 bp; *EPAS1: F* 5' ATGTGTGAGCCAATCCAGC 3'; *R* 5' TCCAAGATTCTGTCGTCACAG 3'; *Probe* FAM-5'-atgtgtgagccaatccagc-3'- BHQ1; amplicon of 116 bp; *Rps18: F* 5' CCGCCATGTCTCTAGTGATC 3'; *R* 5' GTGATGGCGAAGGCTATTT 3'; *Probe* FAM-5'- ccgccatgtctctagtgatc-3'- BHQ1; amplicon of 97 bp). PCR was performed in three replicates in a volume of 15 µl containing 250 µM dNTPs (SibEnzyme, Russia), 300 nM forward and reverse primers, 200 nM probe, 2.5 mM $MgCl_2$, 19 x SE buffer (67 mM Tris–HCl, pH 8.8 at 25 ºC, 16.6 mM $(NH_4)_2SO_4$, 0.01 % Tween-20), 2.5 U Hot Start Taq DNA polymerase (SibEnzyme, Russia), and 50 ng cDNA. The two-step amplification program included 1 cycle of initial denaturation for 10 min at 94 ºC; 40 cycles – step 1 for 10 sec at 94 ºC and step 2 for 20 sec at 60 ºC. The *Actb* (actin beta) gene was used as a reference gene. Relative gene expression was calculated using the Pfaffl method [12] and expressed in units. Calibrator values were averaged values obtained from the RNA samples extracted from mouse tissues immediately after euthanasia.

For the histologic examination, the mouse tissue samples were placed in 10% neutral buffered formalin with subsequent fixation of the material for 24 hours. The histologic processing of the material was carried out according to the standard method in the ASP 6025 automated vacuum tissue processor (Leica Microsystems, Germany) with the preparation of paraffin blocks. Histologic sections with 4–5 µm thickness were obtained from the paraffin blocks using the HM 430 sliding microtome (Thermo Fisher Scientific, Germany). Microslides were stained with a ready-made solution of hematoxylin and eosin in the Varistain™ Gemini automated slide staining (Thermo Fisher Scientific, United Kingdom). Morphological examination and photography of the histologic microslides were carried out using the Eclipse Ni upright microscope (Nikon, Japan) and Nikon digital camera (Japan) with the NIS-Elements D 5.20.00 image analysis tool (Nikon, Japan). The morphological study included an assessment of changes in organs and tissues for signs of autolysis.

Experimental data were processed using the GraphPad Prism 8 software (GraphPad Software, USA). All results were presented as the mean and the standard deviation ($M \pm SD$). The normality of distribution was checked using the Shapiro – Wilk test. The significance of differences between the study groups was tested by the analysis of variance adjusted by the Benjamini – Hochberg correction. The relationship between the features was assessed using the Pearson's correlation coefficient. The differences were considered statistically significant at $p < 0.05$.

RESULTS

In the present study, the quality of RNA isolated from the mouse kidney, liver, and brain was assessed under conditions similar to those used in human autopsy, and RQN was used as an indicator of RNA integrity.

It was found that in the mouse tissues taken from the kidneys, liver, and brain immediately after euthanasia, the RQN values were 8.86 ± 0.49, 8.48 ± 0.44, and 8.36 ± 0.61, respectively (Fig. 1A, 1C, 1E). When calculating RQN, we considered the fractions of the areas in the 18S and 28S peaks on the electropherogram compared to the total area under the curve, the proportion of large molecules compared to smaller ones, and the height of the 18S and 28S peaks, which allowed to obtain comprehensive information about the degree of degradation in RNA molecules [13]. RQN values range from 1 to 10, where 10 corresponds to the highest integrity of the isolated RNA. The obtained results indicate high integrity of RNA molecules in the selected samples and are consistent with literature data [14].

