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Morphogenesis of the Spleen and Cloacal Bursa of a Chicken Embryo under the Influence of "Ligfolum" and "Placenta Denatured Emulsified"

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ABSTRACT

The increasing consumption of poultry products and the intensification of production have prompted the investigation of the effect of solutions of "Ligfolum" and "Placenta denatured emulsified" on the development and morphology change of the spleen in chicken embryos, which is the goal of this study. Morphometric and histological methods were used to investigate the features of the development of chicken embryos under the influence of these solutions. Findings showed a significant superiority of the relative bursa mass of embryos of the second experimental group by 37.7% and 6.86% compared to the control group. The absolute weight of the spleen of the first experimental group exceeded that of the control group, while in the second experimental group, the difference between the relative weight of the cloacal bursa in the embryos of the experimental groups and the control group decreased. The histological structure of the spleen and cloacal bursa in embryos of the experimental groups indicates a more intensive development of these organs and their functional activity. The functional activity of the spleen and cloacal bursa in the experimental groups related to their more intensive development. This study has practical significance, as it allows increasing the overall resistance of the body by unlocking the potential of the immune system under the influence of these solutions at the beginning of chicken embryo development.

Key words: Chicken, Immune System, Spleen, Antenatal Ontogenesis, Ligfolum, Histological and Morphological Analysis, BOF.

INTRODUCTION

Both antenatal ontogenesis, through the use of medications and changes in incubation conditions, and postnatal ontogenesis, through the use of various dietary supplements and vaccines, involve active research on the possibility of influencing the immune system. Modern histological and haematological methods are used to investigate the influence of various factors on the organs of the immune system and the morphological composition of blood (Biyashev et al. 2016; Vashchyk et al. 2020). The immune system of a bird undergoes quantitative and qualitative changes in the process of ontogenesis: the laying, development and involution of the organs of the immune system, changes in the morphological composition of the blood take place. The immune system is a collection of organs, tissues, and cells that protect the body from foreign substances, viruses, microorganisms, cancer cells with antigenic properties. The haematopoietic and immune systems have a common origin and functions, so changes in the immune system are reflected in qualitative and quantitative changes in the composition of white blood (Sanketi and Kurpios 2022; Khaliq et al. 2023).

The organs of the immune system are divided into central, where T- and B-lymphocytes differentiate, these include the red bone marrow, cloacal sac, thymus, yolk sac (in the embryonic period), and peripheral, where the immune response is organised after the antigen is detected: the spleen, lymphatic plaques of the caecum and ileum, Meckel's diverticulum, pharyngeal clusters of lymphatic elements in the submucosa of the respiratory tract, the third eyelid gland. The central link of the immune system of birds, unlike mammals, is separated from the hemocytopoiesis system, that is, proliferation, maturation and differentiation of lymphocytes occur in separate specialised organs (thymus, bursal sac) **(**Fan et al. 2020; Qianru et al. 2021; Lu et al. 2023). The organ of the

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immune system, which is possessed exclusively by birds, is the cloacal sac, the diverticulum of the cloaca, the "cloacal thymus", the bursal sac.

The wall of the bursal sac is three-layered, the mucous membrane consists of a covering multi-row cylindrical epithelium and its own plate, in chickens it forms 12-14 folds. Its own plate consists of a network of thin collagen and reticular fibres, between which the follicles are located. The embryonic kidney of the cloacal sac is laid on the 4th-5th day of incubation. On the 10th day, folds appear in the cloacal sac, by the 12th-13th day, reniform growths of the mucosal epithelium appear, and lymphopoiesis begins in them. By the 14th-16th day, these growths become follicles and consist of cortical and cerebral matter. The cortical zone is filled with small and medium-sized lymphocytes, the cerebral one, formed by epithelial tissue, is the receptacle of large and medium-sized lymphocytes (Bastaki et al. 2022; Kolberg et al. 2022).

In addition, eosinophilic granulocytopoiesis occurs outside the follicles, reaching a maximum on the 14th-18th day of incubation. The maximum size of the bursal sac is reached in chickens by the age of 90 days (Oláh et al. 2022). There are stages of early involution, the signs of which are visible at 9-15 weeks, late involution – up to 25-30 weeks and a residual stage. In a day-old chicken, the size of the cloacal sac does not exceed a pea, at 3-4 months of age it is the size of a cherry. The cloacal sac is not detected in chickens by 12 months (Fu et al. 2022; Sun et al. 2023).

