SLICE AREA ASSESSMENT OF THE GUT-ASSOCIATED LYMPHOID TISSUE OF SACCLUS ROTUNDUS IN HIPLUS RABBITS

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Summary


The development of rabbit breeding requires research in various fields to ensure a high-grade product. One of the most important areas is the study of gut-associated lymphoid tissue. For the study, sacculus rotundus samples of clinically healthy rabbits of 1, 10, 20, 30, 60 and 90 days of age were collected. Microslide pictures were taken from the obtained intestine material. From the photos of the microslides, the characteristics of the slice area of aggregated lymphatic nodules, germinal centres and interfollicular regions were studied. The results can be used as indicators of the normal condition of healthy rabbits from the Hiplus meat breed.

Key words: aggregated lymphatic nodules, germinal centres, interfollicular region, lamina propria lymphocytes

INTRODUCTION

Gut-associated lymphoid tissue (GALT) is a critical component in rabbit’s healthy digestion and plays an essential role in protecting rabbit body against pathogens (Senda et al., 2019) and toxins (Higashizono et al., 2018). It also plays a role in the postnatal formation of the immune system (Arrazuria et al., 2018). Besides, GALT is involved in maintaining the homeostasis of the immune system and contains almost 70% of the immune cells of the cellular composition of the immune system as a whole (Camps-Bossacoma et al., 2017). There are two structurally and functionally separate segments in GALT: the diffuse effector site and the organised inductive site. In turn, the diffuse site includes the lamina propria lymphocytes of the mucosa and the intraepithelial lymphocytes. In contrast, the organised site is formed by mesenteric lymph nodes, solitary lymphatic nodes, and aggregated lymphatic nodules (Camps-Bossacoma et al., 2017; Seeger et al., 2017). A feature of rabbit GALT is that its structure is more developed than that of other mammalian species, which is expressed in the presence of two lymphoid formations:
sacculus rotundus and appendix. These two lymphoid organs involve more than 50% of the total lymphoid tissue of the rabbit body (Zgair et al., 2016; Arrazuria et al., 2018).

Saccus rotundus is located in the ileocecal junction (Fortun-Lamothe & Boullier, 2007). It has a rounded shape and thickened walls due to its high GALT content (Besoluk et al., 2006) and macrophage aggregation (Johnson-Delaney, 2006). This specific organ is formed by aggregated lymphatic nodules (Beyaz et al., 2017).

GALT of sacculus rotundus is formed by follicles, a subepithelial dome, an interfollicular region and a follicle-associated epithelium (Beyaz et al., 2017). Besides, germinal centres are part of the secondary lymphoid organs. B-cell clonal expansion occurs in there, as well as somatic hypermutation and affinity-based selection, which produces high-affinity antibodies (Victora et al., 2012).

Acquisition of primary data on morphology, histology, and morphometry of sacculus rotundus of meat rabbits is vital for further research in breeding, keeping, feeding, obtaining better products, diagnosis, prevention, and treatment of rabbit diseases.

MATERIALS AND METHODS

The study dealt with rabbits of Hyplus meat breed grown at the privately-owned farm in Berdyansk, Ukraine. The rabbits were housed in a closed rabbitry with a controlled microclimate. The rabbits were kept in cages, equipped with automatic drinkers and feeders to ensure a continuous supply of fresh water and feed. The rabbits were fed balanced food twice a day (morning and evening) according to the feeding standards. The selected rabbits were not submitted to any prophylactic treatment against infectious and invasive diseases.

Altogether six age groups were formed from clinically healthy animals of 1, 10, 20, 30, 60 and 90 days of age. Each group consisted of 5 rabbits. These age groups were formed by taking into account the significant critical periods of rabbit development. A transition from the colostrum to dairy type of nutrition occurred on the 10th day of post-natal development. The rabbits received supplementary feed from the 10th to 20th day and were transferred to complete feed on the 20th day. Weaning and movement into individual rabbit cages happened on the 30th day. The physical formation of the rabbits was completed by the 90th day. As the gap between the 30th and 90th days of age was quite large, an intermediate age group of 60 days old animals was formed. All manipulations with animals were carried out in compliance with ethical standards, as well as with international and Ukrainian legislation.

