Kocatepe Veterinary Journal

Kocatepe Vet J. (2023) 16(1):47-56

DOI: 10.30607/kvj.1223940

RESEARCH ARTICLE

The Effects of In Ovo Administered Monosodium Glutamate on the Embryonic Development of Skeletal Muscle in Chickens

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ABSTRACT

The aim of this study was to investigate the effects of monosodium glutamate (MSG) in ovo administration on embryonic skeletal muscle development in chicken embryos, using histological, histopathological, and immunohistochemical methods. In this study, a total of 410 fertilized chicken eggs were used and were divided into five group as control, vehicle control, low-dose group (0.12 mg/g egg MSG), medium-dose group (0.6 mg/g egg MSG), and high-dose group (1.2 mg/g egg MSG). At incubation days 18 and 21, eggs from each group were opened and six live embryos were obtained. The embryos were sacrificed by decapitation, and skeletal muscle tissue samples (musculus fibularis longus and musculus sternocoracoideus pectoralis) were obtained. Sections were stained with hematoxylin-eosin. Moreover, caspase-3 reactivity was determined using the immunohistochemical method. Muscle development was delayed in the MSG groups and the number of caspase-3-positive cells was higher ($p < 0.05$) than in the controls. Histopathological examinations revealed degenerative and necrotic changes in the skeletal muscle. Muscle degeneration (edema, muscle fiber degeneration, Zenker's necrosis, and mononuclear cell infiltrations) were observed in all groups, except for the control groups. It was concluded that MSG could adversely affect the development of the skeletal muscle.

Keywords: Caspase-3, Embryotoxicity, Monosodium glutamate, Skeletal muscle

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Yumurtaya Verilen Monosodyum Glutamat'ın Tavuklarda İskelet Kasının Embriyonik Gelişimi Üzerine Etkileri

ÖZ

Bu çalışmanın amacı yumurtaya verilen monosodyum glutamat (MSG)'ın civcivlerde iskelet kasının embriyonik gelişimi üzerindeki etkilerinin histolojik, histopatolojik ve immunohistokimyasal yöntemlerle araştırmaktı. Bu çalışmada 410 adet döllü tavuk yumurtası kullanıldı ve kontrol, taşıyıcı kontrol, düşük doz grup (0.12 mg/g MSG), orta doz grup (0.6 mg/g MSG) ve yüksek doz grup (1.2 mg/g MSG) olarak 5 gruba ayrıldı. Kuluçkanın 18 ve 21. günlerinde, her bir gruptan yumurtalar açıldı ve 6 canlı embriyo elde edildi. Embriyolar dekapitasyon ile sakrifiye edildi ve iskelet kası (musculus sternocoracoideus pectoralis, musculus fibularis longus) doku örnekleri alındı. Kesitler Hematoksilen-Eozin boyama yöntemi ile boyandı. Caspase-3 reaktivitesi ise immünohistokimyasal yöntemle belirlendi. Kas gelişiminin MSG uygulanan gruplarda geri kaldığı ve caspase-3 immunpozitif hücrelerin kontrol grupları ile karşılaştırıldığında daha fazla olduğu dikkati çekti ($p<0.05$). Histopatolojik incelemelerde iskelet kasında dejeneratif ve nekrotik değişiklikler saptandı. Kontrol ve distile su grubu hariç tüm gruplarda kaslarda dejenerasyon (ödem, kas fibrillerinde dejenerasyon, Zenker nekrozu ve mononükleer hücre infiltrasyonu) gözlendi. MSG'nin iskelet kası gelişimi üzerine olumsuz etkilerinin olabileceği sonucuna varılmıştır.

Anahtar Kelimeler: Embriyotoksisite, İskelet kası, Kaspaz-3, Monosodyum glutamate

To cite this article: Bölükbaş F. The Effects of Monosodium Glutamate on Skeletal Muscle Development in Chicken Embryos. Kocatepe Vet J. (2023) 16(2):47-56

Submission: 25.12.2022 **Accepted**: 24.02.2023 **Published Online**: 03.03.2023
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INTRODUCTION

Food additives are substances used in the food industry to extend the shelf-life of foods, increasing their flavor, color, durability, and consumer acceptability (Chakraborty 2019). People are exposed to these chemicals in their daily food consumption (Adeyemo & Farinmade 2013). Monosodium glutamate (MSG, E621), a sodium derivative of L-glutamate, is one of the most widely used food additives as a flavor enhancer in processed and convenience foods worldwide due to its umami taste and flavor-enhancing properties (Hamza and Diab 2020). Many processed foods contain MSG (e.g., fast food, frozen food, instant soup, broth (bouillon), preserves, processed meats such as sausage and salami, salad dressing, flavored snacks, and potato chips) (Abdel Moneim et al. 2018).

