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ORIGINAL ARTICLE

Effect of Oligopeptides-Homologues of the Fragment of $ACTH_{15-18}$ on Morphogenetic Markers of Stress in the Adrenal Glands on the Model of Acute Cold Injury in Rats

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ABSTRACT

Objectives: Aim of the current study was to evaluate the stress-protective effect of oligopeptides-homologues of the adrenocorticotropic hormone (ACTH) fragment 15-18 on morphogenetic signs of stress reaction of the adrenal glands under acute cold exposure (CE) in rats.

Materials and Methods: The acute cold stress was reproduced by placing random-bred male rats in a freezer at a temperature of -18°C for 2 hours. The peptides-homologous of $ACTH_{15-18}$ acetyl-(D-Lys)-Lys-Arg-Arg-amide (KK-1) and acetyl-(D-Lys)-Lys-(D-Arg)-Arg-amide (KK-5) and the reference medicine (Sema) were administered intranasally in a dose of 20 mg/kg 30 minutes before and after CE. Rectal temperature was measured before and 10 min after CE. *Zona glomeruloza*, *zona fasciculata*, *zona reticularis*, and the area of cells and nuclei of adrenocorticocytes of the *zona fasciculata* were measured.

Results: KK-1 significantly prevented structural changes in the adrenal cortex and medulla and stabilized the secretory activity of glucocorticoid-producing cells. However, the congestion of the capillaries of the *zona fasciculata* and *zona reticularis* remained in some locations. *Zona fasciculata* cells had a marked tendency to decrease, and the area of nuclei significantly decreased ($p<0.05$) recovering the width to control animals' markers. KK-5 had a more marked recovery of the adrenal glands (a greater saturation of cytoplasm of adrenocorticocytes of *zona glomerulosa* and *zona fasciculata*). The number of chromaffin cells at rest was increased in the *adrenal medulla*. KK-5 statistically significantly normalized both the area of cells ($p<0.05$) and the area of nuclei ($p<0.05$) of the *zona fasciculata*, unlike KK-1, which reliably restored only the marker of the nuclei area. Some morphometric parameters of acute stress hypertrophy remained in the adrenal glands of rats receiving Sema.

Conclusion: KK-1 and KK-5 prevented the manifestation of acute stress reactions in the adrenal cortex of rats. KK-5 had a more marked stress-protective effect compared with the peptide KK-1. Both study substances exceeded the reference medicine Sema. KK-5 is a promising stres-sprotector and frigoprotector.

Key words: Stres sprotectors, acute cold stress, cold trauma, adrenal gland morphology, neuropeptides, oligopeptides-homologues of the fragment of $ACTH_{15-18}$

INTRODUCTION

Stress underlies the pathogenesis of many diseases of the cardiovascular, immune, central nervous, and other systems.[1] Prolonged stress exposure reduces the adaptive capabilities of the body and leads to the development of adaptation disease.[2] One of the leading links in the harmful effects of stress is the disruption of the peptidergic system,[3,4] which makes it relevant to search for stress detectors among these substances. The range of peptidergic drugs having stress-protective activity is narrow. It is known that stress activates the release of adrenocorticotropic hormone (ACTH), which led to glucocorticoid hormone release by adrenal glands. Taking into account the leading role of the hypothalamus-pituitary-adrenal axis in regulating the body's response to stress,[5,6] it seems promising to search for stress detectors among oligopeptides-

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homologues of the fragment of ACTH. Peptides-homologues of the fragment of $ACTH_{15-18}$ (Lys-Lys-Arg-Arg) were obtained at the Institute of Highly Pure Biopreparations (St. Petersburg, Russia). In previous studies, we have established the stress-protective effect of peptides on models of acute immobilization[7,8] and acute cold stress on the effect on behavioral reactions, anxiety, and physical endurance of animals,[9] markers of carbohydrate metabolism,[10] blood systems.[11] The issue of the morphogenesis changes in the adrenal response to acute cold stress and the influence of frigoprotectors on these processes has been insufficiently studied, but such data are important for optimizing the frigoprotective effect. Taking into account that adrenal glands have one of the main roles in the body-response to stress, purpose of the current study is to evaluate the stress-protective effect of oligopeptides-homologues of the ACTH fragment 15-18 on morphogenetic signs of stress reaction of adrenal glands under acute cold exposure (CE) in rats.

