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Research Article

Stereological, Embryological and Histomorphometric Studies on Embryonic Development of Cerebellum and Cerebellar Purkinje Cells in Chicks

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ABSTRACT

The cerebellum which is widely used in studies related to the motor system is responsible for maintaining balance, muscle tone and coordination. In present study, we aimed to evaluate the cerebellum at different incubation periods in terms of stereology, embryology and histomorphometry. Twenty-four Babcock White Leghorn chick embryos were used. All data regarding embryo, egg and cerebellum were measured and calculated. All histologic and histomorphometric examinations were evaluated on the preparations stained with Crossmon's trichrome stain, Kluver-Barrera stain, and silver stain. It was determined that the cerebellum weight, which was 0.0191±0.0064 g on the 10th day of incubation, increased gradually towards to hatching day. On the 10th day, it was seen that a four-layered primitive substantia grisea structure began to form. While primary foliation seen on the 10th day, secondary foliation started on the 13th day. On the 16th day, Purkinje cells forming the stratum gangliosum were arranged almost in a single row. At day 21^{th,} the general structure of the cerebellum was almost similar to that of the adult cerebellum. While the stratum moleculare and granulosum thicknesses were measured in all embryonic periods, the stratum gangliosum thickness could be measured on the 16th and hatching days. As a result, it was thought that the data obtained from this study might be a reference for studies on the cerebellum and especially on motor control disorders.

Keywords: Cerebellum, Cerebellar Purkinje cell, Chick embryo, Embryonic development

Introduction

The cerebellum, which contains more than half of all neurons in the central nervous system, has special importance among other central nervous system organs [1,2]. The cerebellum, responsible for the control and coordination of motor nerves, voluntary motor movements and declarative memory, is known as temporal regulation machine or one of the neuronal clocks [3,4]. It also plays an important role in determining the parasagittal lines of sensory perception, respiration, eye movements, nociception and gene expression [5,6]. Recent studies have moved the cerebellum to a multitasking "neuronal machine" rather than a "small brain" [6]. The cerebellum plays a regulatory role between the brain and organs. The failure of this organ to be fully formed is not life-threatening. However, studies have reported that normal motor behaviors are significantly affected [7,8].

In chickens, the cerebrum, which begins to form on the first day of incubation, is fully functional on the 7th day [9]. It has been reported that there is a narrowing between the mesencephalon and the rhombencephalon on the 4th day of incubation [10], and towards the end of the embryonic period, the cerebellum forms from the dorsolateral of the alar plates of the metencephalon [2,11]. AbidÂ and Al-Bakri [12] reported that the cerebellum surrounding the roof of the IV ventricle is a spherical shaped part of the rhombencephalon and metencephalon. It is connected to the medulla oblongata and pons with the feet called the cerebellar peduncle and is separated from these two structures via the IV ventricle [13,14].

The cerebellum consists of the right and left cerebellar hemisphere and the vermis cerebelli which connects the two hemispheres to the midline [2,7,15,16]. In mammals and birds, there are transverse grooves, called sulci cerebelli, on the outer surface of the hemispheres, and deep folds

that defined as folia cerebelli among these grooves [12,17]. It is reported that due to its curved structure, it has a large surface area of 75% of the brain surface area [1,2]. The degree of folding of the cerebellum, which is one of the most significant differences among vertebrates, is the same in all bird species [18-20]. The vermis cerebellum consists of nine cerebellar folia, separated from each other by sulci. The cerebellum consists of anterior, posterior, and flocculo-nodular lobes and are named cerebro cerebellum, spino cerebellum, and vestibulo cerebellum, respectively [12,21]. It is also reported that the general restructuring of the cerebellum is completed shortly after hatching [10].

Nucleoli are nucleus regions where ribosomal subunits are synthesized. The regions that contain the genes that synthesize ribosomal RNA and form the nucleolus are called nucleolus organizer regions (NORs). Since these regions are argyrophilic, they are stained with silver methods, and these regions are called silver staining nucleolus organizer regions (AgNORs). NORs are indicators of cell proliferation rate according to ribosome formation and protein synthesis of cells [22-24].