Keeping mice after euthanasia at room temperature for 3 hours did not result in a decrease in RNA integrity in any of the organs studied (Fig. 1A, 1C, 1E). The integrity of RNA isolated from the mouse kidneys after further storage at +4 °C for 12 hours was reduced by 34.9% ($p < 0.0001$), the integrity of liver RNA decreased by 15.8% ($p = 0.0443$) (Fig. 1A and 1C). A further increase in the postmortem interval to 24, 48, and 72 hours at +4 °C was accompanied by a consistent decrease in the RNA integrity for the tissues of both organs; the end RQN values for them were 4.00 ± 0.86 and 4.81 ± 0.35, respectively.

Thus, RNA isolated from mouse kidney tissue was more susceptible to degradation with increasing postmortem interval. At the same time, brain RNA samples did not show a significant change in RQN at all time points (Fig. 1E). It is important to note that the 260 / 280 ratio, which characterizes the purity of the isolated total RNA, was in the range from 2.0 to 2.3 for all RNA samples, and no significant changes in this parameter were observed immediately after

euthanasia of the animals and at different time points of the postmortem interval.

A negative linear relationship was found between RQN and the duration of the postmortem interval for mouse liver and kidney samples (Fig. 1B and 1D). In the meantime, such a correlation was not identified for mouse brain tissue (Fig. 1F). The obtained results indicate that the degree of RNA degradation depends on the type of tissue and the duration of the postmortem interval.

