Peculiarities of microstructure of the suprarenal glands of rabbits with different types of autonomic tone

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Introduction

The suprarenal (adrenal) glands near the thyroid and parathyroid glands are peripheral endocrine organs (Zhedevov, 1987). During embryogenesis, the adrenal glands form from the epithelium tissue (cortex of the suprarenal glands) and neuroendocrine chromaffin cells of the nervous (medulla) (Huber et al., 2002; Huber et al., 2009). Chromaffin cells together with sympathetic neurons develop from the same predecessors – sympathoadrenal cells (Huber, 2006; Unsicker et al., 2013). Therefore, already in that period, a close relationship between the suprarenal glands and nervous system is established, especially its sympathetic section.

The histological structure of the adrenal gland has been studied many times in different classes of animals taking into account their biological peculiarities and habitat (living conditions). Some species of amphibians have cells located chaotically and therefore the gland is not biological peculiarities and habitat (living conditions). Some species of amphibians have cells located chaotically and therefore the gland is not distinguishable. However, the structure of the adrenal gland, subcapsular layer, central and peripheral zones are distinguished (Humayun et al., 2012; Ye et al., 2018). The glands of ruminants were studied in the age and sex aspects (Jelinek & Konecny, 2011; Fidatou, 2015), and also with respect to breed (Paul et al., 2016) or species peculiarities (Barsocz et al., 2016). The mentioned factors also influence the structure of the suprarenal glands of numerous species of rodents (Sheikhian et al., 2014; Olukole et al., 2016; Santos et al., 2016; Fincu & Hammer, 2018). Significant species peculiarities were determined in the structure of the suprarenal glands of cetaceans (Vukovic et al., 2010) and primates (Tachibana et al., 2015; Raharison et al., 2017). Among the studied animals, surveys frequently detected rabbits in which the structure of the suprarenal glands was studied from the perspective of embryogenesis (Sokolov, 1969; Hussein et al., 2015), blood circulation (Kigata & Shibata, 2018), physiological and pathological conditions (Vinso et al., 1985; Baine et al., 2014) and impact of chemical substances (Chandra, 1975; McCreedy & Harmon, 1992). Special attention in morphological studies is paid to the cellular context of the suprarenal glands. Their cells are found to have different structure and function, depending on the zone to which they belong (Piliakoji et al., 2015; Vinso, 2016). Each zone produces hormones responsible for a broad spectrum of functional processes in the animal organism, including secondary sex features, blood pressure, mineral and carbohydrate metabolisms, and also stress (Dumbell et al., 2016; Gallope et al., 2017; Nicolaides et al., 2017; Sunwoo et al., 2019). Innervation of the suprarenal glands from the sympathetic section of the autonomic nervous system is performed through the nerves of the vertebral column, and also abdominal, mesentric and renal pleureses. Para-

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sympathicotonic innervation is provided by the vagus nerve (Ramey & Goldstein, 1957; Parker et al., 1993).

The different tones of the sympathicotonic and parasympathicotonic centers condition formation of separate types of autonomic regulation in the animal organism, which affect its morphology. Similar effects on the structure of additional suprarenal glands have been observed in rabbits (Zakrevska & Tybinka, 2019). However, none of the previous studies have found a relationship between the morphology of the “main” suprarenal glands and typological peculiarities of the tone of the autonomic centers. Therefore this became the objective of our study.

Materials and methods

For the study, according to the principle of analogues, 27 four month old male rabbits (Oryctolagus cuniculus) of breed Blanc de Termonde were selected. The animals underwent electrocardiographic and variation-pulsometric analyses, which allowed determination of their individual types of the autonomic tone (Baevskij et al., 1984). The speed of ECG during recording of the cardiosignal was 250 mm/s. According to the results of these studies, the rabbits were divided into three groups: sympathicotonic (ST) – 19 rabbits, normotonic (NT) – 5 rabbits and parasympathicotonic (PS) – 3 rabbits. All the animals were weighed with accuracy of up to two grams. For selection of histological material, the rabbits were euthanized by inhalational overdosing with chloroform. Then, pieces of the organs were washed and embedded in gelatin. On a freezing microtome MZ-2, 20 µm thick sections were made, installed on the microslide and embedded in polyvinyl alcohol. The prepared preparations were examined under the microscope with lenses of series HCX PL FLUOTAR with the zooms of 5x/0.15, 10x/0.3, 20x/0.5, 40x/0.75 and 100x/1.3. Digital images of preparations were obtained using a camera with the resolution of 5 megapixels. To perform morphometric surveys we used Aperio Image Scope software (Leica Microsystems GmbH, Germany, 2012). The morphometric method revealed the following parameters: thickness of the capsule of the suprarenal gland, thickness and area of separate zones (glomerulosa, fasciculata, reticularis, medulla), area of the cells and their nuclei in these zones, and also the number of cells per unit area (1,000 µm²). For all the zones we determined nuclear-cytoplasmic ratio of the cells. In the cortex zone we also determined the area of accumulation of corticosteroids, and in the medulla – of catecholamines (adrenaline, noradrenaline). This study was undertaken by determining optical density of these areas using WCIF Image program (WCIF, Canada, 2000).