The research object was the sacculus rotundus, which was preserved in 10% formalin. Histological slices were made using different types of staining: haematoxylin-eosin (Cardiff et al., 2014) and azure II eosin, as well as silver impregnations according to generally accepted methods. Following these techniques, microslides of the gut-associated lymphoid tissue (GALT) with the intestinal mucosa in sacculus rotundus were obtained. The further study incorporated the examination of the microslides with a Leica DM 1000 light microscope. The microphotographs were taken via the LAS V4.12 programme. The microphotographs of 5 sacculus rotundus slices from each age group were used in the further measurements. The morphological traits of the obtained
slice micrographs were measured utilising the Leica QWin and ImageJ software. Altogether, 650 slice micrographs for the above age groups were used. In addition to the common boxplot analysis, the ratio of the slice areas of the aggregated lymphatic nodules to the interfollicular region areas was examined for each age group. The assessed area values of the aggregated lymphatic nodules and the interfollicular region were summed up within each age, and then their ratio was calculated as
\[ \delta_i = \frac{S_n_i}{S_i + S_{n_i}} \]
where \( S_n_i \) – the total assessed area value of all aggregated lymphatic nodules of the \( i^{th} \) slice microphotograph, \( S_i \) – the assessed area of the interfollicular region of the \( i^{th} \) microphotograph and \( j = \{10, 20, 30, 60, 90\} \) – the index of the age group.

The ratios of the assessed areas of the germinal centres to the area values of the aggregated lymphatic nodules with germinal centres were computed by the following equation:
\[ \varepsilon_j = \frac{S_{c_j}}{S_n_j} \]
where \( S_{c_j} \) – the assessed area of the germinal centre of the \( j^{th} \) observed nodule of the \( j^{th} \) group, \( S_n_j \) – the assessed area of the \( j^{th} \) aggregated lymphatic nodule including the \( j^{th} \) germinal centre.

Pearson’s correlation coefficients and their standard errors were estimated (Kobzar, 2006).

RESULTS

Fig.1 shows microslides of the two GALT sections: 1) the diffuse effector site of newborn rabbits in the form of lamina propria lymphocytes and 2) the organised inductive site in the form of interfollicular region and aggregated lymphatic nodules. The microslides were obtained by staining GALT sections of 1, 10, 20, 60 day-old rabbits with haematoxylin-eosin and azure II eosin.

Visual analysis of the microphotograph slices, depicted on Fig. 1, showed that the GALT with the intestinal mucosa was represented as a diffuse effector site in sacculus rotundus of the newborn rabbits. Furthermore, single intraepithelial lymphocytes and lamina propria lymphocytes were observed.

It was noticed that an organised inductive site of GALT was formed by the 10th day of the postnatal rabbit development. At this age, in addition to the presence of intraepithelial cells, small-sized aggregated lymphatic nodules and interfollicular regions were formed. Fig. 1 illustrates the formed aggregated lymphatic nodules of the 10-day-old group. The nodules were observed as a denser cluster of cells. At the same time, the formation of large interfollicular regions between the aggregated lymphatic nodules was noticed.

In the samples obtained from 20-day-old rabbits, the aggregated lymphatic nodules were found to be adjacent to each other with a thin fibrous connective tissue capsule (Fig. 1). The aggregated lymphatic nodules were also found to be covered with follicle-associated epithelium at their apical parts adjacent to the intestinal lumen. However, unlike the samples from the 10-day-old rabbits, some of the aggregated lymphatic nodules had distinguishable germinal centres. The germinal centres were located mainly in the central or basal part of the aggregated lymphatic nodules. The aggregated lymphatic nodules in the part of the sacculus rotundus, which was opposite to its coalescence with the ileum, were more numerous and bigger than in the region near this coalescence. Thus, closer to the coalescence,
Fig. 1. Microphotographs of fragments of sacculus rotundus slices containing GALT:
1 – lamina propria lymphocytes; 2 – aggregated lymphatic nodules without germinal centres;
3 – aggregated lymphatic nodules with germinal centres.
Slice area assessment of the gut-associated lymphoid tissue of sacculus rotundus in Hiplus rabbits

The aggregated lymphatic nodules decreased in size and amount while the interfollicular regions increased.

The slice micrographs for the 30-day-old rabbits were visually similar to the micrographs for the 20-day-old group. The germinal centres were observed in half of the aggregated lymphatic nodules within the age groups of 20, 30, and 60 days. Besides, the micrographs for the 90-day-old group were similar to the micrographs for the 60-day-old group, with no significant dissimilarities detected.

Fig. 2 shows a boxplot of δij values alongside the boxplot of 1- δij values. The latter are the ratios of the interfollicular region area estimates to the GALT area estimates.

Analysis of the mean values shows that the maximum value of the interfollicular region area relative to the area of the aggregated lymphatic nodules was observed for the 10-day-old group. In the same age group, the minimum value of the area of the aggregated lymphatic nodules was noticed. The interfollicular region area decreased with age. This indicator declined quite sharply from the 10th to the 20th and from the 30th to the 60th day: by 26% and 28%, respectively. It allowed concluding that the growth of the aggregated lymphatic nodules from the 20th to the 30th day was less intensive than from the 10th to the 20th and from the 30th to the 60th days of the postnatal development. It was found that by the 30th day of age, the area of the interfollicular region decreased by only 6%. The difference between the 60th and 90th days was not significant. The opposite pattern was observed for the area of the aggregated lymphatic nodules: their maximum increase was observed from the 10th to the 20th and from the 30th to the 60th day of the postnatal development.