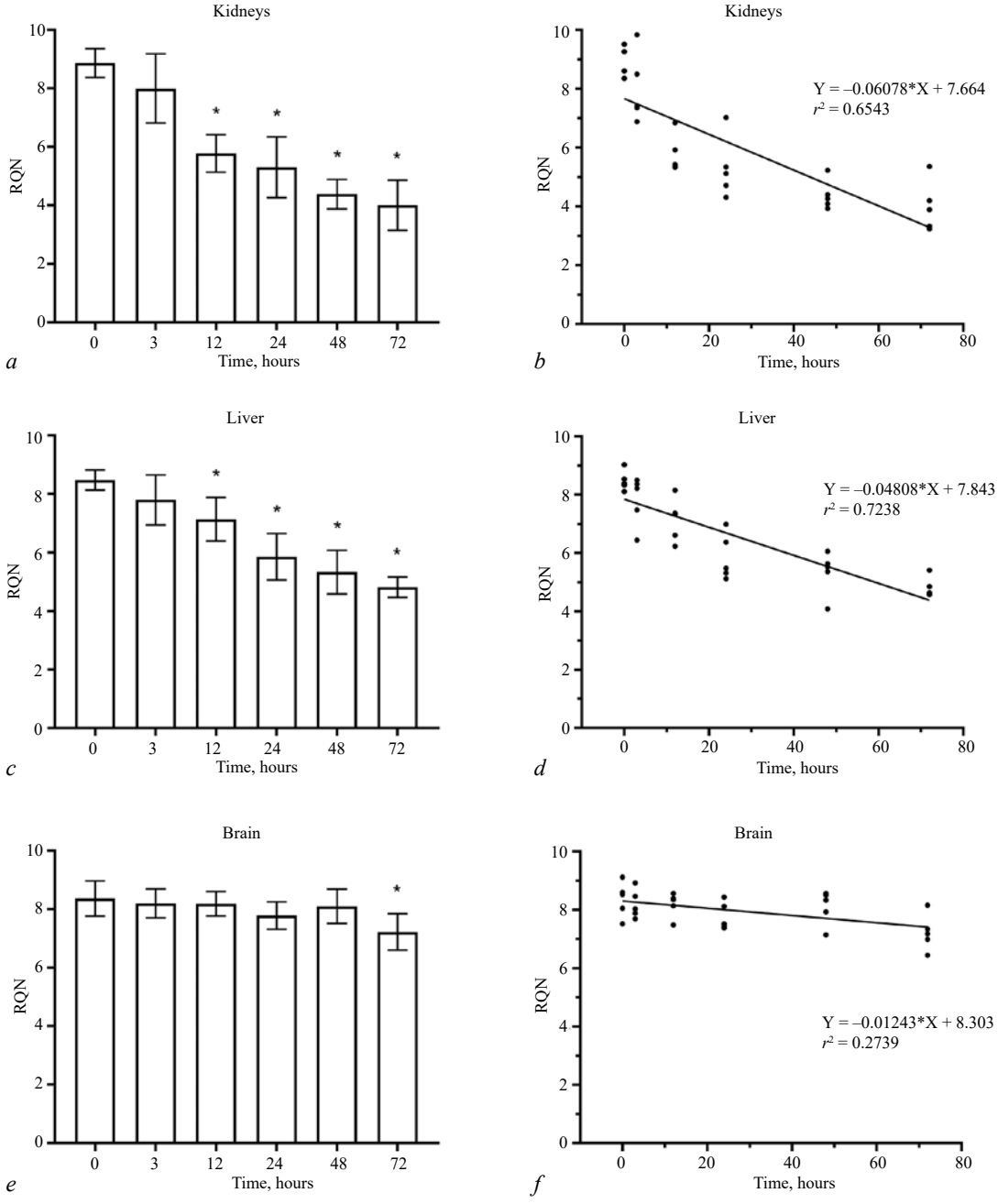

Fig. 1 – RQN and its relationship with time after euthanasia in the kidneys (A and B), liver (C and D), and brain (E and F) of mice. Mouse cadavers were kept at room temperature for the first three hours after euthanasia; then they were moved to the refrigerator (+4 ˚C). The number of animals in each group was $n = 5$. * – the differences were statistically significant ($p < 0.05$) compared to the group of animals subjected to necropsy immediately after euthanasia (0 h)

