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BIOCHEMICAL DETECTION OF APOPTOSIS BY DNA FRAGMENTATION IN THE TISSUES OF THE STOMACH AND KIDNEY UNDER CONDITIONS OF INDUCED STRESS

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The purpose of the study was to investigate the activity of apoptosis in the gastric mucosa and renal by the level of fragmented DNA under conditions of experimental restraint stress. The restraint stress model was chosen as an experimental model of the pathophysiology of stress-related mucosal disease and acute renal failure in rats. According to the level of fragmented DNA, which indicates the level of apoptosis in the tissues of the gastric mucosa at different times after immobilization stress, the phases of the pathological process can be distinguished. The dynamics of changes in apoptosis activity by the level of fragmented DNA in the tissues of the renal cortex after immobilization stress had a two-phase nature. Experimental immobilization stress has a significant effect on DNA fragmentation levels in the renal medulla. The effects of stress increase during the first two weeks, after which adaptation or recovery processes begin. Even after 21 days, DNA damage levels remain significantly higher than normal, indicating long-term effects of stress on kidney cells.

Key words: fragmented DNA, gastric mucosa, kidney.

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БІОХІМІЧНА ДЕТЕКЦІЯ АПОПТОЗУ ЗА ДНК-ФРАГМЕНТАЦІЄЮ В ТКАНИНАХ ШЛУНКУ ТА НИРОК В УМОВАХ ІНДУКОВАНОГО СТРЕСУ

Мета дослідження – дослідити активність апоптозу слизової оболонки шлунка та нирок за рівнем фрагментованої ДНК в умовах експериментального стримувального стресу. Модель стримувального стресу була обрана як експериментальна модель патофізіології пов'язаного зі стресом захворювання слизової оболонки та гострої ниркової недостатності у щурів. За рівнем фрагментованості ДНК, який свідчить про рівень апоптозу в тканинах слизової оболонки шлунка в різні терміни після іммобілізаційного стресу, можна виділити фази патологічного процесу. Динаміка змін активності апоптозу за рівнем фрагментованої ДНК у тканинах кори нирок після іммобілізаційного стресу мала двофазний характер. Експериментальний іммобілізаційний стрес має значний вплив на рівні фрагментації ДНК у мозковій речовині нирки. Наслідки стресу посилюються протягом перших двох тижнів, після чого починаються процеси адаптації або відновлення. Навіть через 21 день рівень пошкодження ДНК залишається значно вищим за нормальний, що вказує на довготривалий вплив стресу на клітини нирок.

Ключові слова: фрагментована ДНК, слизова оболонка шлунка, нирки.

The study is a fragment of the research project "Development and implementation of innovative technologies for the diagnosis of oncogynecological and oncourological diseases based on liquid biopsy data of extracellular DNA and stem cells", state registration No. 0123U101248.

Our previous study has demonstrated that refugees have a higher risk of poor mental health, both as a consequence of adverse or traumatic premigration experiences and as a result of post-migration difficulties. Post-traumatic stress disorder (PTSD) and other mental disorders affect at least one in three refugees [5].

From a biological standpoint, the stress response comprises a series of reactions involving the hypothalamic-pituitary-adrenocortical and sympatho-adrenomedullary axes. Although this stress response is essential for human functioning, excess acute activation or chronic activation is associated with several physical and mental disturbances. Acute stress is associated with different types of pain conditions; gastrointestinal symptoms such as pain, indigestion, diarrhea, acute renal injury [8]. Chronic stress is also associated with several disorders and diseases, including mental health disturbances such as anxiety and depression; neurodegenerative diseases such as Alzheimer's disease; cardiovascular diseases; metabolic disorders such as obesity, metabolic syndrome, and type 2 diabetes mellitus; and restless leg syndrome; and several cancers [8].