The results were statistically analyzed in ANOVA with the use of StatPlus program (AnalystSoft Inc., USA, 2008). At the same time, we determined: x – selective average and SE – standard error. The differences between the groups of animals were considered significant at P < 0.05 taking into account Bonferroni correction.

Results

The results of morphometric studies indicate presence of relationship between the structure of the suprarenal glands and typological peculiarities of the autonomic tone. The thickest connective tissue capsule of the suprarenal gland was determined in the representatives of the sympathicotonic group (Table 1). In the animals of two other groups its thickness was less by 41.2 µm in NT rabbits and 33.8 µm in PS rabbits. These differences were caused by different amounts of fat inclusions and massive vessels in the capsule (Fig. 2, 3).

| Parameter                                      | sympathicotonic, n = 19 | normotonic, n = 5 | parasympathicotonic, n = 3 |
|------------------------------------------------|-------------------------|------------------|---------------------------|
| Thickness of the capsule, µm                   | 88.1 ± 24.0              | 46.8 ± 16.6      | 54.3 ± 9.3                |
| Thickness of the zona glomerulosa, µm          | 301 ± 18                 | 333 ± 90         | 455 ± 152                 |
| Thickness of the zona fasciculata, µm          | 1463 ± 98                | 1410 ± 281       | 1308 ± 205                |
| Width of the medulla, µm                       | 6.74 ± 5.33              | 0.34 ± 0.10      | 0.76 ± 0.45               |
| Area of the medulla, mm²                       | 2,707 ± 378              | 2,941 ± 1,243    | 2,897 ± 1,521             |
| Width of the medulla, µm                       | 809 ± 144                | 307 ± 56         | 434 ± 242                 |
| Area of the cortex zone, occupied by corticosteroids, µm² | 1,129 ± 248             | 1,485 ± 501      | 395 ± 178                 |
| Area of the medulla occupied by catecholamines, µm² | 2,410 ± 521             | 2,850 ± 499      | 2,370 ± 102               |
In parasympathicotonic animals the average parameter of the number of cells in this zone was 0.93 units lower than the parameter of the sympathicotonic group. Furthermore, parasympathicotonic rabbits had the smallest area of nuclei of these cells, with difference between them and sympathicotonic animals equaling 0.40 µm². During the study of optical density of corticosteroids in the cortex of the suprarenal glands (Table 1), we determined that their area was the largest in normotonic animals (Fig. 10) and exceeded that found in sympathicotonic ones by 356 µm². In parasympathicotonic rabbits this parameter was the lowest, being 734 µm² less than in sympathicotonic animals.

Table 2

| Indicator                                                                 | Group of rabbits | Group of rabbits | Group of rabbits |
|---------------------------------------------------------------------------|------------------|------------------|------------------|
| Number of cells in the zona glomerulosa per 1,000 µm²                      | 8.36 ± 0.31      | 8.26 ± 0.88      | 7.94 ± 0.79      |
| Number of cells in the zona fasciculata per 1,000 µm²                     | 4.43 ± 0.18      | 5.14 ± 0.45     | 4.82 ± 0.64      |
| Number of cells of the zona reticularis per 1,000 µm²                     | 6.13 ± 0.35      | 4.91 ± 0.77      | 5.20 ± 0.16      |
| Area of cells of the medulla per 1,000 µm²                                 | 8.45 ± 0.51      | 8.53 ± 0.68      | 8.09 ± 1.00      |
| Area of the zona glomerulosa, µm²                                        | 104.5 ± 4.2      | 115.4 ± 21.6     | 117.7 ± 8.3      |
| Area of cells of the zona fasciculata, µm²                               | 210 ± 15         | 173 ± 12         | 156 ± 25         |
| Area of cells of the zona reticularis, µm²                               | 135.1 ± 7.4      | 158.5 ± 3.3      | 181.2 ± 13.2     |
| Area of the nucleus of the zona glomerulosa, µm²                         | 110.6 ± 7.5      | 97.5 ± 13.7      | 90.3 ± 12.6      |
| Area of the nucleus of the zona fasciculata, µm²                         | 30.84 ± 0.93     | 30.76 ± 1.79     | 31.60 ± 3.10     |
| Area of nucleus of the zona reticularis, µm²                             | 34.2 ± 1.2       | 32.2 ± 1.4       | 33.3 ± 1.1       |
| Area of cell nucleus of the medulla, µm²                                  | 32.5 ± 1.1       | 38.8 ± 6.4       | 32.1 ± 5.0       |
| Area of cell nucleus of the medulla, µm²                                  | 32.7 ± 2.0       | 30.8 ± 3.0       | 30.9 ± 3.4       |
| Nuclear-cytoplasmic ratio of cells of the zona glomerulosa                | 0.419 ± 0.019    | 0.363 ± 0.047    | 0.367 ± 0.045    |
| Nuclear-cytoplasmic ratio of cells of the zona fasciculata                | 0.195 ± 0.016    | 0.220 ± 0.023    | 0.271 ± 0.035    |
| Nuclear-cytoplasmic ratio of cells of the zona reticularis                | 0.317 ± 0.018    | 0.324 ± 0.062    | 0.215 ± 0.061    |
| Nuclear-cytoplasmic ratio of cells of the medulla                         | 0.420 ± 0.021    | 0.462 ± 0.025    | 0.494 ± 0.032    |