MSG has been reported to cross the placental barrier in rats. Subcutaneous MSG injections to pregnant rats have been reported to cause neuronal cell death in the rats and their fetuses (Toth et al. 1987). Experimental studies have shown that exposure to MSG during the embryonic period, which is critical for the development of living things, can lead to serious consequences (Banerjee et al. 2021, Bölükbaş and Öznurlu 2021). Studies on experimental animals have shown that MSG is associated with negative side effects such as genotoxicity, neurotoxicity, diabetes, obesity, and hepatotoxicity (Banerjee et al. 2021). MSG has been reported to induce oxidative stress in different organs including the kidneys, liver, genitals, and thymus (Pavlović et al. 2007, Zanfirescu et al. 2019). In addition, MSG administration has been associated with biochemical and morphological changes in cardiac tissue and changes in heart rhythm (Baky et al. 2009, Kumar and Bhandari 2013, Kingsley et al. 2013, Liu et al. 2013).

Muscle development occurs in hypertrophy and hyperplasia phases: Hypertrophic phase is characterized by the formation of new myofibrils in the muscle fibers (Remignon et al. 1995, Velleman 2007). Hyperplasia is characterized by an increase in the number of myoblast cells during the embryonic period (Latshaw 1987, Moore et al. 2005). The hyperplasia phase is largely completed during the incubation period, and the number of muscle fibers remains constant thereafter (Öznurlu et al. 2022). Therefore, the factors affecting muscle formation in the embryonic period may also affect the developmental performance of the chick in the future.

The aim of this study was to investigate the possible effects of MSG injected into the yolk on skeletal muscle development in chickens, using histological, histopathological, and immunohistochemical methods.

MATERIALS and METHODS

Animal Procedures

This study was conducted with the approval (Date: 02.06.2022, Protocol number: 2022/59) of the Ethics Committee of Selcuk University Faculty of Veterinary Medicine Experimental Animals Production and Research Center. 410 fertile chicken eggs obtained from the commercial Babcock breed were used. MSG (Sigma-Aldrich, St. Louis) was weighed with a precision scale, centrifuged, and diluted by distilled water that had been sterilized in an autoclave. MSG doses for each group were determined according to the 50-55 g egg weight (0.12 mg/g MSG (6–6.6 mg/egg), 0.6 mg/g MSG (30–33 mg/egg), and 1.2 mg/g MSG (60–66 mg/egg)).The eggs were disinfected in a closed cabinet for 15 min before the injection procedure (21 g of potassium permanganate + 42 mL of formaldehyde/m3). The eggs were randomly selected and divided into five groups. While no treatment was applied to the eggs in the control group (n = 40), the vehicle control group (n = 62) received a volume of 100 µL of distilled water by injection. MSG (100 µL) was injected into the eggs in the experimental groups at doses of 0.12 mg/g (low-dose, n = 80), 0.6 mg/g (medium-dose, n = 90), and 1.2 mg/g (high-dose n = 138) per egg. The test solutions were injected into the yolk by sterile insulin injectors at the beginning of incubation. Then, the injection site was sealed with paraffin wax. Incubation processes were incubated by turning 180º once every 2 hours under optimal conditions (37.5 ºC temperature and 65% relative humidity) in the incubator (Imza Technical Equipment).

Histological and Immunohistochemical Investigations

On the 21st and 18th days of incubation, eggs were randomly selected and opened until six viable embryos were obtained from each group. Pectoral muscle (musculus sternocoracoideus pectoralis) and leg muscle (musculus fibularis longus) tissues were taken and fixed in 10% neutral buffered formalin solution (pH 7.4) for 24 h. Tissue samples processed by routine histological methods were embedded in paraffin. The sections taken from the blocks (thickness = 6 µm) were examined using the hematoxylin and eosin (H & E) staining method to determine the general histological structure.

Tissue samples used for immunohistochemical procedures were stained by applying a procedure based on the streptavidin-biotin-peroxidase complex (SABC). Briefly, 6-µm-thick sections were placed on poly-L-lysine-coated slides and dried in an oven at 37 °C overnight.