Stress-related mucosal disease (SRMD) commonly known as stress ulcer is described as a continuum condition ranging from superficial mucosal damage to deep focal mucosal damage. SRMD is observed in critically ill patients during a serious illness such as surgery, trauma, sepsis, severe burns, etc., within twenty-four hours of their admittance to the intensive care unit (ICU) [11].

The kidney is reportedly one of the most vulnerable organs in stress. Kidney injury can lead to renal insufficiency (Acute Stress-induced renal injury, ASRF), hyperkalemia, water intoxication, fatal arrhythmia, and cerebral edema, which are often life-threatening. However, kidney injury induced by acute stress is rarely reported. Therefore, urgent study is needed regarding potential mechanisms and effective treatment targets of kidney injury caused by acute stress [2].

The purpose of the study was to investigate the activity of apoptosis in the gastric mucosa and renal by the level of fragmented DNA under conditions of experimental restraint stress.

Materials and methods. Experimentations were peformed on male Wistar rats (180–200 g). The animals were kept in polypropylene cages (five rats per cage). The temperature of 25±10ºC, relative humidity of 50–55 %, and a 12/12 h light/dark cycle were maintained. The rats were fed commercial food pellets as well as water ad libitum/ Research was carried out in compliance with the main provisions of the Resolution of the First National Congress on Bioethics "General ethical principles of experiments on animals" (2001), orders of the Ministry of Health of Ukraine No. 690 from 23.09.2009, No. 944 from 14.12.2009, No. 616 from 03.08.2012. Research permission was obtained from the Bioethics Committee of the Lugansk State Medical University (number 03.09.2024/1).

The restraint stress model was chosen as an experimental model of the pathophysiology of stress-related mucosal disease and acute renal failure in rats.

In a restraint stress (CRS) model, the rats (n=72) fasted overnight and were immobilized by tying the fore and hind limbs separately on a foam block with a soft elastic band (Fig. 1) after overnight fasting at room temperature [11].

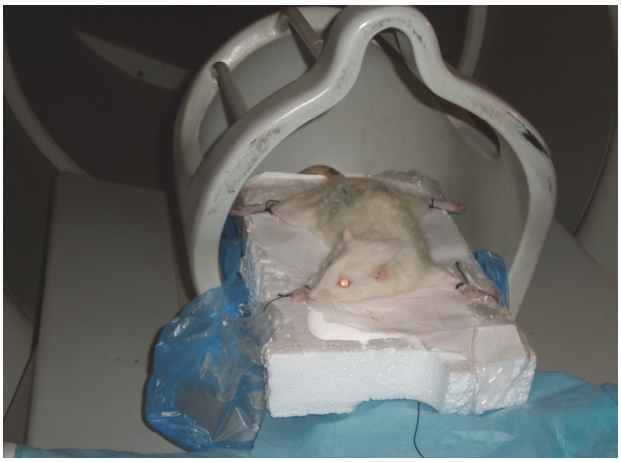


Fig.1. Immobilization of experimental rats.

The animals were divided into two groups: intact (control) and stress group 1 (SRMD and ASRF formation). All studies were conducted dynamically on days 3, 7, and 14 after SRMD and ASRF formation in the following biosubstrates: blood serum, urine, stomach mucosa, renal cortex, and medulla. Each group included 12 animals. The day before decapitation, daily urine was collected from the rats. 10% homogenate of stomach mucosa and renal tissue was prepared in cooled lysis buffer (Triton X-100, Tris-HCl, EDTA) and sucrose isolation medium (sucrose, EDTA).

Ulcer index. Ulcer scoring was done as per Sanyal [11].

Test of experimental acute renal failure.

Renal function was assessed based on the level of creatinine and urea in the blood serum. Urea was determined using a standard set of reagents from the company "Felicit Diagnostics" (Ukraine) using the diacetyl monooxime method, level of creatinine – also with reagents from the company "Felicit Diagnostics" (Ukraine).

