RESEARCH ARTICLE

F.U. Vet. J. Health Sci.
2020; 34 (2): 97 - 101
http://www.fusabil.org

Morphohistological Evaluation of Embryonic Development of Bursa Fabricius in Chicks

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Bursa Fabricius (BF), the primary lymphoid organ of poultry, is responsible for humoral immunity. In poultry, it is very important for BF to complete its embryonic development and reach to a certain maturity in terms of maintaining a healthy life of the animal. In this study, BF development and its morphometric measurements were investigated in chick embryos. BF was taken from 24 Babcock white Leghorn chick embryos on the 10th, 13th, 16th and 21st days of incubation. The average BF weight was 0.006±0.001 g on the 10th day, while this value was 0.037±0.007 g on the 21st day. Average cranio-caudal diameter was different according to the determined incubation days, statistically. While this value was 2.39±0.10 mm on the 10th day, it was 7.55±0.66 mm on the 21st day. The average latero-lateral diameter was ranged from 1.94±0.15 mm to 4.71±0.96 mm, while the average dorso-ventral diameter was in the range of 1.48±0.28 mm and 2.29±0.42 mm. Histologically, a central lumen and plica development were observed on the 10th day of incubation in the bursal sections. 13-day-old embryos were found to have epithelial buddings, two different epithelial structures and three layers of wall in the BF. On the 21st day, we were observed that histologic development of BF was completed. In conclusion, obtained these data concluded that BF is an important lymphoid organ in terms of maintaining a healthy life of the poultry. It is also believed that the obtained morphometric measurements can be used as reference data for further studies on this organ.

Key Words: *Bursa fabricius, chick, embryonic development, lymphoid organ, immun system*

Civcivlerde Bursa Fabriciusun Embriyonik Gelişiminin Morfohistolojik Değerlendirmesi

Kanatlı hayvanların primer lenfoid organı olan Bursa Fabricius (BF) humoral bağışıklıktan sorumludur. Kanatlılarda, BF'nin embriyonik gelişimini tamamlaması ve hayvanın sağlıklı bir yaşamını sürdürmesi açısından belirli bir olgunluğa ulaşması çok önemlidir. Bu çalışmada, civciv embriyolarında BF gelişimi ve morfometrik ölçümleri araştırılmıştır. BF, inkübasyonun 10, 13, 16 ve 21. günlerinde 24 Babcock beyaz Leghorn civciv embriyosundan alındı. Bu çalışmada ortalama BF ağırlığı 10. günde 0.006±0.001 g iken, bu değer 21. günde 0.037±0.007 g idi. Ortalama kraniyo-kaudal çap belirlenen inkübasyon günlerine göre istatistiksel olarak farklıydı. Bu değer 10. günde 2.39±0.10 mm iken, 21. günde 7.55±0.66 mm idi. Ortalama latero-lateral çap 1.94±0.15 mm ile 4.71±0.96 mm arasında değişirken, ortalama dorso-ventral çap 1.48±0.28 mm ve 2.29±0.42 mm arasındaydı. Histolojik olarak bursal kesitlerde inkübasyonun 10. gününde merkezi bir lümen ve plika gelişimi gözlendi. 13 günlük embriyoların BF'lerinde epitelyal tomurcuklanmalar, iki farklı epitel yapısı ve üç katmanlı duvar tabakasının olduğu bulundu. 21. günde BF'nin histolojik gelişiminin tamamlandığı gözlemlendi. Sonuçta, elde edilen bu veriler BF'nin kümes hayvanlarının sağlıklı yaşamını sürdürmesi açısından önemli bir lenfoid organ olduğu sonucuna varıldı. Ayrıca elde edilen morfometrik ölçümlerin bu organ üzerinde yapılabilecek çalışmalar için referans veriler olarak kullanılabileceği düşünülmektedir.

Anahtar Kelimeler*: Bursa fabricius, civciv, embriyonik gelişim, lenfoid organ, immün sistem*

Received : 30.12.2019
Accepted : 09.06.2020

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Introduction

Lymphoid organs play an important role in the body's defense against pathogens. B-cells are responsible for humoral immunity in living organism (1). These cells are producted in Bursa Fabricius (BF) which is the primary lymphoid organ of poultry (2-5). B-cells develop in the BF, differentiate and immunoglobulin isotropic exchange is performed (2-4) here. BF is controlled by bursin hormone and various cytokines (6).

BF begins to develop as a bump in the dorsal diverticulum of the cloacal proctodeum. On the 5th day of embryogenesis, a lumen is formed in the organ and connected to the proctodeum with a small handle (7). BF was found to have a T-lymphocyte area on the dorsal side of the canal opening to the cloaca and therefore, this organ was considered a secondary lymphoid organ. However, atrophy of the organ and low antibody production does not make this feature very important (2, 4, 8). It is reported that traces of cicatrix are seen in the 28th week of the organ which starts to regress after 8 weeks (9).