The sections were deparaffinated in xylol, rehydrated in alcohol, and boiled in citrate buffer solution (pH 6) for 5 min in a microwave oven to reveal antigenic epitopes (antigen retrieval). To inhibit endogenous peroxidase activity, sections were kept in 3% hydrogen peroxide solution for 20 min and incubated in blocking solution (Thermo Fisher Scientific Inc., UK) for 5 min, thus blocking nonspecific binding sites. The sections were then incubated at room temperature for 1 h with a primary antibody (anti-CASP3 antibody (STJ97448), St John's Laboratory, London) followed by a biotinylated goat polyvalent antibody (Thermo Fisher Scientific Inc., UK) for 30 min. Then, all sections were washed with buffered phosphate saline (PBS, Biotech) were incubated with streptavidin-peroxidase (HRP, Thermo Fisher Scientific Inc., UK) at room temperature for 30 min. 3-3'-Diaminobenzidine (DAB, Thermo Fisher Scientific Inc., UK) was used as a chromogen, whereas Mayer's hematoxylin solution was used for nucleus staining. Negative controls were prepared by incubating tissue sections with PBS instead of primary antibodies. All stained sections were examined under a light microscope and digital images of the required areas were recorded.

Immunohistochemical Scoring Method

Allred scoring system was considered for immunohistochemical scoring as previously described (Qureshi and Pervez 2010, Özgermen et al., 2022) Ten different photographs of each animal, including the musculus sternocoracoideus pectoralis and musculus fibularis longus, were taken under a light microscope of 400× magnification. The Allred score between 0 and 8 was determined for each animal by combining the staining intensity score and the staining prevalence
value. The mean value of 10 staining surface areas of each slide was accepted as the score of the relevant case.

Statistical Analysis

The collected data were analyzed by one-way ANOVA (one-way analysis of variance) using IBM SPSS Statistics v.22. Tukey's HSD test was performed to determine the significance of the differences between the mean values of the groups ($p < 0.05$).

RESULTS

On the 18th day of incubation, the myotube organization formed by the combination of myoblasts in both leg and pectoral muscle sections was completed in the control groups. The nuclei of the myoblasts that shaped the myotubes were oval and located in the center of the myotube. In addition, with the formation and organization of myofibrils, the transverse banding began to become evident, and the nuclei began to be pushed to the periphery (Figures 1A and 2A). On the other hand, It was observed that myotube organization was delayed (irregular myotubes) and transverse banding was weak in MSG groups. (Figures 1B-D and 2B-D). In contrast with the control groups, the MSG groups exhibited degenerative and necrotic changes such as muscle fiber degeneration, Zenker's necrosis, and mononuclear cell infiltrations (Figures 1B-D and 2B-D). Leg and pectoral muscle sections stained with immunohistochemical method of 18-day embryos, the most intense caspase-3 positive staining was observed in the MSG groups (Figure 3B-D, 3F-H).

On the 21st day of incubation, the transverse banding became quite evident in the control groups, the muscle fibers gained their normal histological structure, and the nuclei were just below the sarcolemma (Figures 4A and 5A). Compared to the control groups, the MSG groups exhibited an irregular formation of myotubes and weak transverse banding. In addition, histopathological evaluations revealed degenerative and necrotic changes such as muscle fiber degeneration, Zenker's necrosis, and mononuclear cell infiltrations in the MSG-treated groups (Figures 4B-D and 5B-D). Leg and pectoral muscle sections stained with immunohistochemical method of 21-day chick, the most intense caspase-3 positive staining was observed in the groups treated with 0.6 mg/g MSG and 1.2 mg/g MSG. The number of caspase-3-positive cells in the skeletal muscle was significantly higher in the MSG-administered groups than in the control groups ($p < 0.05$, Figure 6A-B, and Figures 7B-D and 7F-H).