DNA fragmentation in tissues was measured with the diphenylamine assay as reported previously [10]. Stomach and kidney tissues were washed five times in ice-cold phosphate-buffered saline (PBS) to clear the blood and then homogenized using a homogenizer in 9 vol of a lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5 % Triton X-100) for 30 min at 4°C. The amount of fragmented DNA (fDNA) was calculated in percentage as the ratio of the amount of extracted DNA (in the supernatant) to the total amount of DNA in the sample.

Data Processing. Statistical and graphical analyses were done using STATISTICA 7.0 (StatSoft Inc. USA, version 7.0) and and MedCalc Version 20.218 64 bit (MedCalc Software, Ostend, Belgium). Parametric data were summarized as mean (standard error) (Mean±SEM). Kolmogorov–Smirnov test was applied to examine the normality of data distribution. To examine group-wise differences, unpaired Student's t-test was used. Nonparametric results are expressed as median (Me) and standart deviation (SD). The difference between study groups was tested by a nonparametric Mann–Whitney U test was used. Receiver operating characteristics (ROC) curve analysis was performed to estimate optimal cut-off values, maximizing sensitivity and specificity according to the Youden index. The appearance of Ulcer index and stage of ARF analysis was performed using the Kaplan–Meier method; univariate analyse were undertaken using log rank test and Cox's regression model, respectively. A *p*-value below 0.05 was considered statistically significant. The Cox proportional hazards regression model was used to assess the effect of tissues DNA levels on the Ulcer index and stage of ARF in survival analysis.

Results of the study and their discussion. Descriptive statistics and comparison results of experimental animal groups for the studied parameters included in the analysis of predictors of the occurrence of stress ulcers of the gastric mucosa after 24-hour restraint stress are presented in Table 1.

Table 1

Main data of Ulcer index and fDNA, % in the rats after restraint stress (mean ± SEM)

Groups (n=12)	Ulcer index	p level	fDNA. %	p level
intact rats –control	0		9.50±0.198	
after restraint stress – Day 1	10.75±0.88	p^1<0.0000001	17.7±0.53	p^1<0.0000001
after restraint stress – Day 3	30.58±2.04	p^1<0.0000001 p^2<0.0000001	30.2±0.95	p^1<0.0000001 p^2<0.0000001
after restraint stress – Day 7	35.75±1.01	p^1<0.0000001 p^2<0.0000001 p^3=0.033277	39.78±1.16	p^1<0.0000001 p^2<0.0000001 p^3=0.000002
after restraint stress – Day 14	31.67±0.78	p^1<0.0000001 p^2<0.0000001 p^3=0.624781 p^4=0.004131	46.49±0.85	p^1<0.0000001 p^2<0.0000001 p^3<0.0000001 p_4=0.000118
after restraint stress – Day 21	22.50±1.19	p^1<0.0000001 p^2<0.0000001 p^3=0.002431 p^4=0.0000001 p^5=0,000002	35.78±0.87	p^1<0.0000001 p^2<0.0000001 p^3=0,002431 p^4=0.011228 p^5<0.0000001

Note: Data are Means ± SEM for Gaussian variables Intergroup by the T-test Students ANOVA showed high statistical significance between groups ($p < 0.001$) F-statistic = 145.44 indicates a strong relationship between time after stress and ulcer index p^1 – significant differences between controi group and groups after restraint stress p^2 – significant differences between group – Day 1and other groups after restraint stress p^3 – significant differences between group – Day 3and other groups after restraint stress p^4 – significant differences between group – Day 14and other groups after restraint stress p^5 – significant differences between group – Day 14and other groups after restraint stress.

Intact rats show no ulcers (Ulcer index = 0). Ulcer Development Dynamics:

Day 1 after restraint stress – initial ulcer formation (mean value 10.75±0.88); Day 3 after restraint stress – significant increase in ulcer index (30.58±2.04); Day 14 after restraint stress – peak of ulcer formation (35.75±1.01); Day 14 after restraint stress there was a slight decrease in Uhcer index (31.67±0.78); and at 21 days after restraint stress there was a marked decrease in Uhcer index (22.50±1.19).