MATERIALS AND METHODS

Experimental animals and ethical clearance

30 adult (3 months old) random-bred male rats (249 ± 5 g) were taken from the vivarium of the National University of Pharmacy (Kharkiv, Ukraine). The animals were housed in standard polypropylene cages at 22-24°C and 50% humidity in the well-ventilated room with a 12 hour light/dark cycle and free access to food and water. The work was carried out in the Educational and Scientific Institute of Applied pharmacy of the National University of Pharmacy in compliance with Directive 2010/63/EU of the European Parliament and the Council "On the protection of animals used for scientific purposes" (Brussels, 2010). All experimental protocols were approved by the Bioethics Commission of the National University of Pharmacy (no: 6, 8 June, 2021).

Drugs and chemicals

Peptide homologs of the fragment of $ACTH_{15-18}$ (Lys-Lys-Arg-Arg) under the laboratory codes KK-1 [acetyl-(D-Lys)-Lys-Arg-Arg-amide] and KK-5 [Acetyl-(D-Lys)-Lys-(D-Arg)-Arg-amide] were synthesized at the Institute of Highly Pure Biopreparations by A. A. Kolobov. The peptides were obtained by solid-phase synthesis methods using Boc-technology and purified by preparative reverse-phase chromatograph; their purity was at least 98%. In these compounds, one (KK-1) or two (KK-5) natural amino acids are replaced by the corresponding D-stereomer. Peptides do not relieve any hormonal activity and due to the presence of D-amino acids have increased resistance to human serum proteases, are practically non-toxic substances.[12]

Study design

The model of acute cold stress was reproduced by placing animals in a freezer "NordInter-300" at a temperature of -18° C for 2 hours in individual plastic pencil cases with a volume of 5 dm^3 without limiting airflow and motor activity.[13,14] This experimental model was chosen according to the aim study and the results of a previous investigation that showed a positive influence of peptide homologs of the fragment of $ACTH_{15-18}$ on behavioral reactions, anxiety, and physical endurance of animals,[9] markers of carbohydrate metabolism,[10] and blood systems.[11] The peptides-homologous of the fragment of $ACTH_{15-18}$ were administered intranasally (*i/n*) in the form of a solution in an effective stress-protective dose of 20 mcg/kg 30 min before and after CE. This dosage was chosen to take into account the cerebroptotective and stressprotective properties relieved in previous investigations.[7-11] The reference medicine, Sema (peptogen, RF), was administered *i/n* at a dose of 20 mcg/kg in a similar treatment. Heptapeptide semax (Met-Glu-His-Phe-Pro-Gly-Pro) is a synthetic analog of ACTH fragment 4-7 without hormonal activity. It is used to increase the adaptive capacity of the body under stress.[15]

The animals were divided into 5 groups (6 rats in each group). Group 1: control; group 2: CE (rats were exposed to acute cold stress); groups 3-5 - animals with CE who received experimental therapy: group 3: CE + KK-1, group 4: CE + KK-5, group 5: CE + Sema. Rectal temperature was measured with a WSD-10 thermometer before and 10 min after CE.

Adrenal gland histopathology

Animals were removed from the experiment 2 h after cold stress exposure by decapitation under thiopental anesthesia (40 mg/kg). Adrenal glands of the animals were fixed in a 10% formalin solution, dehydrated in alcohols of increasing concentration, and poured into paraffin. The sections were stained with hematoxylin and eosin.[16]

The photographs were processed on a Pentium 2.4 GHz computer using the Toup View program. In the photographs, using the Toupcam Granum program, the width of zones of the adrenal gland cortex (microns, μm) was measured: the *zona glomeruloza* (WZG), the *zona fasciculata* (WZF), the *zona reticularis* (WZR); the area of cells and nuclei (microns, μm^2) of adrenocorticocytes of *zona fasciculata* (AcZF, AnZF).

Statistical analysis

Statistical processing was carried out by methods of variational statistics using a standard package of statistical programs "Statistica, V. 6.0".[17-19] The results are expressed as mean ± standard error of mean (the level of statistical significance was considered $p<0.05$) or Me (LQ;UQ). Statistical differences between groups were analyzed using the parametric Student's *t*-test in cases of normal distribution and non-parametric Mann-Whitney *U* test and Kruskal-Wallis test in cases of its absence.