Since chicken egg does not have a placental barrier, which shows an embryonic development independent of maternal effects, is accepted as one of the most suitable materials for embryotoxic and teratogenic studies [25-28].

In present study, it was aimed to evaluate the cerebellum taken from chick embryos on different days of incubation (10., 13., 16. and 21. days) in terms of stereological, embryological development and histomorphometry.

Material and Methods

Ethical Statement

Ethical approval was taken from The Ethical Committee of Health Sciences of Karamanoglu Mehmetbey University (protocol number: 2022/19).

Material

For the present study, cerebellums were obtained from 24 Babcock White Leghorn chick embryos on the 10th, 13th, 16th and 21st days of incubation considering organogenesis. According to the determined embryonic days, the randomly-selected eggs were opened from each of the groups until six live embryos were obtained for evaluation in terms of cerebellum development.

Morphometric Measurements

First, the embryo weight, pre-hatching, and initial egg weight were weighed with precision scales and the relative embryo weights (REW, %) were calculated by the following formula (Equation 1) [27].

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

Then, a dissection of the cerebellum was performed from the peripheral cerebrum tissues. Cerebellums taken from embryos according to incubation periods were weighed on precision scales and their weights were recorded. Relative cerebellum weights (RCW, %) were calculated with the following formula (Equation 2) [29].

$$RCW = \frac{\text{Cerebellum weight}}{\text{Embryo weight}} x100$$

Histological Processing of Cerebellum

Routine histological preparation procedures were performed on cerebellums fixed in 10% neutral buffered formalin solution. Using a rotating microtome, three sagittal serial sections of 5 µm thickness were taken at regular intervals from each block for histological examinations and 10 µm thickness sections were taken every 50 sections for cerebellum volume calculation [29]. The sections were oven-dried at 37°C for 24 h and were stained with Crossmon's trichrome stain [29], Kluver-Barrera stain, and silver stain [30]. After examining the stained sections with a camera-attached microscope (Leica DM-2500 attached to a DFC-320 digital camera), digital images of the necessary regions were recorded. All measurements were analyzed with an ImageJ Analysis Program [31]. For the histomorphometric measurements related to Purkinje cells, 25 Purkinje cells having nuclei with definite nucleoli were evaluated. The number of Purkinje cells per unit length (1 mm) of the ganglionic cerebellar layer was counted on digital images. In the examination of cerebellar development in different embryonic periods, cerebellar layer thicknesses and folia

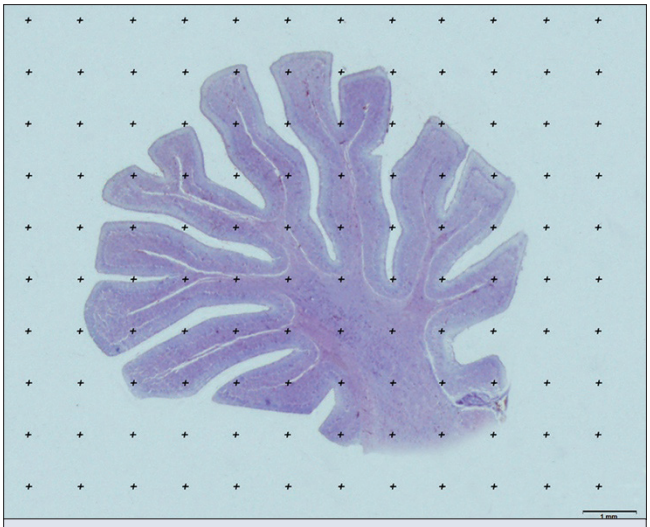

Fig 1. Application of point counting grid on histological sections of the cerebellum (area per point = 1 mm^2)

widths (in six folia) of each animal were measured from six different areas.

Volume Calculations in Cerebellum

The grid function of ImageJ was used in volume calculations and a point counting grid (d=1mm) was placed on the sagittal-section. The points on grey and white matter were counted *(Fig. 1)*. The volumes were estimated as $V = a(p) \times \Sigma p \times t$ formula. In this formula, V is the volume of the structure concerned, a(p) is the area of the one point on the grid (this value is 1 mm^2 in the study), Σp is the sum of the points on the structure of interest and t is the section thickness [29,32].