These data indicate that immobilization stress causes significant ulcer formation in rat stomachs, with a peak on day 7 and subsequent gradual recovery by Day 21, although complete return to baseline conditions is not achieved.

Let us consider the dynamics of changes in fragmented DNA in the gastric mucosa of rats after 1–21 days of immobilization stress). The level of fragmented DNA in the gastric mucosa of intact rats was 9.50±0.198 %. A progressive increase in DNA fragmentation was observed in the gastric mucosa of rats from the first day after immobilization stress: Day 1 – 17.7±0.53 %; Day 3 – 30.2±0.95 %; Day 14 – 39.78±1.16 %. The peak of DNA fragmentation in the gastric mucosa was reached on the 14th day after immobilization stress (46.49±0.85 %). By the 21st day, there was a partial decrease in fDNA (decrease to 35.77±0.87 %). All time points show statistically significant differences with the control group.

Analysis of the ROC curve and the Kaplan-Meier survival curves in the rats after restraint stress is shown in Fig. 1.

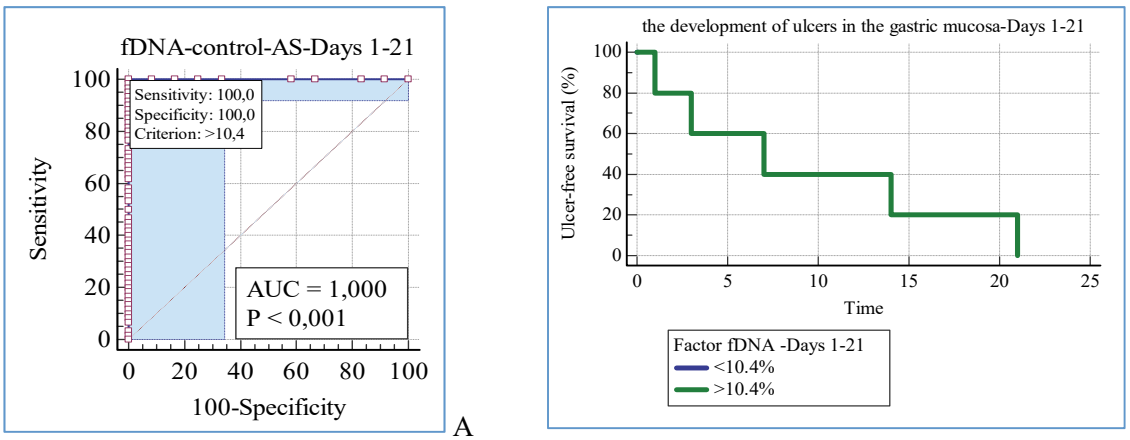
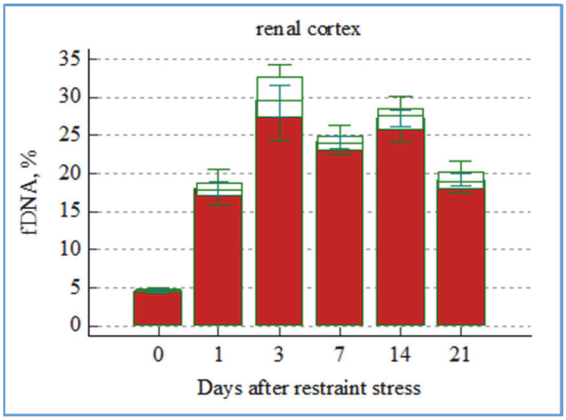
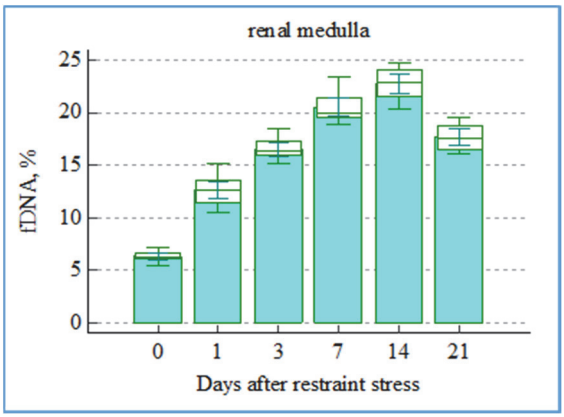