RESULTS

After the 24 h acute CE in rats of the group with CE, the rectal temperature significantly decreased by an average of 3°C ($p<0.05$), or 8% relative to the initial level (Table 1). All three peptides decreased the severity of hypothermia compared to the CE group: body temperature decreased by 1.0°C, or 2.7% (KK-1); by 0.3°C, or 0.8% (KK-5), and 0.4°C, or 1.1% (Semax). This decrease was statistically significant for all groups, respectively ($p<0.05$). The temperature of rats treated with the oligopeptides being studied did not differ from that of intact animals.

The histostructure of the adrenal cortex and medulla of the rats of the intact control group fully corresponds to the status of a physiologically functioning organ (Figure 1).

According to morphometry, functional state of adrenal glands of control rats is characterized by the following indicators: WZG was 44.64 microns, WZF-174.1 microns, WZR-78.4 microns; AcZF-168.8 microns 2, and AnZF-34.1 microns (Table 2).

In the majority of rats in the CE group, focal disorientation of connective tissue fibers in the capsule, their plasma impregnation, and tearing were observed (Figure 2A, B).

In *zona fasciculata*, linearity of corticocyte strands is broken in places, there is a decrease in their vacuolization, appearance of areas of complete cell defatting, which sometimes extends to the outer zone up to *zona glomerulosa*. Some nuclei had not a rounded, but a more elongated, "lobed" shape. The boundary between *zona glomerulosa* and *zona fasciculata* is not always clear, while the capillary net is expanded and congested (Figure 2C-E). The cells of *zona reticularis* have not been visually changed. The expansion and congestion of the venous-capillary net of *zona reticularis* were observed (Figure 3F). In the *adrenal medulla*, number of the cells with basophilic cytoplasm increased markedly with a decrease in the number or absence of vacuoles (Figure 2G).

According to morphometric measurements, WZF in the rats of CE group had significantly increased. There was a clear tendency toward an increase in AcZF and a significant increase

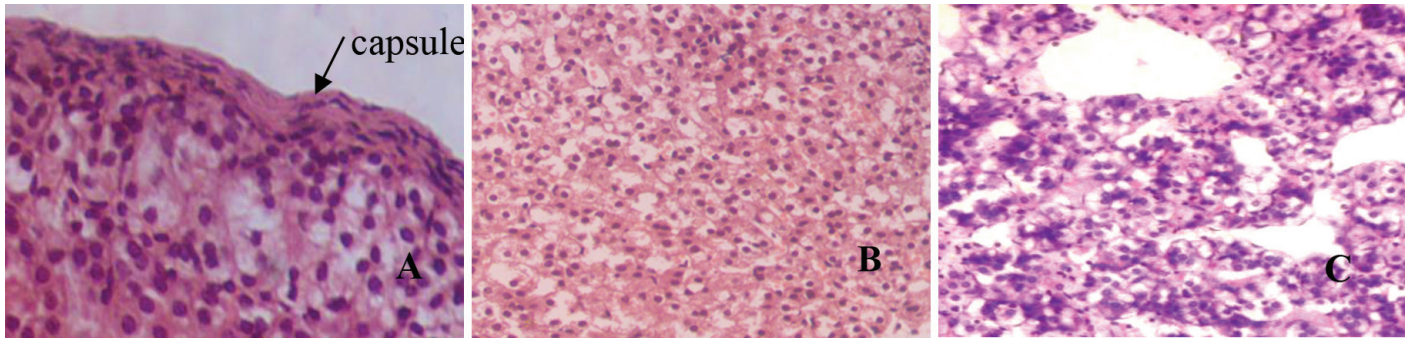

Figure 1. The adrenal gland of a control rat. (A) Capsule and cells of *zona glomeruloza* (arrow), hematoxylin-eosin, x400; (B) *zona fasciculata*, hematoxylin-eosin, x400; (C) chromaffin cells of the *adrenal medulla*, hematoxylin-eosin, x250

Table 1. The effect of the peptides KK-1, KK-5, and Sema on the body temperature of rats before and after a 24 h cold exposure at -18°C

Observation period	Group of animals, body temperature (°C)				
	Control	CE	CE + KK-1	CE + KK-5	CE + Sema
Before CE	36.8 ± 0.21	37.4 ± 0.38	37.5 ± 0.5	36.6 ± 0.35	37.1 ± 0.45
10 minutes after CE	-	34.4 ± 0.21*	36.5 ± 0.4#	36.3 ± 0.4#	36.7 ± 0.57#

*p<0.05 compared with intact, #p<0.05 compared with the CE group (Student's criterion). CE: Cold exposure

Table 2. The effect of acute cold stress on the morphometric markers of the adrenal glands of rats (Me (LQ; UQ)