The peripheral organs of the immune system of birds include the spleen, Meckel's diverticulum, Harderian gland, and a network of lymphatic vessels and lymphoid tissue scattered in the body. The spleen is formed from a cluster of mesenchymal cells on the 4th-5th day of incubation. During the embryonic period, granulocyto-poiesis and, to a lesser extent, erythropoiesis take place in it (Stepanova 2016). By the 12th-14th days of incubation, lymphopoiesis begins in the spleen, at the same time the arterial network develops. This organ is a cluster of reticular tissue, the stroma is poorly developed, there are no trabeculae. In the spleen, a white pulp is isolated – a cluster of lymphocytes at different stages of differentiation, and a red pulp, in which red blood cells, granulocytes, macrophages, and other blood cells are found. The spleen participates in the production of T- and B-lymphocytes, destroys old red blood cells, participates in immune reactions. The study by E.V. Stepanova (2016) on the age morphology of the spleen showed that there were no lymphoid nodules in the spleen of 1–15-day-old chickens, indicating the functional maturity of the organ. They appeared on average on the 25th day of postnatal development. At the time of hatching, there is no differentiation of the spleen parenchyma into red and white pulp. From the initial to the pre-slaughter period, heterochronous growth of the structural components of the spleen occurs. Its absolute mass in the process of postnatal development of a bird increases by 16.3 times, length – by 3.8 times, width – by 4.6 times (Jax et al. 2022).

Currently, a lot of work is being done to investigate the effects on the immune system of chickens in the antenatal period by introducing various substances to increase the overall resistance of poultry (Saint-Martin et al. 2022).

"Ligfolum" and "Placenta denatured emulsified" are two drugs that have been studied for their effects on the morphogenesis of the spleen and cloacal bursa in chicken embryos. "Ligfolum" is a medication that contains the active ingredient glycine, which is known to have anti-inflammatory and antioxidant properties. It has been suggested that "Ligfolum" may have a positive effect on embryonic development by reducing oxidative stress and inflammation. "Placenta denatured emulsified" is a drug derived from placental tissue and is believed to have immunomodulatory and regenerative properties. Studies have suggested that "Placenta denatured emulsified" may have a positive effect on the development of the spleen and cloacal bursa by promoting tissue regeneration and modulating the immune response. Further research is needed to fully understand the potential benefits of these drugs in embryonic development. The purpose of this study is to investigate the effect of solutions of "Ligfolum" and "Placenta denatured emulsified" on the process of emergence and shape change of the spleen and cloacal bursa in chicken embryos.

MATERIALS AND METHODS

This study involving the use of chicken eggs with live embryos was conducted in accordance with the ethical principles and guidelines for animal research established by the Shakarim University Institutional Animal Care and Use Committee (IACUC). The experiment was conducted with the highest level of animal welfare and care.

The experiment was carried out on chicken eggs with live embryos in the laboratory of Shakarim University. The sanitary and epidemiological situation of poultry content corresponded to the standards prescribed in the Research and Technological Poultry Institute. For the experiment, 35 eggs were used for two experimental and one control groups, and tested for their suitability for incubation. There are three groups in total: control, first and second experimental. At the same time, the eggs were checked for the degree of contamination, the degree of marbling of the shell, and the weight of the eggs was recorded. Incubation was carried out in an ILB-0.5 incubator, with automatic egg rotation and temperature and humidity control. To introduce the solution into the egg, the deep treatment method was used. Before starting the experiment, the incubator and eggs of experimental group 1 were heated to 37.8°C. These eggs were then exposed to the "Ligfolum" solution. The same thing was done with the eggs of the experimental group No. 2, only they were immersed in a solution of the denatured emulsified placenta. The temperature of both solutions corresponded to 16°C, the exposure time of the solutions was 20 minutes (Migachev and Suleimanov 2021). After holding the appointed time, the eggs were removed from the solution, allowed to dry in the air and placed in an already warmed incubator to form a chicken embryo.