There are three layers in the wall of BF which are tunica mucosa, tunica muscularis and tunica serosa respectively from inside to outside (4). In BF, lymph follicles containing cortex, medula and corticomedular regions are located in the

evagination of the tunica mucosa towards the lumen (plica) (10). There are two types of epithelium as follicle-related epithelium (FAE) and interfollicular epithelium (IFE) in BF. FAE, which has a pseudostratified columnar epithelium without basal lamina, covers the lumen-facing surfaces of the developing follicles. IFE, a single layer of columnar epithelial layer, covers between two follicular structures (11).

It is very important for this organ to complete its embryonic development and reach a certain maturity in terms of maintaining a healthy life of the animal. The purpose of this research was to evaluate organogenesis and morphometric measurements (organ weight, cranio-caudal, latero-lateral and dorso-ventral diameters, organ volume) of BF in chick embryos. Also, this study will be able to model embryological studies in humans by using chick embryos during incubation. It is thought that the data and information obtained from the study may support the studies planned on this organ.

Materials and Methods

Ethical approval for this study was taken from The Ethical Committee of Health Sciences of Karamanoglu Mehmetbey University (protocol number: 2019/51).

The Eggs: BF used in this study were obtained from 24 *Babcock* white Leghorn chick embryos on the 10^{th}, 13^{th}, 16^{th} and 21^{st} days of incubation. Embryonic periods determined in the present study were selected considering organ development. 40 fertilized eggs were purchased for this study. For the disinfection of these initially visibly clean eggs and incubator, formalin fumigation was carried out with a mixture of 40 mL formalin (40%) and 20 g potassium permanganate per cubic meter for 30 minutes (12). These eggs were divided into four groups to be opened at determined embryonic periods for this study. 10 eggs were placed in each group because some of the eggs placed in the incubator were thought to be infertile. The eggs were numbered and placed in hatching machine (temperature: 37.4-37.6 ^{0} C; humidity: 55%-65%). According to the determined embryonic days, the eggs were opened from each of these groups until 6 live embryos were obtained for evaluation in terms of organ development.

Macroscopic Evaluation of Eggs and Embryos: In this study, embryo weight, the pre-hatching egg weight, and first egg weight were weighed with precision scales. The relative embryo weights (%) were calculated by the following formula (Equation 1). Also, daily egg weight loss was obtained.

$$\text{REW (\%)} = \frac{\text{Embryo weight}}{\text{Pre} - \text{hatching egg weight}} \text{x100}$$

Collection and Anatomical Assessment of BF Samples: The dissected BF were removed from the peripheral tissues and their weights were measured with precision scales. Relative organ weights (%) were calculated with formula (Equation 2). Cranio-caudal, latero-lateral and dorso-ventral diameters were measured by digital caliper (OEM KMP150, 0-150 mm, Loyka).

$$\text{ROW (\%)} = \frac{\text{Organ weight}}{\text{Embryo weight}} \text{x100}$$

Histological Processing of BF Samples: BF taken on the 10^{th}, 13^{th}, 16^{th} and 21^{st} days of incubation was fixed in 10% neutral-buffered formalin solution. These samples washed overnight in running water to remove the fixation solution. Then, these samples were dehydrated in graded alcohol series, cleared in xylene (3 times), and embedded in paraffin blocks. Serial sections of 5 µm thickness were taken at regular intervals using a microtome from these blocks. For histological examination, the sections were stained with Crossmon's trichrome stain and examined with a light microscope (Leica DM-2500 attached to a DFC-320 digital camera) (13).

Statistical Analysis: The statistical analyses was performed using SPSS software version 21. The variables were investigated using visual (histograms, probability plots) and Kolmogorov-Smirnov/Shapiro-Wilk's test were performed whetherthe data are normally distributed or not. Data are expressed as means±standard deviation (SD). One-way ANOVA was used to compare parameters. Levene test was used to assess the homogeneity of the variances. Statistically, significance was accepted as $P<0.05$. When an overall significance was observed, further post-hoc tests were performed using Tukey's test (14).

Results

Anatomical Changes: The data were given in Table 1. Statistically, the lowest pre-hatching egg weight was on 21^{st} day whereas the highest value was on 10^{th} day ($P<0.05$). The embryo weight was different according to the determined incubation days. On the 10^{th} and 21^{st} days, it was found as 3.07±0.33 g and 41.19±3.94 g, respectively. Statistically, the organ weights were similar on 10^{th}-13^{th} days and 16^{th}-21^{st} days. The daily egg weight loss was in the range of 0.53%-0.67%. In the statistical analysis of diameter measurements, whereas the smallest values were on the 10^{th} day, the highest values were on the 21^{st} day ($P<0.05$).