Group of animals	Markers				
	WZG, μm	WZF, μm	WZR, μm	AcZF, μm^2	AnZF, μm^2
Control	44.64 (38.03; 45.15)	174.1 (163.6; 181.3)	78.4 (72.4; 86.9)	168.8 (159.7; 187.6)	34.1 (32.9; 35.1)
Cold exposure	43.90 (43.09; 45.10)	187.5 (179.1; 191.6)*	86.2 (74.3; 88.4)	186.4 (175.3; 199.1)	39.0 (38.8; 39.4)*
Cold exposure + KK-1	41.56 (33.34; 50.63)	179.8 (169.3; 190.1)	75.7 (61.3; 88.4)	177.6 (156.3; 191.6)	33.3 (30.3; 38.8)^
Cold exposure + KK-5	41.08 (39.85; 42.28)	173.2 (166.7; 182.8)^	77.4 (75.4; 80.3)	157.0 (149.1; 161.1)^	32.9 (32.4; 36.3)^
Cold exposure + Sema	40.47 (39.07; 47.84)	181.7 (180.3; 186.8)	81.6 (68.1; 85.5)	205.2 (188.2; 219.0)*#	36.1 (34.9; 39.1)*#
p	0.9235	0.2468	0.8303	0.0075	0.0011

p: The level of statistical significance when comparing samples (Kruskal-Wallis criterion), *p<0.05 compared with the control group (Mann-Whitney U criterion); ^p<0.05 compared with the cold exposure group (Mann-Whitney U criterion); #p<0.05 compared with the group of animals treated with KK-5 peptide (Mann-Whitney U criterion)

in AnZF, whereas WZG and WZR did not change in comparison with the markers of control (Table 2).

Changes in the connective tissue fibers of the adrenal capsule of rats that received KK-1 peptide under acute general hypothermia was expressed insignificantly. There is only a weakly pronounced focal tearing of the capsule. The cells of *zona glomerulosa* retain their location and their cytoplasm contains a different number of vacuoles. Cytoplasm vacuolization was increased in corticocytes of *zona fasciculata* and there were no marks of defatting (Figure 3A). The congestion of the capillaries of *zona fasciculata* and *zona reticularis* is preserved in some locations. The number of cells with basophilic cytoplasm and the presence of vacuoles in the medulla is increased (Figure 3B).

Results of the morphometric analysis confirmed positive effect of KK-1 peptide on prevention of adrenal gland hypertrophy under acute cold stress. Thus, AcZF had a marked tendency to decrease and AnZF significantly decreased compared with rats of the CE group. All this contributed to the recovery of WZF to the markers of control animals (Table 2).

Peptide KK-5 had a more marked positive effect on the histostructure of the adrenal glands. This was expressed in greater and evenly saturation of the cytoplasm of adrenocorticocytes both of *zona glomerulosa* and *zona fasciculata* (Figure 4A). More chromaffin cells of the medulla were in a state of functional rest (Figure 4B).

Morphometric markers of the adrenal cortex confirmed the marked positive effect of KK-5 peptide on the adrenal glands under acute stress hypertrophy. WZF, AcZF, and AnZF were significantly reduced in comparison with the markers of the CE group and practically reached the level of control (Table 2). Normalization of AcZF and AnZF significantly exceeded such markers of the reference drug.