The assessed area values of the aggregated lymphatic nodules were divided into two groups: 1) the aggregated lymphatic nodules without germinal centres, and 2) the aggregated lymphatic nodules containing germinal centres. The results of the statistical computation carried out for the two groups, is displayed on Fig. 3.

Fig. 3 shows an increase in the mean values and medians of the assessed areas.
with age. Median and mean values were close. In the group of the aggregated lymphatic nodules without germinal centres, the minimum mean value was observed for 10-day-old rabbits (0.005 mm²). By the 20th day of life, the mean value was almost doubled: 0.012 mm². By the 30th and 60th days of development, the mean assessed areas of the aggregated lymphatic nodules without germinal centres were also doubled to 0.020 mm² and 0.044 mm² correspondingly. However, by the 90th day, the mean area of the aggregated lymphatic nodules without germinal centres increased slightly to 0.048 mm².

There was a tendency to an increase in the interquartile range (IQR) of the area of the aggregated lymphatic nodules without germinal centres. Thus, the minimum value of 0.004 mm² of this scatter index was observed on the 10th day of postnatal development. By the age of 20 days, IQR increased almost twice to 0.007 mm². Its highest increase was observed by the age of 30 days (0.020 mm²). On day 60, the IQR increased to 0.036 mm² and at day 90, IQR increased slightly compared to the previous age group (0.041 mm²).

As shown on Fig. 3, the aggregated lymphatic nodules with germinal centres were absent on the 10th day of postnatal development and appeared on the 20th day. The minimum mean area of 0.019 mm² was observed on the 20th day of the postnatal development. By the 30th day, it increased to 0.036 mm². On the 60th and 90th day, the mean area was almost the same – 0.110 mm², about three times higher than the mean area of the aggregated lymphatic nodules of rabbits on the 30th day of life. An increase in IQR with age was observed in the group of aggregated lymphatic nodules with germinal centres. Its smallest value of 0.009 mm² was detected on the 20th day. By the 30th day of life, IQR increased almost four times, reaching 0.032 mm². By the age of 60 days, it increased 2.5 times to 0.082 mm² and by the 90th day, IQR did not increase so much and was 0.102 mm².

Comparison of the two groups (Fig. 3), showed that the mean values and medians of the assessed areas in the group of aggregated lymphatic nodules with germinal centres significantly exceeded the esti-

Fig. 3. Boxplot of the assessed area values of the aggregated lymphatic nodules without and with germinal centres of rabbit sacculus rotundus on postnatal development days 10, 20, 30, 60 and 90.
Slice area assessment of the gut-associated lymphoid tissue of sacculus rotundus in Hiplus rabbits

The mean value was 1.7 times higher on the 20th day in the former group, 1.8 times on the 30th day, 2.4 times on the 60th day and 2.3 times on the 90th day; the median was 1.5 times higher on the 20th day, 1.7 on the 30th day, 2.5 on the 60th day and 2 times on the 90th day.

Analysis of IQR estimates of aggregated lymphatic nodule areas of the two groups also showed that the IQRs in the group of aggregated lymphatic nodules with germinal centres significantly exceeded those in the other group. On the 20th day, IQR estimates of the areas of aggregated lymphatic nodules with and without germinal centres were almost the same. By the 30th day, the IQR value for the group of aggregated lymphatic nodules with germinal centres was 1.6 times higher; by the 60th day – 2.3 times, and by the 90th – 2.5 times. Besides, in both groups, there was a conspicuous spike in IQR on the 30th day of postnatal development.

Germinal centres are of particular interest because of their functions. Fig. 4 shows a boxplot of the assessed areas of the germinal centres of aggregated lymphatic nodules. The mean values and medians (Fig. 4) indicated an increase in the area of germinal centres with age. The smallest estimates were obtained for the 20-day-old age group (mean 0.003 mm$^2$ and median 0.002 mm$^2$). By the 30th day, these estimates were almost doubled: the mean value reached 0.006 mm$^2$, and the median: 0.005 mm$^2$. An increase of about three times is observed by the 30th day (0.018 mm$^2$ and 0.014 mm$^2$ respectively).

By the 90th day of life, the increase in the estimates was quite small viz. mean 0.020 mm$^2$ and median 0.018 mm$^2$. The IQR estimates of the germinal centre areas also increased with age. The smallest IQR (0.003 mm$^2$) was detected on the 20th day. A two-fold increase in IRQ up to 0.006 mm$^2$ was observed by the 30th day of postnatal development. By the 60th day, the IRQ also rose two times to 0.017 mm$^2$ while by the 90th day, it increased slightly to 0.020 mm$^2$.