Fig. 1. A – ROC analysis: receiver operating characteristic (ROC) curves for fragmented DNA and B – Kaplan–Meier curves of the time of the development of ulcers in the gastric mucosa measured in the rats at different times after immobilization stress.

According to the receiver operating characteristic curve, the area under ROC curve (AUC=1.000; optimal cut-off values of fDNA – >10.4 %; p<0.001) values of fDNA to predict the development of ulcers in the gastric mucosa measured in the rats at different times after immobilization stress.



A



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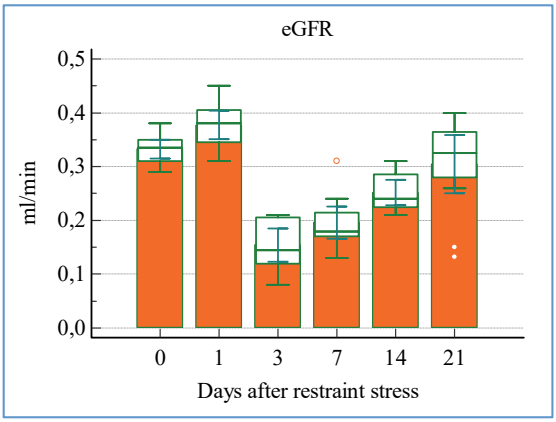

C

Fig. 2. Main data of fDNA, %: A – renal cortex; B – renal medulla; and C – eGFR, ml/min, in the rats after restraint stress. fDNA data are Means ± SD for Gaussian variables, intergroup by the T-test Students; eGFR – intergroup by the Mann–Whitney U test.

The Kaplan–Meier survival curves, after classifying the rats on the basis of Youden cut-offs obtained by ROC curves, showed the maximum number and size of ulcers (Ulcer index) in the gastric mucosa developed on average by day 9.2 (95 % CI for the mean 7.3 to 11,1). The fDNA value >10.4 % in rats after immobilization stress can be used as a reliable diagnostic criterion for determining the presence of ulcers in the gastric mucosa. Given the ideal sensitivity and specificity, this marker can be recommended for both screening and confirmatory diagnostics of the presence of ulcers in the gastric mucosa of rats.

Next, we performed a Cox proportional hazards regression analyses of predictors for the development of ulcers in the gastric mucosa measured in the rats at different times after immobilization stress. In univariate analysis, fDNA was significantly associated with the development of ulcers in the gastric mucosa measured in the rats at different times after immobilization stress in all observation groups. The Cox model shows that fDNA is a significant factor in increasing the time of development of ulcers in the gastric mucosa (p<0.0001), since Exp(b) (Hazard ratio)=0.91 (less than one); while the Harrell index C = 0.801, as a value close to 1, which indicates the high efficiency of the Cox model.

Next, we studied the level of apoptosis according to fragmented DNA in kidney tissues (cortex and medulla) in the same rats after 24-hour immobilization stress for the same observation periods depending on the level of developed acute renal failure by the level of eGFR (Fig. 2).

Differences were observed in eGFR levels between all groups of rats and control group. Thus, the baseline level (control) of eGFR in rats was 0.33±0.03 ml/min; on Day 1 after immobilization – 0.38±0.04 ml/min (compared to the control group p=0.006812); on Day 3, a sharp decrease in eGFR was observed to 0.15±0.005 ml/min (compared to the control section p=0.000001). Gradual recovery follows of eGFR: on Day 14 – 0.2±0.05 ml/min (compared to the control section p=0.000005); on Day 14 – 0.25±0.04 ml/min (compared to the control section p=0.000072); on Day 1– 0.30±0.085 ml/min (compared to the control section p=0.712535).