*Note.* \( ^{a} \) – statistically reliable difference compared with the group of sympathicotonic rabbits (\( ^{b} P < 0.05; ^{aa} P < 0.01; ^{aaa} P < 0.001 \)), \( ^{b} \) – statistically reliable difference compared with the group of normotonic rabbits (\( ^{b} P < 0.05; ^{aa} P < 0.01; ^{aaa} P < 0.001 \)).

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Studies of the internal structure of the suprarenal glands revealed significant differences between thickness of the zona glomerulosa and zona fasciculata, and also fluctuations in sizes and shapes of the medulla. Thickness of the zona glomerulosa in the parasympathicotonic group (Fig. 4) was 154 µm greater than that of the sympathicotonic groups. However, according to the data in Table 2, in parasympathicotonic rabbits, the number of cells per 1,000 µm² in this zone (Fig. 5) was 0.42 units lower than in the same area in sympathicotonic rabbits. Much smaller divergences were observed in the group of normotonic rabbits. Thickness of the zona glomerulosa in these animals was 32 µm greater than in the sympathicotonic group, and the differences in the number of cells per unit area between these groups equaled 0.10 units.

The largest zone of the cortex of the suprarenal gland is the zona fasciculata. Its maximum thickness was observed in sympathicotonic animals and exceeded this value by 53 µm in normotonic animals (Fig. 6) and by 155 µm in parasympathicotonic animals. In the normotonic groups (Fig. 7), the highest number of cells in this zone per unit area was observed and they exceeded the number for sympathicotonic rabbits by 0.71 units (\( P < 0.01 \)). Parasympathicotonic rabbits by number of cells in 1,000 µm² exceeded the sympathicotonic ones by 0.39 units. Therefore, in animals with normotonic type of autonomic nervous system, the zona fasciculata of the cortex was characterized by higher saturation with cells compared with the same zone in animals of other groups.

Special attention should be paid to the zona reticularis of the suprarenal gland, which interweaves with or surrounds the medulla (Fig. 8), and sometimes is completely absent. In this zone, the lowest quantity of cells per 1,000 µm² was observed in normotonic rabbits – 4.91 ± 0.77 units. This was 1.22 units less than in sympathicotonic animals (Fig. 9).

![Fig. 3. Suprarenal gland of normotonic rabbit: 1 – capsule, 2 – zona glomerulosa, 3 – zona fasciculata, 4 – additional suprarenal gland; hematoxylin and eosin](image1)

![Fig. 4. Suprarenal gland of parasympathicotonic rabbit: 1 – zona glomerulosa, 2 – capsule, 3 – zona fasciculate; hematoxylin and eosin](image2)

![Fig. 5. Cells of the zona glomerulosa of parasympathicotonic rabbit: 1 – cell cytoplasm, 2 – nucleus; hematoxylin and eosin](image3)
Fig. 6. The suprarenal gland of normotonic rabbit: 1 – zona fasciculata, 2 – zona glomerulosa, 3 – medulla; hematoxylin and eosin

Fig. 7. Cells of the zona fasciculata of normotonic rabbit: 1 – cell’s cytoplasm, 2 – cell’s nucleus; hematoxylin and eosin

Fig. 8. The suprarenal gland of sympathicotonic rabbit: 1 – medulla, 2 – zona reticularis, 3 – zona fasciculata, 4 – central vein; hematoxylin and eosin