The area of the germinal centres, as well as area of the aggregated lymphatic nodules showed minimal mean values and medians on the 20th day of postnatal development. The medians and mean area
values of the germinal centres, as well as aggregated lymphatic nodules increased with age. The same pattern was also observed for areas of the aggregated lymphatic nodules with germinal centres, as stated above.

The sample correlation coefficients between the assessed area values of the aggregated lymphatic nodules and the assessed areas of their centres were computed for each age group of rabbits. There was a positive correlation between the assessed area values of the aggregated lymphatic nodules and the assessed areas of their centres with correlation coefficients about 0.7 for the 20-, 30- and 60-day-old groups, whereas coefficients were lower (about 0.5) for the 90-day-old group.

Fig. 5 illustrates the boxplot of ratios of the assessed areas of germinal centres to the assessed areas of aggregated lymphatic nodules of rabbit sacculus rotundus on postnatal development days 20, 30, 60 and 90.

**DISCUSSION**

Along the entire gastrointestinal tract, GALT is present in the form of aggregated and solitary lymphatic follicles (Liebler-Tenorio & Pabst, 2006; Newberry, 2008). Moreover, rabbits, unlike other animal species, have a well-developed GALT structure included in sacculus rotundus and appendix (Cesta, 2006). Studies by Shi-Jin & Nan-Hui (2007) indicated that a sacculus rotundus plays a role similar to an appendix, and together with GALT provide anti-infection protection and immune function of the intestinal mucosa.

In rabbits weighing from 1 to 1.5 kg, the GALT of the sacculus rotundus was represented by the zones of the dome and the germinal centre, however, the interfollicular region was not detected (Ranjan et al., 2016).

Comparison of the GALT of germfree and conventional rabbits on the 14th day of postnatal development showed that aggregated lymphatic nodules had not yet been formed in the GALT of the sacculus rotundus of the germfree species. At the same time, the conventional rabbits at that
age, had aggregated lymphatic nodules, and their sizes corresponded to the aggregated lymphatic nodules of germfree animals on the 62nd day of development. On the 62nd day of life, the aggregated lymphatic nodules of sacculus rotundus and the appendix of the conventional rabbits were 10 and 15 times larger than in the group of the germfree rabbits. Besides, germinal centres (from birth to the 103rd day) were not found in the germfree rabbits. In contrast, the lymphoid tissue of sacculus rotundus and appendix was represented by large aggregated lymphatic nodules with germinal centres of the conventional rabbits on the 62nd and 103rd days (Štěpánková et al., 1980).

Al-Haaik & Al-Saffar (2017) found well-defined aggregated lymphatic nodules that had a germinal center in the GALT of sacculus rotundus of newborn rabbits. Moreover, the diameter of lymphatic nodules actively increased from birth to 10 days old, did not change up significantly to the 15th day, and increased intensively by the 40th day of development (Al-Haaik et al., 2017).

In the sacculus rotundus of adult rabbits of the Angora breed, the following three regions of the aggregated lymphatic nodules were formed: a subepithelial dome region, a germinal centre located in the middle and the crown which split the previous two areas. Besides, an interfollicular region was observed between the aggregated lymphatic nodules (Beyaz et al., 2010).

In the current research, a well-developed sacculus rotundus of rabbit ileum including aggregated lymphatic nodules was observed, in line with the results published by Cesta (2006). However, in comparison with the germ-free rabbits by Štěpánková et al. (1980), in the species subject to the current investigation, first aggregated lymphatic nodules appeared by the 10th day of life. In contrast with the research by Al-Haaik & Al-Saffar (2017), the germinal centres were not found on the 10th day. The developed germinal centres appeared by the 20th day only, which was still significantly earlier than it was observed for the germ-free rabbits by Štěpánková et al. (1980). Contrary to Al-Haaik & Al-Saffar (2017), an increase in the area of aggregated lymphatic nodules was observed from the 10th till the 20th day of life. Ranjan et al. (2016) did not found an interfollicular region in the sacculus rotundus of rabbits weighing 1–1.5 kg. However, the current investigation showed that the interfollicular region was observed for the all investigated age groups. Moreover, the area of the region decreased as the age increased.

CONCLUSION

The GALT in the sacculus rotundus of the ileum of newborn rabbits was represented by single intraepithelial cells and lamina propria lymphocytes. The first aggregated lymphatic nodules were observed on the 10th day of postnatal development of rabbits. Meanwhile, the first aggregated lymphatic nodules containing germinal centres were noticed by the 20th day.

The decrease in the area of interfollicular region and an increase in the area of aggregated lymphatic nodules with age was observed. Spikes in changes of the assessed area values were detected from the 10th to the 20th, and from the 30th to the 60th days of postnatal development.

The means, medians and IQRs of the cross-sectional areas of the aggregated lymphatic nodules with germinal centres significantly exceeded estimates computed for the aggregated lymphatic nodules.
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