Statistical Analysis

In the present study, the analysis of the data obtained from the chick embryos was performed using the SPSS software version 21.0 statistical package program. Since the variables showed normal distribution, they were compared among groups using one-way ANOVA test. In cases where the P value was significant, pairwise post-hoc comparisons between statistically significant results were made using the Tukey test. Descriptive analyzes were given using the mean and standard deviation. A value of $P<0.05$ was accepted as the significance limit.

Results

Macroscopical Evaluation

The weights of all eggs, embryos and cerebellums used in the study are given in *Table 1*. According to the data obtained, although there was no statistical difference in both egg weights among different incubation periods, it was observed that the embryo and relative embryo weights increased gradually according to the advancing incubation days ($P<0.001$). The weight of the cerebellum, which was 0.0191±0.0064 g on the 10th day of incubation, increased gradually towards to hatching day, while the relative cerebellum weight decreased ($P<0.001$). When the measurements in *Table 1* are examined, it is seen that the cerebellum volume gradually increases during the advancing incubation periods and, it is statistically significant ($P<0.001$).

Embryonic Development of Cerebellum

The embryonic development of the cerebellum on certain days of incubation are given in *Fig. 2*. The external granular layer, rich in granular cells, was seen on the 10th day of incubation. The marginal layer that will form into the stratum moleculare in future incubation periods was just below of external granular layer. The inner cortical layer and internal granular layer were seen. The presence

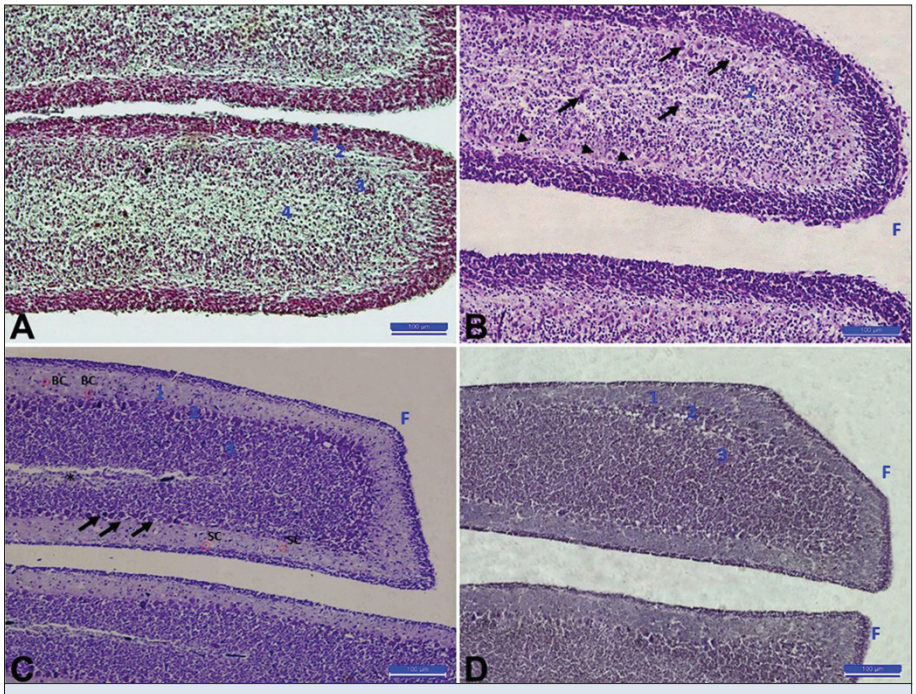

Fig 2. Embryonic development of cerebellum in different embryonic periods. **A:** The cerebellum section from a day 10 chick embryo, 1: External granular layer, 2: Marginal layer, 3: Inner cortical layer, 4: Internal granular layer, Crossmon's trichrome staining. **B:** The cerebellum section from a day 13 chick embryo, 1: Primitive stratum moleculare, 2: Primitive stratum granulosum, Arrows: Precursor Purkinje cells, Arrowheads: Granular cells, F: Foliation, Kluver-Barrera staining. **C:** The cerebellum section from a day 16 chick embryo, 1: Stratum moleculare, 2: Primitive stratum gangliosum, 3: Stratum granulosum, Star: Substantia alba, Arrows: Purkinje cells, F: Foliation, SC: Stellate cells, BC: Basket cells, Kluver- Barrera staining. **D:** The cerebellum section from a day 21 chick embryo, 1: Stratum moleculare, 2: Stratum gangliosum, 3: Stratum granulosum, F: Foliation, Crossmon's trichrome staining