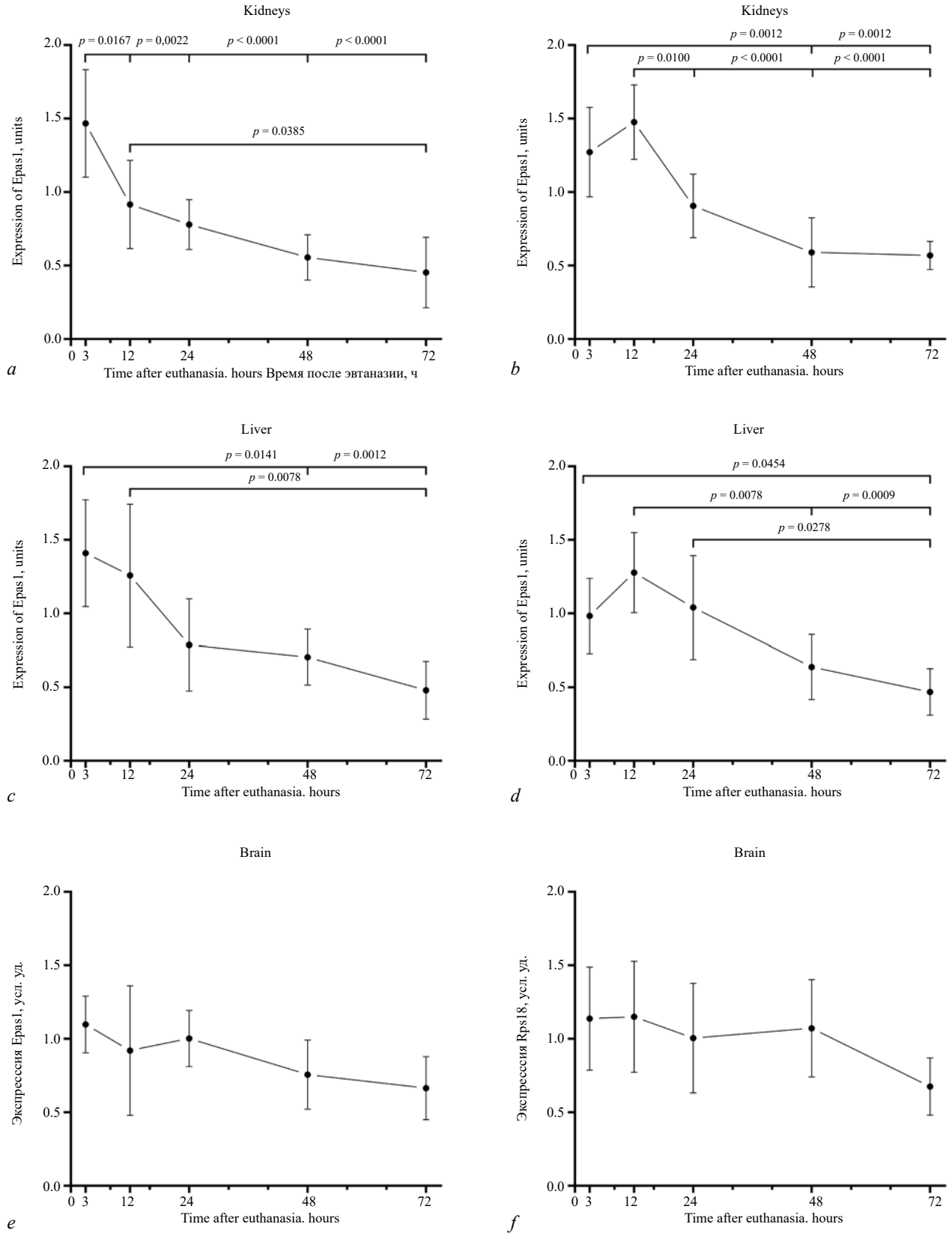

Fig. 2 – Expression of *Epas1* and *Rps18* genes in the kidneys, liver, and brain of mice at different time points after euthanasia, assessed with respect to the *Actb* (actin beta) reference gene. Mouse cadavers were kept at room temperature for the first three hours after euthanasia; then they were moved to the refrigerator (+4 ˚C). The number of animals in each group was $n = 5$. * – the differences were statistically significant ($p < 0.05$) compared to the group of animals subjected to necropsy immediately after euthanasia (0 h)

It is known that the integrity of RNA molecules is important in experiments aimed at assessing gene expression [14]. To assess the relationship between RNA integrity in mouse tissue and the results of molecular biology studies in selected samples of kidneys, liver, and brain using RT-PCR, the expression of housekeeping genes *Actb*, *Epas1,* and *Rps18* was assessed. *Actb* was chosen as a reference gene due to its high stability in the postmortem tissue samples [15].

Following the experiments, it was established that in the kidney tissues of mice after storing for 12 hours (3 hours at room temperature and 9 hours at +4 °C), the expression of the *Epas1* gene significantly decreased from 1.5 ± 0.4 units to 0.9 ± 0.3 units (p = 0.0167). In the liver, the expression of this gene decreased only 48 hours after euthanasia (1.4 ± 0.4 units after 3 hours and 0.7 ± 0.2 units after 48 hours, respectively, p = 0.0141). Increasing the postmortem interval resulted in a further decrease in *Epas1* gene expression in the kidneys and liver (Fig. 2A and 2C).

It is worth noting that the kidney and liver tissues showed a slight increase in *Rps18* gene expression 12 hours after euthanasia (by 15.4% in the kidneys and by 30.0% in the liver compared to autopsy material collected 3 hours later) (Fig. 2B and 2D). Subsequently, the expression of this gene decreased, and after 72 hours of tissue storage, it was 0.6 ± 0.1 units in the kidneys (1.3 ± 0.3 units after 3 hours, p = 0.0012) and 0.5 ± 0.2 units in the liver (1.0 ± 0.3 units after 3 hours, p = 0.0454).

At the same time, the expression level of the *Epas1* and *Rps18* genes in the brain of the animals remained stable at all time points and did not show a significant decrease 72 hours after euthanasia (Fig. 1E and 1F).

Therefore, the demonstrated dynamic changes in gene expression patterns in mice are consistent with the above results of RNA integrity assessment and depend on the organ studied and the duration of the postmortem interval.