In this study, a statistical analysis was conducted to assess the differences in organ development between the control group and the experimental groups. The analysis included measures of central tendency and variability, such as mean and standard deviation, as well as inferential statistics, such as t-tests and ANOVA, to determine the significance of the observed differences. The statistical

analysis was performed using appropriate software and a significance level of P<0.05 was used to determine whether the differences between groups were statistically significant. To calculate the relative weight of the organs, the weight of the organ was divided by the weight of the whole embryo and expressed as a percentage. The data were then analyzed using t-tests and ANOVA to compare the mean relative weight of the organs between the control and experimental groups at different stages of embryonic development. Overall, the statistical analysis was important for determining whether the observed differences in organ development between the control and experimental groups were statistically significant, and to provide evidence to support the conclusions drawn from the study (Verma et al. 2019).

According to the data obtained, conclusions were drawn about the effectiveness of processing. The control group also included 35 eggs that were not treated with one of these solutions. The incubation regime corresponded to all sanitary and epidemiological standards for the incubation of poultry eggs. To apply the active solutions, the timing of laying chicken organs was considered: to investigate the effect of the Ligfolum solution, it was administered on the second day from the start of incubation, and the denatured emulsified placenta solution was applied on the fourth day from the start of incubation. Egg weight data was used to calculate the relative weight of the embryo, and the relative mass of organs was obtained based on the data of the weight of the embryo. The equation was used to calculate the relative growth rate.

Every day, 5 eggs were opened at the same time. The studied organs were extracted from the embryos: the spleen and cloacal bursa (Fig. 1). Laboratory electronic scales were used to measure the bodyweight of the embryo and the weight of the extracted organs. A caliper was used to measure the embryo. The samples obtained correspond to the incubation periods from 9 to 20 days. And for histological studies, a 10% formalin solution was used, paraffin for filling. Then the sections with a thickness of 7 microns were stained with haematoxylin and eosin. Microphotographs of organs were made using a "Mikmed-3" microscope. An eyepiece-micrometre was used for micrometry of histological components of the cloacal bursa and spleen. This study investigated the absolute and relative weight of the spleen in two experimental and control groups from 9 to 20 days of embryonic development, to identify the difference, and to establish the effectiveness of the solutions used (Verma et al. 2019). Cloacal bursa is an immune lymphoepithelial organ that is laid for the first time on the 5th day of antenatal development. Therefore, the cloacal bursa was examined only from the 8th day of embryonic development, and in the spleen, the relative and absolute masses of the cloacal bursa were studied (Rana et al. 2019; Stefaniak et al. 2020).

RESULTS

The morphological characteristics of the spleen were studied from the 9th day of embryonic development (Table 1). The absolute mass of the spleen in the control group from day 9 to 11 of embryonic development was inferior to the mass of the organ of the first experimental group with a significant difference of 26.42; 79.10; 16.04%, respectively. On the 15th day of incubation, it was possible to detect a difference in the weight of the spleen in embryos of experimental group No. 1 in relation to the control group, its indicators significantly exceed those of the control group, but were similar towards the end of antenatal ontogenesis. The

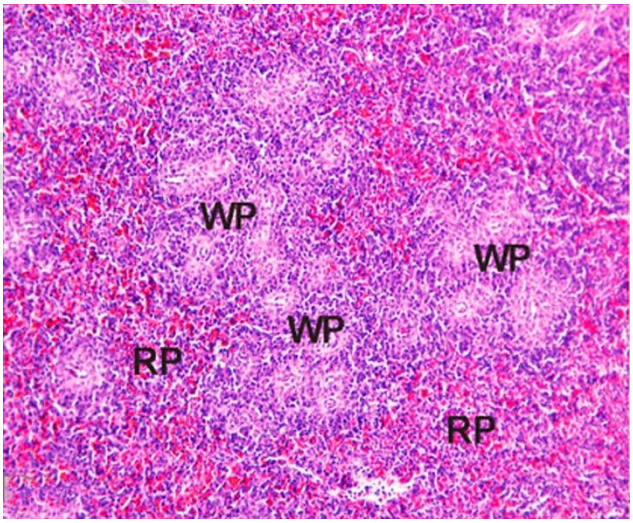

Fig. 1: Histological structure of the chicken spleen. WP: white pulp; RP: red pulp.