of all these four layers on the 10th day showed us that the primitive substantia grisea structure began to form. During this period, precursor Purkinje cell clusters began to be found. Furthermore, the presence of deep folds that are known as folia cerebelli revealed the initiation of primary foliation. No distinguishable substantia alba structure was observed *(Fig. 2-A)*.

On the 13th day, it was noted that the thickness of the external granular layer decreased. We determined that a primitive stratum moleculare structure was formed. Precursor Purkinje cell rows, arranged in one-two rows, were seen in the inner cortical layer. The dark granular cell clusters migrating from the extra granular layer to the internal granular layer were evident. As such, we can say about a primitive stratum granulosum structure. In addition to the primary foliation, it was observed that secondary foliation started or even progressed. It was observed that the substantia alba, consisting of axons and myelinated nerve fibers, began to become prominent *(Fig. 2-B)*.

In the 16-day-old embryos, the layers forming the substantia grisea could be easily distinguished. In this period, it was observed that the stratum moleculare became prominent. It was noteworthy that Purkinje cells forming the primitive stratum gangliosum were almost arranged in a single row. The inner granular layer was filled with granular cells and was replaced by the stratum granulosum. The substantia alba was clearly visible *(Fig. 2-C)*.

Stratum gangliosum, formed by Purkinje cells with large flask-shaped bodies, central nuclei, dark nucleoli, and dendrites extending into the molecular layer, was easily seen in 21-day-old embryos. In this period, it was remarkable that the stratum gangliosum was similar to the adult cerebellum. At day 21st, the general structure of the cerebellum was almost similar to that of the adult cerebellum *(Fig. 2-D)*. Also, stellate cells were found superficially in the molecular layer. Basket cells, mostly located close to Purkinje cells, were also observed *(Fig. 2-C)*.

Histomorphological Evaluation of the Cerebellum

All histomorphometric measurements done in the cerebellum in this study are given in *Table 1*. While the stratum moleculare and stratum granulosum thicknesses of the cerebellum were measured in all embryonic periods, the thickness of the stratum gangliosum formed by the Purkinje cell row could be measured on the 16th and hatching days. As a result of measurements, it is seen that there is a statistical difference among the stratum moleculare thicknesses during the incubation. The highest value was recorded as 123.55±19.66 µm on the 21st day (P<0.001). Although there is no statistical difference in the thickness of the stratum granulosum, it is noteworthy that there is an increase in this layer thickness throughout incubation (P>0.001).

Among the incubation periods, substantia grisea and substantia alba thicknesses of the cerebellum were statistically significant (P<0.001). While the thickness of the substantia grisea increases throughout all incubation periods, it is seen that this ratio decreases gradually in the substantia alba. Ratio substantia grisea/substantia alba thickness and folia width were statistically significant, and this ratio was highest on the 16th and 21st days (P<0.001). Ratio stratum gangliosum/substantia grisea thickness did not differ statistically (P>0.001).

Purkinje cell count in a unit length (1 mm) was counted from the 13th day of incubation. The highest values were determined on the 16th day of incubation and hatching day (P<0.001). On the 13th day of incubation, the lowest transverse diameter body and nucleus of the Purkinje cell were measured as 4.44±0.47 µm and 2.22±0.41 µm, respectively, (P<0.001). Statistically, the highest nucleus area of the Purkinje cell was on the 21st day of incubation (P<0.001). While the nucleolus area of the Purkinje cell could be detected from the 13th day of incubation, the highest values were observed on the hatching day (P<0.001) *(Fig. 3)*.