To characterize postmortem changes in autopsy samples (kidneys, liver, and brain) of mice, pathomorphological and histologic studies were carried out (Fig. 3).

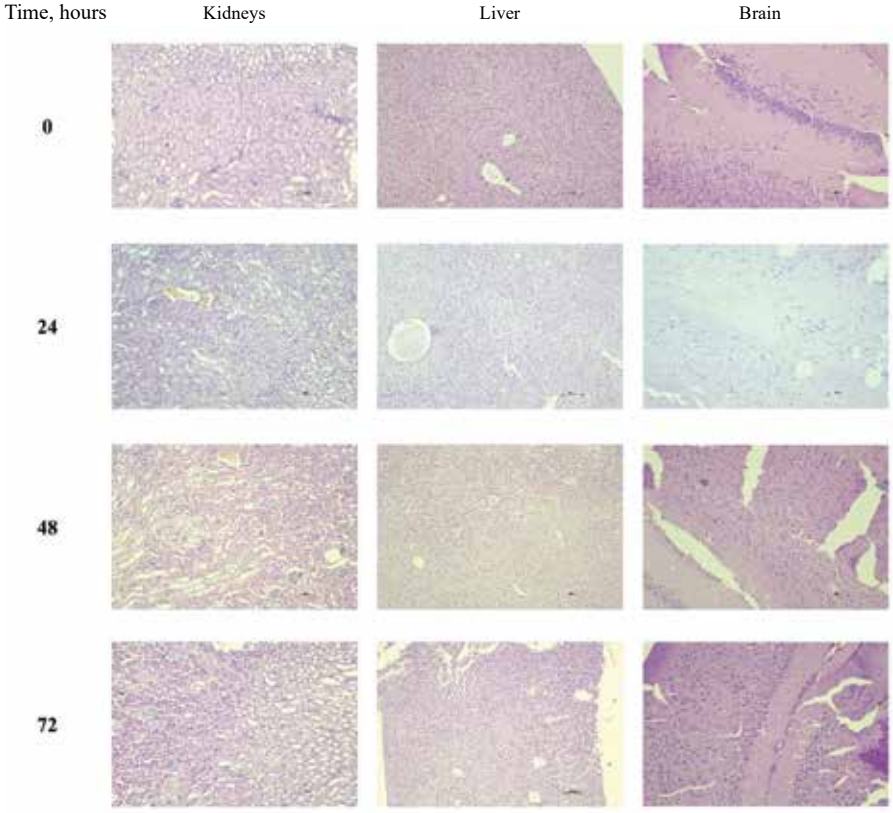

Fig. 3. Microscopic image of the kidneys, liver, and brain of mice at different time points after euthanasia. For the first three hours after euthanasia, mouse cadavers were kept at room temperature; then they were moved to the refrigerator (+4 °C). Staining with hematoxylin and eosin, ×10

In tissue samples obtained from the experimental animals at different time points (at 0, 3, 12, 24, 48, and 72 hours after euthanasia), the histologic structure of organs was preserved. In the kidneys at all stages of the experiment, the cortex and medulla were clearly differentiated, the renal tubules appeared normal, and no autolysis phenomena were detected at the optical level (Fig. 3). When examining the liver samples, the histologic structure of the organ was also preserved; some of the specimens contained cytoplasmic lipid inclusions in hepatocytes and showed signs of granular dystrophy. No signs of autolysis were detected in any of the samples studied (Fig. 3). Mild pericellular edema was observed in the brain, macro- and microglia were preserved, and no autolysis was detected in any of the samples studied (Fig. 3).

Thus, at the microscopic level, no obvious morphological changes, such as autolysis and degenerative changes, were detected, which does not exclude the presence of ultrastructural pathological changes [16].