The primary endpoint of this study was incident acute kidney disease (AKD), defined as the incident-estimated glomerular filtration rate (eGFR)<0.33 mL/min during the follow-up period.

Thus, immobilization stress significantly affects kidney function, and function recovery occurs slowly. The most critical period is day 3 after stress.

The initial response to stress shows an increased eGFR (13.5 % higher than the baseline). Maximum dysfunction was observed on Day 3 (a decrease of 54.5 % from the baseline). Slow initial recovery of kidney function was observed on Days 3–14, and a moderate rate of recovery was observed on Days 14–21; on the 21st day – almost complete restoration of kidney function (the recovery curve shows

an asymptotic approach to the initial values). The recovery process shows a predictable experimental model.

The level of fDNA in the renal cortex at all observation periods is higher than in the control group. Namely, Day 1: sharp increase to 18.03±1.35 % (almost 4-fold compared to control 4.58±0.23 %, p<0.0000001); Day 3: peak values 29.54±3.22 % (compared to the control group, p<0.0000001); Day 7: decrease to 24.07±1.17 % (compared to the control group, p<0.0000001); Day 14: secondary increase to 27.27±1.77 % (compared to the control group, p<0.0000001); Day 21: decrease to 19.13±1.29 % (compared to the control group, p<0.0000001).

These data indicate an active inflammatory process with maximum expression on Day 3 and a secondary rise on Day 14, followed by a tendency toward normalization by Day 21, although complete return to baseline values does not occur.

The level of fragmented DNA in rats of the control group was 6.38±0.50 %. Namely, Day 1: sharp increase to 12.63±1.31 % (98 % increase from control, p<0.0000001); Day 3: further increase to 16.55±1.06 % (159 % increase from control, p<0.0000001); Day 7: continued growth to 20.55±1.37 % (222 % increase from control, p<0.0000001); Day 14: peak values – 22.73±1.43 % (256 % increase from control group, p<0.0000001); Day 21: decrease to 17.70±1.29 % (177 % increase from control, but 22 % decrease from peak value group, p<0.0000001).

In the renal medullary layer, a clear tendency toward an increase in the level of fragmented DNA is observed during the first 14 days of the experiment. The maximum level of DNA fragmentation is achieved on the 14th day, which may indicate a peak in DNA damage in the cells of the renal medulla. After the 14th day, a decrease in the level of fragmented DNA is observed, which may indicate the activation of adaptation or DNA reparation mechanisms. Despite the decrease by the 21st day, the level of fragmented DNA remains significantly higher than the control values.

Analysis of the ROC curve and the Kaplan–Meier survival curves in the rat`s renal after restraint stress is shown in Fig. 3.