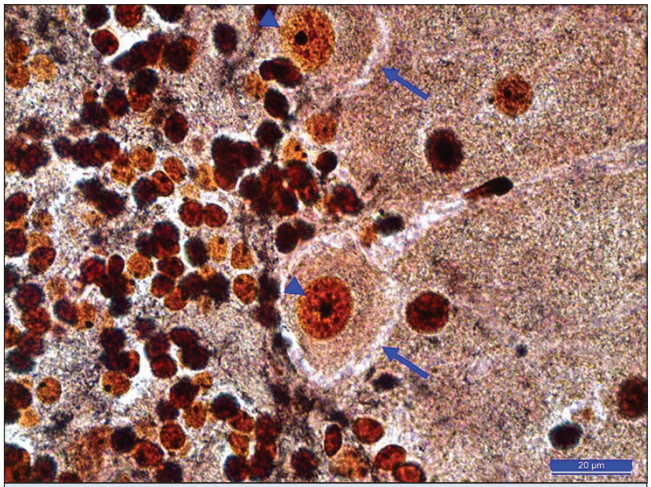

Fig 3. Purkinje cell's histological appearance, Arrows: Purkinje cells, Arrowheads: Nuclei, Silver staining method

Discussion

The cerebellum, which is considered the motor center of the brain, is important in balance and coordination of muscle movements [7,16]. Differences in the volume, morphology and histology of the cerebellum are related to the general anatomical structure and behavioral characteristics of the species [20]. Pal et al.[33] reported that the size and shape of the cerebellum depend on the type of movement, center of

Table 1. *Some morphometric values of the chick embryos according to incubation days (Mean±SD)*

Parameter	Day of Incubation (n=6)			
	10th	**13th**	**16th**	**21st**
Initial egg weight (g)	60.86±6.29	57.91±5.45	58.58±2.76	54.98±3.67
Pre-hatching egg weight (g)	56.40±6.43	53.07±5.09	50.78±3.85	48.87±3.05
Embryo weight (g)	3.06±0.15	8.88±0.57	22.74±2.71	41.17±3.58
Relative embryo weight (%)	5.56[a]	16.90[b]	44.76[c]	84.21[d]
Cerebellum weight (g)	0.0191±0.0064[a]	0.0644±0.0057[b]	0.0844±0.0103[c]	0.0932±0.0146[c]
Relative cerebellum weight (%)	0.72[a]	0.62[b]	0.37[c]	0.23[d]
Cerebellum volume (mm^3)	0.11±0.01[a]	0.31±0.03[a]	0.91±0.15[b]	1.25±0.34[c]
Stratum moleculare thickness (µm)	45.96±9.14[a]	52.69±8.31[ab]	68.48±14.30[b]	123.55±19.66[c]
Stratum gangliosum thickness (µm)	NA	NA	18.32±4.08	22.69±3.77
Stratum granulosum thickness (µm)	94.68±9.62	95.54±10.67	114.69±56.87	159.98±62.82
Substantia grisea thickness (µm)	143.13±24.19[a]	151.97±21.59[a]	199.72±72.34[a]	315.17±97.01[b]
Substantia alba thickness (µm)	NA	83.39±7.62[a]	73.11±13.45[a]	59.67±11.97[a]
Ratio substantia grisea/ substantia alba thickness	NA	1.87±0.28[a]	4.33±1.46[b]	4.74±0.51[b]
Ratio stratum gangliosum/ substantia grisea thickness	NA	NA	0.09±0.03	0.08±0.03
Width of the folia (µm)	344.49±18.63[a]	379.180±51.72[a]	441.06±164.49[ab]	619.26±176.38[b]
Mean Purkinje cell counts in a unit length	NA	NA	42.33±5.72	44.67±7.03
Mean transverse diameters of the Purkinje cell bodies (µm)	NA	4.44±0.47[a]	11.72±1.06[b]	11.79±0.79[b]
Mean transverse nucleus diameters of the Purkinje cells (µm)	NA	2.22±0.41[a]	6.49±0.47[b]	7.69±0.44[c]
Mean nucleus areas of the Purkinje cells (μm^2)	NA	10.31±0.76[a]	30.65±4.39[b]	40.16±3.74[c]
Mean NOR areas of the Purkinje cells (μm^2)	NA	1.87±0.12[a]	4.15±0.51[b]	5.42±1.43[c]

Different letters in the same row [a, b, c, d] indicate significant differences (P<0.001). NA: not available

gravity, and body posture in animals. The more complex the body movements, the more developed the cerebellum. Chicken cerebellum has been reported to be larger in size and weight than that of humans. This situation is related to the balance center and reveals the importance of the cerebellum in birds [34].