Table 1: Changes in spleen weight during egg incubation

Day	Absolute weight of spleen, mg (M±m)			Relative weight of spleen, % (M±m)		
	Control group	Experimental group No.1	Experimental group No. 2	Control group	Experimental group No.1	Experimental group No. 2
9	0.53±0.03	0.67±0.03*	0.60±0.06	0.033±0.002	0.042±0.002*	0.031±0.003
10	0.67±07	1.21±0.12*	1.13±0.03**	0.035±0.03	0.055±0.005*	0.042±0.001
11	1.87±0.3	2.17±0.17	2.27±0.22	0.071±0.0012	0.076±0.006	0.067±0.006
12	3.00±0.6	3.23±0.15	3.03±0.06	0.074±0.0014	0.054±0.002**	0.055±<0.001***
13	5.07±0.7	5.0±0.58	4.97±0.27	0.082±0.001	0.07±0.008	0.068±0.004*
14	6.33±0.03	6.53±0.20	6.30±0.1	0.068±0.0004	0.065±0.002	0.067±0.001
15	8.13±0.12	9.53±0.32*	7.40±0.06**	0.063±0.0009	0.074±0.003*	0.057±0.0004**
16	9.50±0.31	10.40±0.25	9.63±0.13	0.061±0.002	0.068±0.002*	0.061±0.0008
17	10.73±0.23	13.40±0.32**	10.40±0.40	0.057±0.0012	0.073±0.0017**	0.053±**0.002
18	12.4±0.09	13.5±0.25*	12.67±0.18	0.056±0.0004	0.060±<0.001*	0.052±0.0007**
19	13.2±0.3	14.5±0.09*	14.07±0.22	0.052±0.0014	0.053±0.0003	0.051±0.0008
20	13.8±0.09	14.6±0.23*	14.61±0.13**	0.045±0.0003	0.044±0.0007	0.046±0.0004

Note: *– significant difference (P<0.05); ** – significant difference in statistics (P<0.01); *** – high significant difference (P<0.001).

difference was 17%. And accordingly, the indicators were equalised on day 20. As for the absolute values, here they exceeded those of the experimental group No. 2 and amounted to 68 and 5%, respectively, on the 10th and 20th days of development, and on the 15th day there was a reverse trend in the control group, these values exceed by 9%. On the 12th, 14th, and 16th days of incubation, the weight indicators in both experimental groups are similar.

The spleen differs in general by a small specific gravity in the control and in both experimental groups. The study of the effect of the treatment of incubation eggs with solutions of "Ligfolum" and "Placenta denatured emulsified" did not show a high degree of influence on the change in the relative weight of the spleen. The relative mass of the organ of the embryos of the first experimental group exceeds the relative mass of the spleen of the embryos of the control group almost throughout the embryonic development, except for 12 and 13 days of incubation, when the embryo of the first experimental group itself grows with great intensity. On the 9th day of development, the difference in indicators is 27.27%, on the 15th day – 17.46% with a significant difference, and from the 18th day until the end of the period of embryonic development, the difference between the relative weight of the spleen in the control and first experimental groups disappears (Dunislawska et al. 2023).

The relative weight of the spleen of the embryos of the control group at the beginning of the study is almost equal to the relative weight of the organ of the embryos of the experimental group No. 2, but subsequently, almost the entire period of embryonic development exceeds it. On days 12, 15, and 18, –this superiority is statistically and highly reliable and amounts to 34.55; 10.53, and 7.69%, respectively. The decrease in the relative weight of the spleen in the second experimental group during these periods can be explained by the higher growth rate of the embryo itself. On the 16th and 19t-20th days of embryonic development, the difference between the relative weight indicators in the control and second experimental groups practically disappears (Isupova et al. 2019).

Solutions of "Placenta denatured emulsified" and "Ligfolum" positively affected the developing embryo, the development of its cloacal bursa (Nalyotova and Kushkina, 2019). The results obtained during the study are shown in Tables 2, 3.