Histological Evaluation: On the 10^{th}, 13^{rd}, 16^{th} and 21^{st} days of incubation, histological structure of the BF was microscopically given in Figure 1. In the tissue sections, we observed organ drafts containing a lumen on day 10^{th} of incubation. A lot of mucosal protrusions, called plica, were seen on the inner surface of the bursa. Large basophilic cell accumulations and hematopoietic foci were determined under the epithelial layer. On the 13^{rd} day of incubation, the number of plicas in BF increased, and its development was more advanced than the 10^{th} day. There were epithelial buddings occurring by basophilic cell accumulation and the presence of ongoing basophilic cell accumulations in the mucosa layer. Occasionally, lymphocytes were noted. The separation of the BF wall layers was clearly observed. Erythrocytes filled vessels and small

Table 1. Some morphometric values of the embryos used in the study according to periods of incubation (Mean±SD)

Day of incubation	10th	13rd	16th	21st
Initial egg weight (g)	61.54± 4.07	58.51±4.79	57.77±4.32	56.97±3.78
Pre-hatching egg weight (g)	58.17±3.93[a]	53.99±4.50[ab]	51.59±3.87[ab]	51.01±4.67[b]
Embryo weight (g)	3.07±0.33[d]	8.33±0.45[c]	24.27±2.48[b]	41.19±3.94[a]
Relative embryo weight (%)	5.29±0.54[d]	15.49±1.27[c]	47.20±5.04[b]	80.91±5.47[a]
Organ weight (g)	0.006±0.001[b]	0.010±0.002[b]	0.032±0.006[a]	0.037±0.007[a]
Relative organ weight (%)	0.18±0.05	0.13±0.02	0.13±0.02	0.09±0.02
Daily egg weight loss (%)	0.55	0.55	0.67	0.53
Cranio-caudal diameter (mm)	2.39±0.10[d]	3.44±0.29[c]	4.89±0.43[b]	7.55±0.66[a]
Latero-lateral diameter (mm)	1.94±0.15[b]	2.61±0.20[b]	4.39±0.21[a]	4.71±0.96[a]
Dorso-ventral diameter (mm)	1.48±0.28[b]	1.65±0.16[b]	1.95±0.30[ab]	2.29±0.42[a]

Different letters in the same row ([a, b, c, d]) indicate statically significant differences (P<0.05).

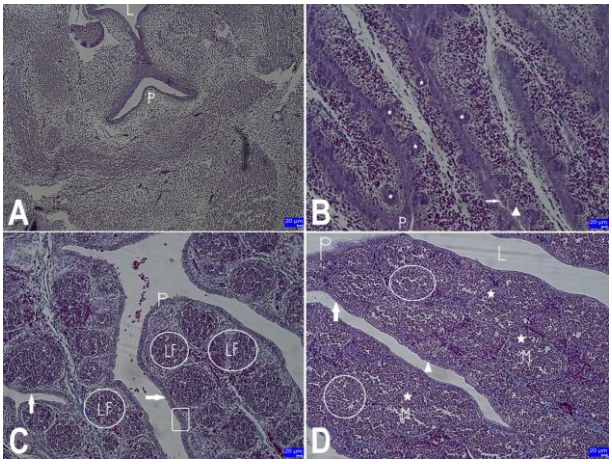

Figure 1. Light microscopic images of BF sections in different embryonic periods. **A:** Light microscopy of BF section from chick embryo (day 10). L: Lümen, p: plica. **B:** Microscopic image of BF section from chick embryo (day 13). p: plica, ☐:epithelial budding, →: IFE, ►: FAE. **C:** Light microscopy of BF section from chick embryo (day 16). p: plica, →: FAE, □:IFE, LF: lymph follicle. **D:** Microscopic image of BF section from chick embryo (day 21). Crossmon's triple staining. p: plica, →:FAE, □: IFE, ○: lymph follicle, ☐: cortex, M: medulla. Bar: 20 µm

hematopoietic areas were found. Furthermore, it was noted that the FAE and IFE began to form in the organ. On the 16th day of the incubation, many small lymph follicles occurring from epithelial buddings were observed in the tunica mucosa. The lymphocyte-rich medulla of these follicles was quite prominent. FAE and IFE were considerably developed according to 13th day of incubation. Interfollicular connective tissue were developing. On the 21st day, histological development of BF was completed. Advanced follicular development, marked separation cortex-medulla-corticomedullar zone, evident FAE and IFE development besides trabeculae were observed in the organ.