In chickens, the cerebellum begins to develop from a thick neuroepithelial layer on the roof of the IV ventricle [10]. Some researchers determined that the substantia grisea develops from the ventricular neuroepithelium and the outer granular layer [24,35]. Feirabend et al.[36] showed that mitotic activity in the outer granular layer started on the 6th embryonic day and intensified until the 18th day. In our study, we found numerous dark granular cell clumps in the outer granular layer from the 10th day of incubation. It was observed that these dark granular cells, which are important for the development of the cerebellum, migrated towards the inner granular layer. This was in line with what some researchers said [24,37]. On the 9th-10th days of incubation, some researchers reported that Purkinje cell clumps begin to appear in the inner cortical layer, which originates from the inner mantle layer. In addition, they have said that these cells take their characteristic arrangement and form the stratum gangliosum during

the incubation periods [16,24]. In present study, it was rarely encountered the inner cortical layer and its precursor Purkinje cell clusters in 10-day-old embryos. It was obvious that a primitive substantia grisea structure consisting of external granular layer, marginal layer, inner cortical layer and inner granular layer was beginning to take shape in the cerebellum *(Fig. 2-A)*.

Feirabend et al.[10] reported that foliation, which is characteristic for the embryonic development of the cerebellum, started to develop on 11th-12th days of incubation. They also found 10 primary folia and reported the secondary foliation in V-IX primary folia in a 13-day-old embryo. Akar and Sur [24] claimed the primary foliation on the 11th day. In present study, primary foliation was first seen in 10-day-old embryos, while secondary foliation was seen in 13-day-old embryos *(Fig. 1)*. Maulana et al.[4] reported that the substantia grisea was formed from the molecular and granular layers on the 7th day of incubation. Studies have shown that Purkinje cells line up in three rows and the granular layer is not yet fully organized [24,35,38]. In this study, it was observed that the primitive substantia grisea structure in 13-day-old embryos developed slightly more than on the 10th day and formed into a substantia grisea consisting of primitive stratum moleculare, primitive stratum granulosum and precursor Purkinje cell lines (two-three rows). Also, some researchers said that the substantia alba, where axons and myelinated nerve fibers are located in the medulla of the cerebellum, become evident on the 14th day of incubation [4,39,40]. On the 13th day of this study, this was consistent with the existing literature *(Fig. 2-B)*.

In chickens, four layers (external germinative layer, the molecular layer, the Purkinje layer, and the internal granular layer) of the cerebellar cortex were seen on 15th-16th incubation days [24,41]. On the 17th day of incubation, it has been reported that Purkinje cells are arranged in a single row and the molecular layer becomes clear [24,35,38,]. In the 16-day-old embryos of this study, there were three layers forming the substantia grisea (stratum moleculare, primitive stratum gangliosum, and stratum granulosum). The single-row arrangement of Purkinje cells forming the primitive stratum gangliosum began to take shape during this incubation period *(Fig. 2-C)*.

Maulana et al.[4] reported that the chicken's cerebellum layer development was completed on the 20th day of incubation and the cerebellum had a loose structure. They stated that they saw the single-row Purkinje cell arrangement between the granular and molecular layers at the hatching and that the development of the cerebellar cortex was completed [24,42]. In the present study, it was seen that the substantia grisea and substantia alba were in the known histological cerebellum structure in 21-day-old embryos. It also detected stellate cells and basket cells in the stratum moleculare, which is made up of small neurons and glial cells, as the researchers said [42-44]. Substantia alba was highly developed compared to other incubation periods *(Fig. 2-C)*.