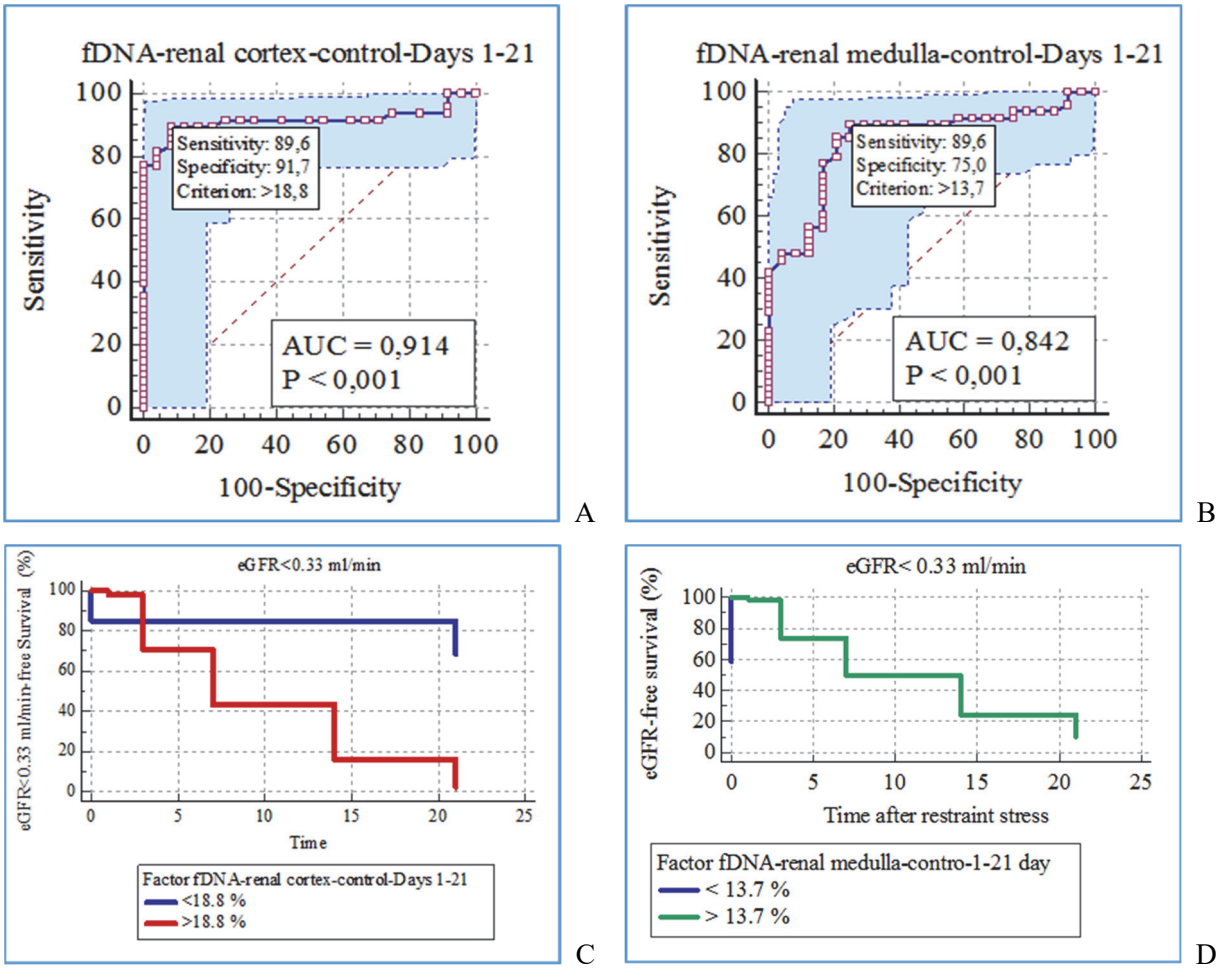

Fig. 3. ROC analysis: receiver operating characteristic (ROC) curves for fragmented DNA: A – renal cortex; B – renal medulla; and Kaplan–Meier curves C – renal cortex; D – renal medulla; of the time of the development of eGFR in rats <0.33±0.03 ml/min measured in the rats at different times after immobilization stress.

According to the receiver operating characteristic curve, the area under ROC curve (AUC=0.914; optimal cut-off values of fDNA – >18.8 %; p<0.001 in the renal cortex; AUC=0.842; optimal cut-off values of fDNA – >13.7 %; p<0.001 in the renal medulla) values of fDNA to predict of the development of eGFR in rats <0.33±0.03 ml/min measured in the rats at different times after immobilization stress..

The Kaplan–Meier survival curves, after classifying the rats on the basis of Youden cut-offs obtained by ROC curves, showed the time to development of eGFR in rats <0.33±0.03 ml/min measured in the rats at different times after immobilization stress. on average by Day 9.9 (95 % CI for the mean 7,9 to 11,8).