DISCUSSION

The integrity of RNA molecules is of paramount importance in experiments aimed at assessing gene expression of isolated RNA, especially in modern high-tech studies using microarrays and multi-omics technologies [13, 17]. Indeed, one of the main problems in working with autopsy tissues is pronounced heterogeneity of samples, since the factors determining molecular parameters before and after death cannot be fully controlled [18, 19].

Understanding the relationship of changes occurring at different times in the postmortem interval when working with autopsy samples with sample quality and the correctness of interpretation of the results of molecular biology studies, including RNA integrity and gene expression profile, is of great importance [20].

Therefore, in the present study, we investigated the patterns of morphological changes, RNA integrity, and gene expression patterns in mouse tissues taken at autopsy under controlled experimental conditions.

The analysis showed the presence of tissue specificity with low RQN at different times in the postmortem interval. In this case, the kidneys and liver demonstrated a negative correlation of RQN with increasing time before sampling. In contrast, brain tissue samples were much less susceptible to postmortem changes resulting in a decrease in the RQN score. Similar results were obtained earlier in experiments studying the effect of the postmortem interval on the quality of total RNA isolated from the brain of Balb/c mice [21]. Assessing RNA integrity is essential to obtain reliable results about gene expression levels [22]. RQN ranged from 1 to 10 [13], where RQN above 8.0 indicated high integrity of RNA samples, RQN of 5.0 to 8.0 indicated moderately degraded samples, and RQN below 5.0 indicated significant degradation [23]. RQN of 5 is often used as a criterion for inclusion of biological samples in a study, although there is no consensus in the literature on this matter [23].

The kidney and liver tissues of mice at the endpoint of the study (72 hours after euthanasia) demonstrated a decrease in the RQN score by more than 50% to 4.0 ± 0.86 and 4.81 ± 0.35, respectively, which is consistent with a significant decrease in the amount of mRNA of stably expressed genes *Epas1* and *Rps18*.

Indeed, some studies have shown that the integrity of ribosomal RNA, expressed in RQN, can be used as an alternative parameter of the quality of messenger RNA (mRNA) [9, 22].

In the meantime, the results of the histologic examination using routine staining did not reflect dynamic tissue degradation in the postmortem period at the molecular level. To identify patterns in the development of ultrastructural changes, studies using electron microscopy methods are required [16].

Therefore, RQN in autopsy tissues serves as a crucial predictor of sample quality for molecular biology studies, including gene expression analysis. Further research will help identify patterns describing the relationship of molecular profiles of tissues obtained from the deceased with biopsy material and standardize protocols for handling autopsy material to obtain valuable results in multi-omics studies.

CONCLUSION

Following the study, it was established that RNA degradation in the postmortem period occurs tissue-specifically and is most pronounced in the kidneys and liver of experimental animals, in contrast to the brain. In mouse kidney and liver tissues, expression of stably expressed *Epas1* and *Rps18* genes was significantly reduced. The expression levels of the *Epas1* and *Rps18* genes in the brain of the animals remained stable at all time points and did not show a significant decrease at 72 hours after euthanasia. The observed dynamic changes in gene expression patterns in mice are consistent with the results of RNA integrity

assessment and depend on the organ studied and the duration of the postmortem interval. Thus, RQN in autopsy tissues serves as a crucial predictor of sample quality for molecular biology studies, including gene expression analysis.

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Buyko E.E. – review of literature, acquisition and interpretation of experimental data, drafting of the article. Perina E.A. – identification of dynamic changes in RNA integrity, drafting of the article. Vasilchenko D.V. – carrying out of histologic studies. Tsydenova I.A. – carrying out of molecular biology studies. Khmelevskaya E.S. – collection of biological material. Ufandeev A.A. – statistical processing of the data. Kaidash O.A. – coordination of sample collection for the comprehensive assessment of parameters of the molecular biology study. Ivanov V.V. – conception and design, coordination of the study, drafting of the article, final approval of the manuscript for publication. Vtorushin S.V. – carrying out of the experiment, analysis and interpretation of the data. Udut E.V. – coordination of the study, final approval of the manuscript for publication.

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