In four sympathicotonic animals, in one normotonic and one parasympathicotonic rabbit the medulla was fragmented (Fig. 11, 12). This is related to peculiarities of embryogenesis of this genus of mammals (Sokolov, 1969). Therefore to characterize its forms we measured not only the areas, but lengths and widths of this zone. The largest medulla by area was observed in sympathicotonic rabbits – $6.74 \pm 5.33 \, \text{mm}^2$ (Table 1). In animals of other groups, this parameter was significantly lower: by $6.40 \, \text{mm}^2$ in normotonic and by $5.98 \, \text{mm}^2$ in parasympathicotonic animals. Number of cells of the medulla in the area of $1,000 \, \mu\text{m}^2$ in ST and NT rabbits differed by 0.08 units with higher number in the animals of the second group. In parasympathicotonic animals this parameter was 0.36 units less than the parameters of sympathicotonic animals (Fig. 13).

Fig. 9. Suprarenal gland of sympathicotonic rabbit: 1 – zona reticularis, 2 – medulla, 3 – central vein; hematoxylin and eosin

Fig. 10. Accumulation of corticosteroids in the cortex zone of normotonic rabbit (1'): phenylhydradine reaction

Fig. 11. Suprarenal gland of sympathicotonic rabbit: 1 – total (not fragmented) medulla, 2 – zona fasciculata, 3 – zona glomerulosa, 4 – capsule; hematoxylin and eosin

Highest values of length of the medulla were observed in normotonic animals ($2,941 \pm 1,243 \, \mu\text{m}$). Width of this zone was the greatest in sympathicotonic rabbits ($809 \pm 144 \, \mu\text{m}$), which was by $502 \, \mu\text{m}$ higher than in normotonic animals, and by $375 \, \mu\text{m}$ than in parasympathicotonic ones.
Fig. 12. Suprarenal gland of sympathicotonic rabbit: 1 – fragmented medulla, 2 – cortex zone; hematoxylin and eosin

Fig. 13. Suprarenal gland of sympathicotonic rabbit: 1 – medulla, 2 – central vein, 3 – zona reticularis; hematoxylin and eosin

Because the sizes of the medulla significantly differed in all examined groups of animals, we determined the ratio between its length and width, which equaled 3.3 in sympathicotonic, 9.6 in normotonic and 6.7 – in parasympathicotonic rabbits. These parameters allow us to understand that in NT rabbits the shape of the medulla was most elongated compared with the other groups of animals.

Despite the fact that the medulla was biggest in ST rabbits, the area of location of catecholamines in it was the largest in NT rabbits. In sympathicotonic animals (Fig. 14) it was smaller by 440 µm².

Fig. 14. The suprarenal gland of sympathicotonic rabbit. 1 – accumulation of hormones in cells of the medulla, 2 – cortex zone; chromaffin reaction according to the method by Hillarp and Hokfelt

The lowest parameters of the area of catecholamines corresponded to parasympathicotonic rabbits, in which they were less by 40 µm² compared with sympathicotonic animals. For more detailed characteristic of the cellular component of the suprarenal glands, we performed morphometry of cells and their nuclei, and also determined nuclear-cytoplasmic ratios in separate zones. In all cases we observed no significant difference between three groups of rabbits by the parameters of both area of cells and their nuclei in the examined zones.

Compared with the previous parameters, the nuclear-cytoplasmic ratio showed significant differences between the animal groups. In the zona fasciculata and medulla the highest value of this parameter belonged to parasympathicotonic rabbits, and the lowest were observed in sympathicotonic animals. The difference between the groups was 0.076 (P < 0.001) and 0.074 units (P < 0.01) respectively. By obtaining average values of nuclear-cytoplasmic ratio in these zones, NT rabbits exceeded ST animals respectively by 0.034 (P < 0.01) and 0.042 unit (P < 0.03).

In the zona glomerulosa, ST rabbits had highest values of nuclear-cytoplasmic ratio. In NT and PS animals this parameter was almost the same and was lower than in the previous group respectively by 0.056 (P < 0.001) and 0.052 units (P < 0.001).

Value of nuclear-cytoplasmic ratio in the zona reticularis was highest in NT rabbits. In sympathicotonic animals it was observed to be lower only by 0.007 units. In parasympathicotonic rabbits this parameter was much lower, differing from ST animals by 0.102 units (P < 0.001). A characteristic feature of only nuclear-cytoplasmic ratio is presence of reliable differences between normotonic and parasympathicotonic rabbits. In the zona fasciculata and medulla of PS rabbits it was higher compared with NT respectively by 0.042 units (P < 0.01) and 0.032 units (P < 0.05), and in the zona reticularis it was lower by 0.109 units (P < 0.001).