Discussion

The BF, which varies in shape and size according to poultry species, is opened by a small canal into the proctodeum region of cloaca (3, 15, 16). Mahanta et al. (3) reported that the BF was caudal 2.15±0.39 mm from the left kidney and 1.53±0.22 mm from the right kidney. In this study, BF of embryos according to certain embryonic periods were oval shaped. This finding was consistent with other studies (2, 4, 15, 17).

Mahanta et al. (3) reported that the average weight of the BF was 0.053±0.02 g. Also, average longitudinal diameter, transverse diameter and organ thickness of Kadaknath chick were 5.78±0.28 mm, 3.59±0.18 mm and 3.09±0.29 mm, respectively. In this study, the average BF weight was 0.006±0001 g on the 10th day, while this value was 0.037±0.007 g on the 21st day. Average cranio-caudal diameter was different according to the determined incubation days, statistically. While this value was 2.39±0.10 mm on the 10th day, it was 7.55±0.66 mm on the 21st day. The average latero-lateral diameter was ranged from 1.94±0.15 mm to 4.71±0.96 mm, while the average dorso-ventral diameter was in the range of 1.48±0.28 mm and 2.29±0.42 mm. These values indicate that the embryo and its organs develop and grow.

Optimal incubation conditions should be met in terms of reliability of the study. The relative humidity of the incubator. It is the water that evaporates from the pores in the egg shell that determines the relative humidity of the incubator. This also leads to daily egg weight loss. This rate should be 0.55%-0.70% during incubation (18). In this study, the daily egg weight loss was between 0.53% and 0.67% (Table 1). These values were within the range of desired values, which indicates that our incubation conditions are optimal. In this study, average embryo weights were 3.07 g, 8.33 g, 24.27 g, and 41.19 g in the incubation on days 10th, 13th, 16th, and 21st, respectively. According to Table 1, the data were similar with the data of Bellairs and Osmond (19). Differences may be due to egg size and/or chick breed.

In the present study, the highest relative embryo weight and the lowest relative organ weight were statistically 21-old-day embryos ($P<0.05$). Consequently, these data might be considered as an indication that the embryo developed.

As a result of the measurements, increased the BF weight and embryo weight were determined in the hatching time of chicks. The increase in BF weight and embryo weight depends on the development and growth of the embryo. It was determined that egg weights decreased as the hatching time of the chicks approached. Therefore, it was thought to be that the nutrients contained in the egg were burned with oxygen and converted into carbon dioxide and water and thrown out of the egg.

BF, a lymphoepithelial organ, is not only the primary but also a secondary lymphoid organ in terms of having a T-lymphocyte area in poultry. B-lymphocytes, which are responsible for the formation of a humoral immune response to diseases, develop and differentiate in this organ (4, 20, 21). The epithelial bursa draft begins to develop in 4^{th}-5^{th} days. B-lymphocyte precursors migrate to this organ draft on the 7^{th} day of incubation (7, 22, 23). On the 9^{th} day of embryogenesis, evaginations into the lumen of the mucosal layer are named as plica. In 10-day-old embryos, the development of lymph follicles is reported to be initiated by the accumulation of large basophilic cells under the epithelial layer (24). In this study, a central lumen and plica development were observed on the 10^{th} day of incubation in the bursal sections.

Dense basophilic cell accumulation forms epithelial buddings in the mucosa layer (24). The wall structure of the BF consists of tunica mucosa, tunica muscularis and tunica serosa (4). There are structurally different epithelium in the BF as FAE and IFE. Whereas FAE is pseudostratified columnar epithelium that covers the luminal surface of follicles, IFE is a single layer prismatic epithelium that covers inter-follicular regions (25). In present study, 13-day-old embryos were found to have epithelial buddings, two different epithelial structures and three layers of wall in BF.

The development of the lymph follicle is reported to be completed in the organ by the $17t^{h}$ day of incubation. Each lymph follicle has a narrow cortex and a large medulla. The corticomedullar border, the continuation of IFE, separates the cortex and medulla (10). In our study, the development of lymph follicle was clearly visible on the 16^{th} day of incubation. On the 21^{st} day, we were observed that histologic development of BF was completed. Advanced follicular development, marked separation cortex-medulla-corticomedullar zone, evident FAE and IFE development, and trabeculae were seen in the organ. According to the results of this study, embryonic development of BF was found to be consistent with the findings of some researchers (4, 22, 26).

As conclusion, obtained results suggest that BF is an important lymphoid organ in terms of maintaining a healthy life of the poultry. Organogenesis and morphometric measurements of BF evaluated with this research by using chick embryos during incubation. It is thought that the data and information obtained from the present study may support the studies planned on this organ.

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