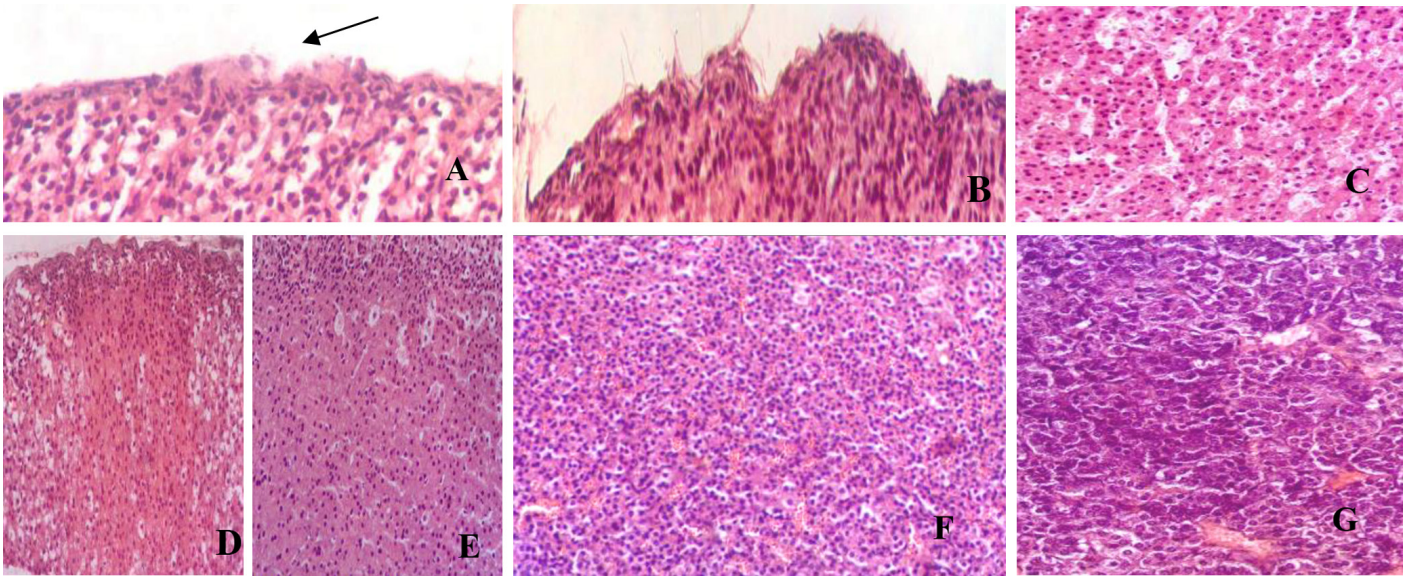

Figure 2. The adrenal gland of the animals of the cold exposure group (acute cold stress): (A) the disorientation of fibers, plasmatic impregnation in capsule (arrow); (B) disruption of arcade-like arrangement of corticocytes, the absence of vacuolization of the cytoplasm of cells; hematoxylin-eosin, x250; (C) reduction of vacuolization of the cytoplasm of corticocytes of *zona fasciculata*, expansion and congestion of the capillaries; hematoxylin-eosin, x250; (D, E) different size plots of complete defatting of corticocytes; hematoxylin-eosin, x250; (F) expansion and congestion of the venous-capillary net of *zona reticularis*; hematoxylin-eosin, x200; (G) an increase in endocrine cells with basophilic cytoplasm of the medulla, decrease or absence of vacuolization of the cytoplasm; hematoxylin-eosin, ×250

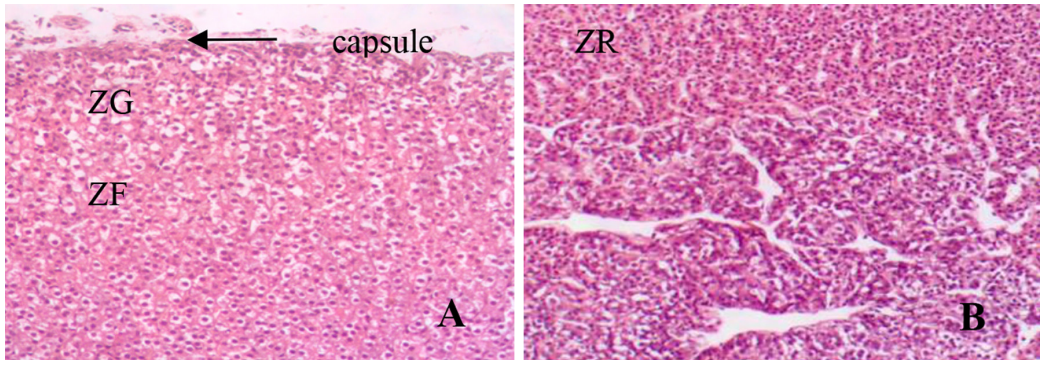

Figure 3. The adrenal gland of a rat received KK-1 peptide under acute cold stress. (A) Recovering of the functional state of *zona glomerulosa* (ZG) and *zona fasciculata* (ZF); hematoxylin-eosin, x200; (B) the normal state of cells and capillary net of *zona reticularis* (ZR), an increase in chromaffin cells of the *adrenal medulla*, which is relevant to the state of rest; hematoxylin-eosin, x200