Histologically, the layers that make up the cerebral cortex are the molecular layer, the Purkinje cell layer, and the granular layer from the outside to the inside, respectively [4,16,42,43]. In female geese aged between 10-12 months, Koral Taşçı and Bingöl [16] found the mean thickness of the molecular and granular layers as 348.53±72 µm and 184.83±48 µm, respectively. In the study of Maulana et al. [4], the molecular layer thickness obtained on the 14th day was higher than the 13th day of our study, while it was lower in terms of the granular layer. Stratum gangliosum thickness was measured from the 16th day until the hatching day, since Purkinje cells did not show a single-row array on the 10th and 13th days of incubation. On 16th-21st days, the values were found 18.32±4.08 µm and 22.69±3.77 µm, respectively. The axons of Purkinje cells reaching the stratum granulosum and being surrounded by myelin sheath in the substantia alba caused an increase in the stratum gangliosum thickness.

In our study, the substantia grisea thickness was higher on the 13th day compared to that of Maulana et al.[4] while the subtantia alba thickness was lower on the 21st day. Whereas the thickness of the substantia grisea was an increase as the incubation progressed, there was a decrease in the thickness of the substantia alba. Because the cerebellum layers were gradually developing during the advancing incubation periods. The pressure formed as a result of the shaping of the layers on the substantia alba, especially the stratum gangliosum, and it caused the substantia alba to shrink. In this study, the ratio substantia grisea/substantia alba thickness was 4.74±0.51% in 21-old-day embryos.

The Purkinje cell is one of the largest neurons of the central nervous system with its dendrites extending to the surface of the cerebellar cortex and myelinated axon extending to the granular layer [45,46]. Sur et al.[42] found the number of Purkinje cells of 1 mm length in turkeys as 16.34±1.47, in ducks 20.98±2.24, in pigeons 27.39±1.05 and in starlings 27.00±0.91. Celik et al.[47] found the number of Purkinje cells in one unit length (486 µm) of the ganglionic layer in rats as 9.38 in males and 9.53 in females. Although there is no embryological study related to this data, we found mean Purkinje cell counts in a unit length (1 mm) as 42.33±5.72 and 44.67±7.03 in 16th day of incubation and hatching day, respectively. The increase in the number of Purkinje cells indicates the functional development of the cerebellum in terms of behavioral and cognitive skills [20].

In laying hens on the hatching day, Turgay-İzzetoğlu et al.[46] nuclear diameter and nuclear area measurements of Purkinje cell are similar to those of Akar and Sur [24].

In addition to these parameters, the mean transverse diameter of the Purkinje cell was also similar with those of Akar and Sur [24]. It is thought that the reasons for the larger data in our study may be some differences such as the incubation conditions, examined Purkinje cells, the image analysis program or the embryos.

Purkinje cells, which establish morpho-functional and synaptic connections that require high metabolic activity, synthesize different amounts of protein at different developmental stages of the embryonic period [48]. NOR areas provide information about the cellular activities of cells, especially protein synthesis [49]. NORs, known as nucleolus forming regions, are associated with the nucleus area. While NOR areas obtained from this study were higher than those of Akar and Sur [24] on the hatching day, this parameter was similar with Turgay-İzzetoğlu et al.[46]. The increases in NOR area, nuclear diameter and area of Purkinje cells are thought to result from increased protein synthesis due to the development of the cerebellum during the advancing incubation days. It is also thought that incubation conditions may cause differences in Purkinje cells, image analysis program or embryos.

As conclusion, the cerebellum which is widely used in studies related to the motor system is responsible for balance, muscle tone and muscle coordination. Although the inability of this organ to fully form is not life-threatening, studies have shown that motor behaviors are significantly affected. It is thought that this study provides new data to the literature about cerebellum development by monitoring the histologic, histomorphometric and stereological developments of chick cerebellum in different incubation periods. In addition, the fact that there has not been a study that gives embryological cerebellum volume data of chicks before increases the importance of the study. Since cerebellum dysfunctions bring along motor control disorders, this organ is used especially in studies on the motor system. The data obtained from this study contribute to the studies that can be done especially on motor control disorders.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (F. Colakoglu).

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Statement

Ethical approval was taken from The Ethical Committee of Health Sciences of Karamanoglu Mehmetbey University (protocol number: 2022/19).

Author Contributions

FC: Conceptualization, methodology, investigation, writing original draft. MLS: Conceptualization, methodology, writing-review and editing.

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