During histological survey of glycogen, glycoproteins and glycosaminoglycans, we determined that PAS-positive substances (Fig. 15) were present only in the intracellular connective tissue and capsule of the suprarenal gland in all groups of rabbits. Acidic glycosaminoglycans in the tissue of the gland and capsule were completely absent.

Fig. 15. PAS-positively stained connective tissue (1) and endothelium of the vessel (2) of the suprarenal gland of sympathicotonic rabbit; PAS-reaction according to Mc Manus

Typological peculiarities of the autonomic tone are also reflected in the parameters of the animals’ body weight. In sympathicotonic and normotonic rabbits this parameter was almost the same – respectively 3.68 ± 0.10 and 3.67 ± 0.24 kg. That is difference between these groups was only 0.01 kg. Parasympathicotonic rabbits had the highest body weight (3.92 ± 0.43 kg), exceeding the weight of ST rabbits by 0.24 kg.

Discussion

The conducted studies revealed that typological peculiarities of the autonomic tone manifests in morphological peculiarities of the suprarenal glands of rabbits. Intensity of autonomic impacts on different parameters was not the same. During the morphometry of the capsule of the gland,

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thickness of the capsule of sympathicotonic rabbits was found to be twice as large as in animals of other groups, which is related to large amount of fatty tissue between the fibers of the connective tissue. Furthermore, in the gland’s capsule we found areas with cells of the cortex zone. This can indicate fast embryogenesis of the suprarenal glands in ST animals, due to which epithelial and nervous tissues of their suprarenal glands do not complete their fusion completely (Keeegan & Hammer, 2002; Rapků, 2002; Shekhtman et al., 2014). This was also confirmed by the data of Vidal et al. (2016), according to which the suprarenal glands contain subcapsular blastaema, cells of which can further differentiate and during regenerative processes become round, accumulate lipid droplets and become cells of the zona glomerulosa. However, not all researchers (Katnieslon, 1968) agree with these data, because consider that during embryogenesis usual cells of the cortex zone intervened in the structure of the capsule, and according to the recent reports, not only they but also cells of adrenogonadal primordium (Bandiera et al., 2013; Dömer et al., 2016). The determined peculiarities allow us to consider the suprarenal gland’s capsule not only as a protective structure, but also indicate its important role in the processes of adaptation of the gland’s morphology to trophic-regulatory impacts from the autonomic tone.

The survey of the structure of the cortex zone of the gland revealed that its division into separate functional zones (glomerulosa, fasciculata and reticularis zones) is not clear and the borders between them are blurred. This is explained by the fact that during the embryonic period the cortex of the suprarenal glands is represented by an interrenal gland with non-differentiated cells (Barwick et al., 2005; El-Nahla et al., 2011; Xing et al., 2015). Formation of the zona glomerulosa and zona fasciculata (Sokolov, 1972; Katnieslon, 1968; Lotfi et al., 2018) begins only in the second half of the embryonic development. A number of authors (Freedman et al., 2013; Pihlajoki et al., 2015) state that the cells of the zona glomerulosa can change and become cells of zona fasciculata. The zona reticularis is considered a derivative of the juxtamедullary zone (homologue of the fetal cortex of mammals and X-zone of mice) and differentiates during the postnatal period (Sedova, 1974; Huang & Kang, 2019). However, Vidal et al. (2016) indicate that this zone forms in people, but is absent in rodents. As for the non-uniformity of the arrangement of the medulla and zona reticularis, or complete absence of the latter which we observed in some rabbits, this was probably caused by embryonic ingrowth of the chromaffin tissue (future cortex zone) into the interrenal gland, which takes place in two stages. During the unification of the progenitors of cortical and medullary substances, they can move, which is obviously the reason for their fragmentary nature (Sokolov, 1972). At the same time, the medulla underwent fragmentation only in one PS rabbit, and cells of the zona reticularis were found only in two rabbits, which was 67% of all the examined animals of that group. In NT animals, the zona reticularis was found in 40% of animals, and fragmented medulla in 20% of the cases. In the ST group, 74% of the animals had zona reticularis and only 37% were observed to have fragmentation of the medulla. During the study of the cellular composition of the suprarenal glands’ medulla, we found only one type of cells – chromaffin cells (epinephrocytes, A-cells), which produce two types of hormones of this zone. This correlates with the data of other scientists (Krulova, 1975). These cells form separate accumulations, i.e. are represented in the form of cluster structures (Hussein et al., 1984). These cells form separate accumulations, i.e. are represented in the form of cluster structures (Hussein et al., 1984).

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