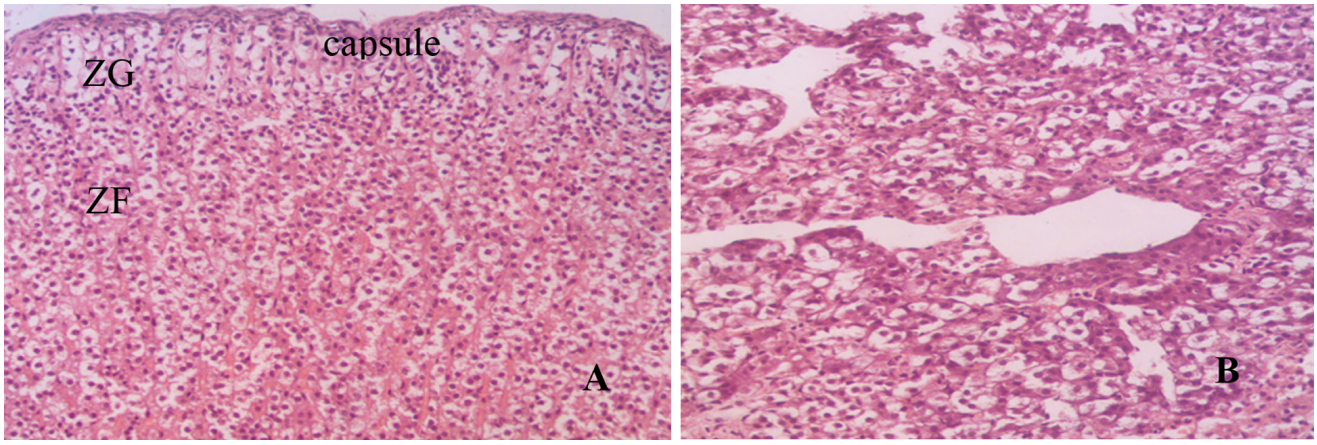

Figure 4. The adrenal gland of the rat received the peptide KK-5 under acute general hypothermia. (A) Complete recovering of the structural and functional state of *zona glomerulosa* (ZG) and *zona fasciculata* (ZF); hematoxylin-eosin, x200; (B) a significant number of chromaffin cells of the *adrenal medulla* in a state of functional rest; hematoxylin-eosin, x250

Small focal segments with disorientation, plasma impregnation, and fibrillation of connective tissue fibers are observed in the adrenal capsule of some rats having received Sema. The structure of *zona glomerulosa* is mostly restored, although there were small areas with indistinctness of arcade-like cell formation. There was not always clear linearity of the location of corticocytes in *zona fasciculata*; there were still enough cells with a decrease/absence of cytoplasm vacuolization. Foci of cell defatting with different severity were also observed in the middle and outer sections of *zona fasciculata* (Figure 5A, B).

In some locations, extension and congestion of capillaries are observed both in *zona fasciculata* and *zona reticularis*. The state of chromaffin cells of the medulla varied in different animals from the condition of rest to functional tension (Figure 5C, D). Morphometric parameters of the adrenal glands of rats after administration of Sema, the reference medicine, indicated that some markers of acute stress hypertrophy remained (Table 2). Thus, AcZF and AnZF had no statistically significant differences compared with animals in the CE group.

DISCUSSION

Stress is essential in the body's adaptation to adverse environmental factors, which, with prolonged exposure, turns into a pathogenic process.[2,20] One of the most common stressful factors is cold stress, which negatively affects health and productivity.[21] Due to the complexity of the pathogenesis of cold injury associated with cardiovascular, endocrine, central nervous, respiratory, immune, and other systems, the treatment is a challenging problem.[1] Considering that stress leads to disruption of the functioning of the peptidergic system,[4] it is advisable to study the stress-protective properties of neuropeptides. Since hypothalamus-pituitary-adrenal system participates in the mechanism of stress,[22] neuropeptides-homologues of ACTH deserve special attention in this aspect. Among such medicines, synthetic $ACTH_{1-24}$ Tetracosactide (Synacthen Depot®) is known, which is used for treating disseminated sclerosis, and a number of allergic diseases;[23] Sema is a synthetic analog of ACTH 4-10 with a nootropic and