Histological examination of cloacal bursa was performed on the 8th day of embryonic development. It was found that these bag weights in embryos of experimental group No. 1 exceed the control group by 40%. On day 10, the data of the absolute weight of the embryo sac of this group exceed the control by 33.0%. On the 14th day of antenatal development, the weight of the organs in both groups is identical, but there are changes on the 15th day and until the end of embryo development in favour of the embryos of the experimental groups, since the weight of the cloacal sac exceeds the weight of the organ in the control by 30%. The relative mass of bursa in embryos of experimental group No. 1 from the 8th to the 10th day of development exceeds the control indicator by 23.13; 22.02, and 17.14%, respectively, with a significant difference. From the 12th to the 14th day of development, a statistically significant superiority of the relative bursa mass in control embryos by 9.43-43.9% is determined. From 15 to 18 days, bursa in embryos of experimental group No. 1 is characterised by more intensive development, as evidenced by an increase in its relative weight compared to the control during this period by 4.63-34.31% with a statistically significant difference.

The absolute mass of cloacal bursa of embryos of experimental group No. 2 also exceeds the bursa mass of control embryos from 8 days of embryonic development by 70.07% with a significant difference. On the 10th and 15th days of development, the difference is highly significant, and the bursa mass in the experiment exceeds the control indicator by 49.26% and 18.11%, respectively. Up to 18 days, such superiority persists with a decreasing difference in indicators, on the 18th and 19th days the bursa mass is equal in both groups, and by the end of embryo development, an unreliable superiority of 1.21% is noted in favour of the embryos of the second experimental group. A significant superiority of the relative bursa mass of embryos of experimental group No. 2 by 37.61 and 6.86% compared to the control is noted on the 9th and 16th days of development, respectively. 15 days are distinguished by the superiority of the relative

Table 2: Change in the absolute weight of the cloacal pouch of a chicken embryo

Absolute weight of cloacal sac, mg (M±m)		
Control	Experimental group No.1	Experimental group No. 2
1.47±0.22	2.07±0.12	2.50±0.12*
1.73±0.09	2.11±0.11*	2.83±0.17**
2.03±0.07	2.70±0.12**	3.03±0.07***
2.90±0.31	3.33±0.12	4.07±0.09*
4.77±0.32	4.90±0.15	6.17±0.15*
8.30±0.06	7.67±0.32	8.27±0.23
10.77±0.09	10.78±0.13	10.67±0.29
12.70±0.21	15.61±0.45**	15.01±0.10***
15.93±0.32	20.80±0.7**	17.17±0.19*
22.40±0.12	24.22±0.72	23.43±0.90
23.83±0.95	25.51±0.21	23.81±0.41
28.71±0.98	28.97±0.64	28.87±0.15
31.47±0.81	31.93±0.12*	31.85±0.35

Note: *– significant difference (P<0.05); ** – significant difference in statistics (P<0.01); *** – high significant difference (P<0.001).

Table 3: Change in the relative weight of cloacal sac of a chicken embryo

Relative weight of cloacal sac, % (M±m)		
Control	Experimental group No.1	Experimental group No. 2
0.160±0.020	0.197±0.011	0.211±0.010
0.109±0.006	0.130±0.006*	0.150±0.009*
0.105±0.003	0.121±0.005*	0.111±0.002
0.110±0.011	0.117±0.004	0.120±0.003
0.118±0.008	0.082±0.003*	0.114±0.003
0.135±0.001	0.108±0.004**	0.113±0.003**
0.116±0.001	0.106±0.001**	0.114±0.003
0.098±0.002	0.121±0.003**	0.115±0.001***
0.101±0.002	0.140±0.005**	0.109±0.001*
0.120±0.001	0.130±0.004*	0.120±0.005
0.108±0.004	0.111±0.001	0.097±0.002
0.111±0.004	0.105±0.002	0.108±0.001
0.100±0.003	0.097±0.0004	0.101±0.001

Note: *– significant difference (P<0.05); ** – significant difference in statistics (P<0.01); *** – high significant difference (P<0.001)

mass of embryos of the experimental group No. 2 by 17.35% with a highly reliable difference. In the remaining periods of the study, there is no significant difference in indicators, and by the time of the end of antenatal ontogenesis, the relative bursa mass in embryos of both groups is equalised (Salautin et al. 2019). The mortality rate for this study was 1%.

DISCUSSION

Studies by Stepanova (2016) on the age morphology of the spleen showed that there were no lymphoid nodules in the spleen of 1–15-day-old chickens, indicating the functional maturity of the organ. They appeared on average on the 25th day of postnatal development. At the time of hatching, there is no differentiation of the spleen parenchyma into red and white pulp. From the initial to the pre-slaughter period, heterochronous growth of the structural components of the spleen occurs. Its absolute mass in the process of postnatal development of a bird increases by 16.3 times, length – by 3.8 times, width – by 4.6 times (Tsareva 2018).