Next, we performed a Cox proportional hazards regression analyses of predictors for the development of development of eGFR in rats <0.33±0.03 ml/min measured in the rats at different times after immobilization stress. In univariate analysis, fDNA was significantly associated with the development development of eGFR in rats <0.33±0.03 ml/min measured in the rats at different times after immobilization stress in all observation groups. The Cox model shows that the level of fDNA in the renal cortex is a significant predictor of the development of eGFR in rats <0.33±0.03 ml/min (p<0.0001), since Exp(b) (risk coefficient)=2.4; while the Harrell index C=0.755, as a value close to 1, which indicates the high efficiency of the Cox model. The level of fDNA in the renal medulla can be used to judge the time of development of acute renal failure, (p=0.0054), since Exp(b) (risk coefficient) = 0.87 is less than 1; while the Harrell index C=0.678, which indicates the adequacy of the Cox model.

Our data show a peak in ulcer formation in the gastric mucosa after immobilization stress on Day 7, which is consistent with the studies of Liu L.et al. (2019), who showed similar dynamics with a peak on Day 7–8 [6]. However, in some studies, for example, Szabó IL. (2017), the peak of ulceration was observed earlier – on Days 3–4 [12]. According to DNA fragmentation data, our data demonstrate a progressive increase in DNA fragmentation with a peak on Day 14. These data differ from some published studies: Wang J. et al. (2020) observed a peak in DNA fragmentation on day 7 [13].

The discrepancy between the peaks of ulcer formation (Day 7) and DNA fragmentation (Day 14) may indicate different mechanisms of these processes. We observed a longer persistence of increased DNA fragmentation compared to the ulcer index and incomplete recovery of both indices by Day 21.

Our results are generally consistent with the literature on the effects of experimental restraint stress on the kidneys. The study by Wei Q. (2018) showed that restraint stress induced a significant increase in apoptosis in rat kidneys, which is consistent with our observations of elevated levels of fragmented DNA [14]. The work of Cerda-Flores RM. et al. (2018) examined the effects of acute and chronic stress on oxidative DNA damage in various tissues including the kidney [1]. They observed a significant increase in DNA damage in the kidneys after acute (2 hours) and chronic (5 days) restraint stress. This finding is consistent with our results showing a rapid increase in DNA fragmentation already on the first day of the experiment. Our data show different dynamics in the cortex and medulla. This is in line with the study by Zhang J. (2019), who also observed differences in the stress response between these two regions of the kidney [15]. The rapid increase in fDNA levels in the first days is consistent with the results of Choi HM. (2020), who found a rapid increase in apoptosis markers within the first 48 hours after acute stress [3]. The decrease in fDNA levels in the cortex on day 7 that we observed on day 7 is consistent with the study by Liu Y. (2017), who also noted an adaptation phase after the initial injury [7]. The secondary increase in fDNA levels observed in our study is less well documented in the literature. However, the study by Ratliff BB. (2016) suggests the possibility of secondary damage with prolonged stress [9]. Our observation of persistent elevated fDNA levels even after Day 21 is consistent with the study by Gu L. (2015) that noted long-term effects of chronic stress on the kidney [4].

Our results are in good agreement with the existing literature on the effects of stress on DNA damage in the kidney. They confirm that immobilization stress causes significant and long-lasting DNA damage in the renal medulla. However, our study provides a more detailed time course of these changes, which may be a valuable contribution to understanding the development of stress-induced renal injury.

Conclusions

1. According to the level of fragmented DNA, which indicates the level of apoptosis in the tissues of the gastric mucosa at different times after immobilization stress, the phases of the pathological process can be distinguished.

2. The dynamics of changes in apoptosis activity by the level of fragmented DNA in the tissues of the renal cortex after immobilization stress had a two-phase nature.

3. Experimental immobilization stress has a significant effect on DNA fragmentation levels in the renal medulla. The effects of stress increase during the first two weeks, after which adaptation or recovery processes begin. Even after 21 days, DNA damage levels remain significantly higher than normal, indicating long-term effects of stress on kidney cells.

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