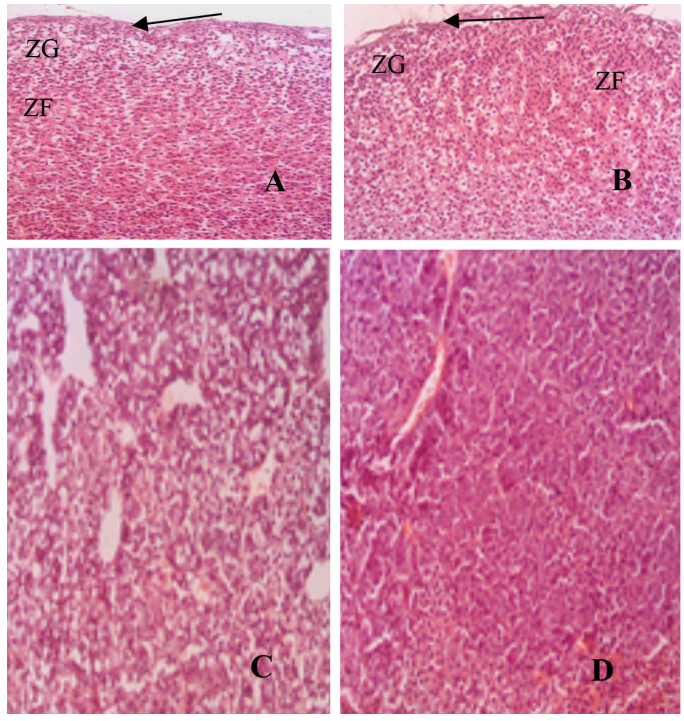

Figure 5. The adrenal gland of a rat that received Sema under acute cold stress. (A) A small area of disorientation of fibers in the capsule, incomplete recovering of the structure of *zona glomerulosa* (ZG), lack of linearity of the location of corticocytes of *zona fasciculata*, a moderate increase in vacuolization of the cytoplasm of cells of *zona fasciculata* (ZF); hematoxylin-eosin, x200. (B) Tearing of the capsule fibers, a site of a violation of the structure of *zona glomerulosa* (ZG), a site of complete defatting of the middle and outer sections of *zona fasciculata* (ZF); hematoxylin-eosin, x200. (C, D) Different functional states of chromaffin cells of the *adrenal medulla*: from a condition of rest (C) to tension (D). Hematoxylin-eosin x200

neuroprotective activity.[24,25] Stress-protective properties of these peptides are being studied currently.[26-29]

In our previous studies on the model of acute immobilization stress in rats, stress-protective properties of oligopeptides homologous to the primary amino acid sequence of $ACTH_{15-18}$ were revealed.[7,8] Under acute cold stress, a positive effect

of peptides on behavioral reactions, physical endurance, and carbohydrate metabolism was shown.[9,10] In the current study, using a model of acute cold injury, we confirmed the frigoprotective properties of these peptides to reduce hypothermia and found their marked protective effect on the histostructure of adrenal glands of rats under cold stress.

Exposure to acute cold stress caused marked morphological changes in all areas of the adrenal glands of rats that did not receive experimental therapy. Thus, there was a disorientation of connective tissue fibers in the capsule, a violation of the arcade-like arrangement of cells in the *zona glomerulosa* was observed, in *zona fasciculata* part of the nuclei had not a rounded, but an elongated shape. All these morphological changes indicate the functional tension of the adrenal glands. It is believed that this is a compensatory mechanism that intensifies metabolic processes by increasing the surface of the junction of the nuclear and cytoplasmic parts.[30]

Hypertrophy of the adrenal cortex was characterized by an increase in the area of adrenocorticocytes of *zona fasciculata* of adrenal cortex and their nuclei, which led to the expansion of this zone. An increase in the zone of adrenocorticocytes is also typically for models of chronic immobilization stress[31,32] and chronic variable/unpredictable stress in rats.[33] However, the level of corticosterone did not change in these stress models. Under acute cold stress in our study, an increase in cell size is accompanied by defatting the adrenal cortex, which indicates functional stress associated with stimulation of glucocorticoid hormone production. An increase in corticosterone production under stress is typically for both acute and chronic heat stress in rats.[34,35] Activation of the adrenal cortex under acute cold stress is accompanied by changes in its vascular system; expansion and congestion of capillaries, which coincides with the data of other scientists.[36]

KK-1 peptide significantly prevented structural changes in the adrenal cortex and medulla under acute cold stress, stabilized the secretory activity of glucocorticoid-producing cells, and contributed to the maintaining of lipid saturation. However, the congestion of capillaries of *zona fasciculata* and *zona reticularis* remained in some locations.

Much more complete recovery of the adrenal glands of rats receiving KK-5 peptide was observed, which was reflected in greater and uniform saturation of the cytoplasm of adrenocorticocytes of *zona glomerulosa* and *zona fasciculata*. The number of chromaffin cells in the state of rest was increased in *adrenal medulla*. The peptide KK-5 statistically significantly normalized both AcZF and AnZF, unlike KK-1 peptide, which reliably restored only the marker of AnZF.