The immune system has been studied relatively recently, at the moment scientists face many more questions and tasks. Promising areas for studying the effect of biologically active substances, electromagnetic waves, ionising and laser radiation on the chicken embryo on the stimulation of the organs of immunogenesis, substantiation and introduction of dietary supplements that stimulate the immune system, the effect of feed components, drugs, and other chemicals (Suleymanov and Migachev 2020). However, it is important to understand that the immune system is a complex mechanism of interaction between cells and organs, and violation of this mechanism can lead to various pathologies, such as oncological, autoimmune diseases, sensitisation of the body, and allergic reactions (Dunislawska et al. 2023). It is also worth noting that the size and histological structure of immunocompetent organs are only indirect signs of an increase in the overall resistance of the animal. To prove the positive effect of dietary supplements or effects in the antenatal period on the immune system, it is possible to use immunological assessment methods, such as assessing the phagocytic activity of leukocytes, determining the total and relative number of subpopulations of T- and B-lymphocytes.

According to Timchenko et al. (2011), during the development of the embryo, there are four periods of increasing the concentration of alpha-fetoprotein in the homogenate of the chicken embryo associated with the development of the embryo itself. These periods fall on the 5th, 10th, 15th, and 18th days of its development. On the 5th day of incubation, active processes of the proliferation of all tissue components of the embryo occur, in all the groups studied by us there is an increase in the intensity of growth of body weight of embryos at this age. On the 10th day of development, skeletal muscles are intensively formed, active proliferation of cellular elements continues at the sites of development of the organs of the reproductive system and intestines, the embryos of the control and first experimental groups have an increase in the relative growth rate of body weight (Vilches-Moure 2019). On the 15th day of incubation, there was an increase in the intensity of growth of the mass and length of the embryo in experimental group No. 2. On the 18th day of incubation in the first and second experimental groups, the intensity of embryo mass growth increases, and in the control group this occurs only on the 20th day of incubation. Current study and (Timchenko et al. 2011) share a common focus on the development of chicken embryos and the changes that occur during specific stages of development. In both studies, changes in the growth rate of the embryo and the development of certain organs are observed during specific periods of embryonic development. However, the current study specifically focuses on the effects of two substances on the development of certain organs, whereas the previous study was a more general investigation of the developmental stages of chicken embryos.

Changes in the immune system and related changes in the morphological composition of blood occur both in the antenatal and postnatal period, and they are associated with the development of the immune system, haematopoiesis, and the adaptation of the body to the conditions of the outside world, the gas composition of the atmosphere, viruses, bacteria, and protozoa. Research on increasing the overall resistance of the body by unlocking the potential of the immune system under the influence of various factors is an important and promising area in poultry farming.

Conclusion

Histological and hematological methods are being used to explore the effects of medications, changes in incubation conditions, and dietary supplements on the immune system and blood composition. This study examined the impact of "Ligfolum" and "Placenta denatured emulsified" solutions on the development of the spleen and cloacal bursa of chicken embryos using morphometric and histological methods. The results indicated that the use of these drugs had a positive effect on the development and functional activity of these organs during specific periods of antenatal ontogenesis in chickens. The relative bursa mass of embryos in the second experimental group showed significant superiority compared to the control group, and the histological structure of the spleen and cloacal bursa in experimental group embryos demonstrated more intensive development and functional activity. The study suggests that unlocking the potential of the immune system at the beginning of chicken embryo development through the use of these solutions can increase overall resistance in poultry farming. The limitation of the current study is the lack of colored figures for the incubated or hatched embryos. These findings have practical significance and represent a promising area for future research. Based on the findings of the study, the authors can recommend the use of these results to positively influence the development of the spleen and cloacal bursa in chicken embryos. Further research can be conducted to explore the potential of these supplements in improving the immune response of chickens.

Conflict of Interest

The authors have no conflicts of interest to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Dauletbek Muratbayev, Akerke Ygiyeva and Yermekkazy Bilyalov. The first draft of the manuscript was written by Olga Zaikovskaya and Assel Zhexenayeva and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

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