The results of our study demonstrate a marked frigo- and stress-protective effects of peptides, which might be connected with a reduction in the effect of ACTH on the synthesis of glucocorticoids in *zona fasciculata* and release of epinephrine in *adrenal medulla*. It is known that ACTH belongs to the melanocortin family.[37,38] The ACTH receptor, known as MC2R, is located in *zona fasciculata* and *zona reticularis* of the adrenal cortex[22,39] and selectively binds only to ACTH.[40] Stimulation of MC2R promotes the production of glucocorticoids and mineralocorticoids by adrenal cortex under stress.[41] The sequence of amino acids of ACTH at positions 15-19 is responsible for its binding to MC2R.[42] In addition, it is supposed that ACTH participates in the regulation of its own secretion through negative feedback *via* MC2R mRNA detected in the pituitary gland.[43] We assume that stress-protective effect of the peptides-homologues being studied of the fragment of $ACTH_{15-18}$ is realized due to affinity to MC2R receptors, which leads to the prevention of the effect of ACTH on the development of histostructure changes in both the cortex and the *adrenal medulla* under stress.

The reduction range of morphogenetic marks of a stress reaction in the adrenal glands does not directly depend on the severity of the antihypothermic effect of the peptides under study. Thus, despite almost the same indicators of body temperature in the groups of animals receiving the peptide KK-5 and Sema under CE, morphological changes in the adrenal glands are almost absent in animals treated with the peptide KK-5, but moderately expressed in rats treated with Sema.

Thus, both peptides under study, especially KK-5, reduce morphogenetic signs of a stress reaction in the adrenal glands of rats under acute cold injury, surpassing Sema, the reference medicine. KK-5 peptide can be considered as the most promising frigoprotector.

Study limitations

The absence of discussion of hormonal status is the limitation of our study.

CONCLUSION

1. On the model of acute cold injury in rats, peptides-homologues of the fragment of $ACTH_{15-18}$ [acetyl-(D-Lys)-Lys-Arg-Arg-amide and acetyl-(D-Lys)-Lys-(D-Arg)-Arg-amide] and Sema, the reference medicine (Met-Glu-His-Phe-Pro-Gly-Pro), prevent hypothermia.

2. Acute cold exposure causes acute stress hypertrophy of the adrenal cortex in rats, which is characterized by an increase in the area of adrenocorticocytes of *zona fasciculata* and *zona reticularis* of the cortex. These changes are accompanied by defatting the adrenal cortex. This indicates functional tension associated with stimulation of glucocorticoid hormone production.

3. Peptides-homologues of the ACTH fragment 15-18 [acetyl-(D-Lys)-Lys-Arg-Arg-amide and acetyl-(D-Lys)-Lys-(D-Arg)-Arg-amide] prevent the manifestation of acute stress reactions in adrenal cortex of rats, which is proven by the absence of structural changes in the cortex. Also, the peptides stabilized the secretory activity of the glucocorticoid-producing cells and contributed to the maintaining of their lipid saturation. Thus, preserving the reserve capabilities of secretory cells, acetyl-(D-Lys)-Lys-Arg-Arg-amide and acetyl-(D-Lys)-Lys-(D-Arg)-Arg-amide peptides increase the resistance of adrenal cortex to the action of a cold factor having a stress-protective effect.

4. According to severity of the stress-protective effect, acetyl-(D-Lys)-Lys-(D-Arg)-Arg-amide has a more marked stress-protective effect compared to acetyl-(D-Lys)-Lys-Arg-Arg-amide, and both study substances exceed Sema, the reference medicine.

Ethics

Ethics Committee Approval: The work was carried out in the Educational and Scientific Institute of Applied Pharmacy of the National University of Pharmacy in compliance with Directive 2010/63/EU of the European Parliament and the Council "On the protection of animals used for scientific purposes" (Brussels, 2010). All experimental protocols were approved by the Bioethics Commission of the National University of Pharmacy (no: 6, 8 June, 2021).

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: O.K., Y.L., Concept: O.K., Y.L., Design: S.S., Data Collection or Processing: O.K., Y.L., Analysis or Interpretation: S.S., O.K., Y.L., Literature Search: O.K., Y.L., Writing: O.K., S.S., Y.L.

Conflict of Interest: No conflict of interest was declared by the authors.

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