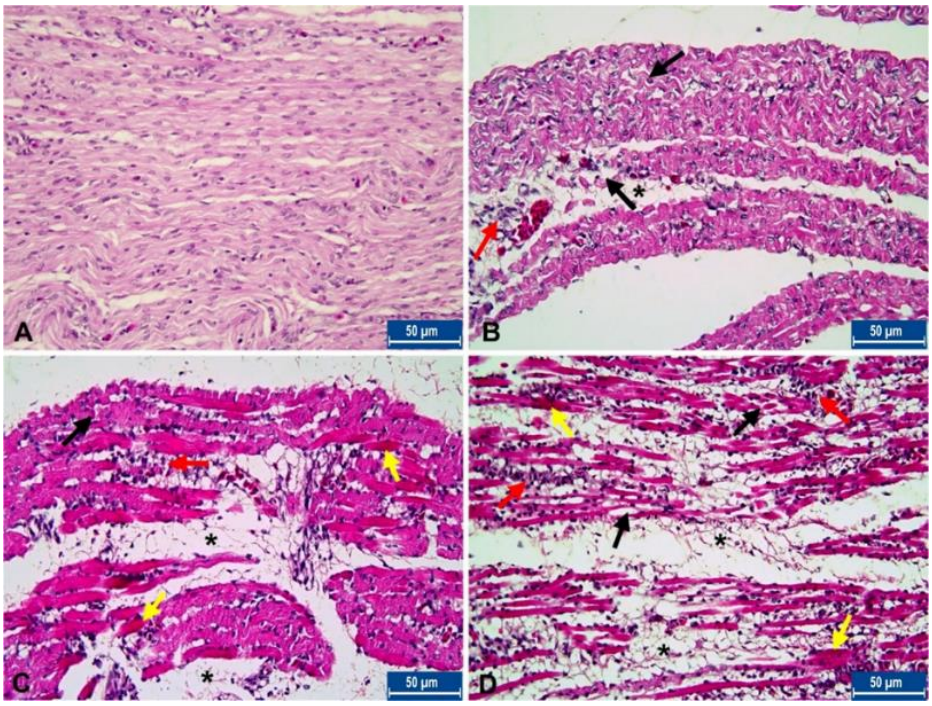

Figure 1. Sections of the leg muscle in the control (A), low-dose MSG group (B), medium-dose MSG group (C), and high-dose MSG group (D) on the 18th day of incubation. Compared to the control groups, myotube organization is delayed and transverse banding is weak in the MSG groups. Degenerating muscle fibers (black arrows), edema (stars), Zenker's necrosis in the muscle fibers (yellow arrows), and mononuclear cell infiltrations (red arrows) between the fibers in the MSG groups. H & E.

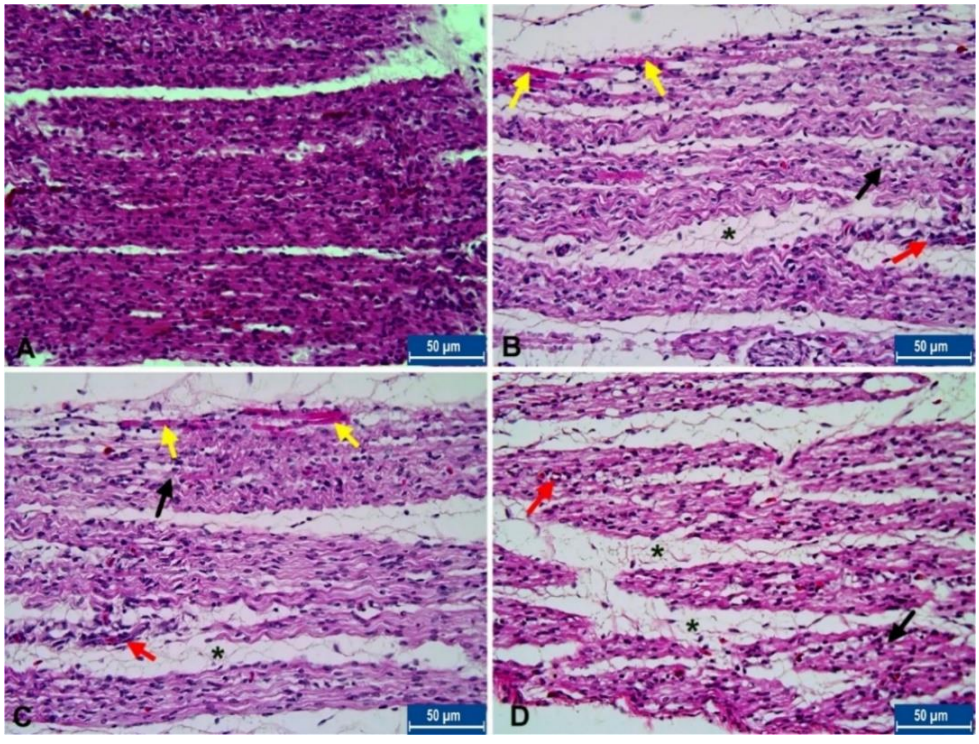

Figure 2. Sections of the pectoral muscle in the control (A), low-dose MSG group (B), medium-dose MSG group (C), and high-dose MSG group (D) on the 18th day of incubation. Compared to the control groups, myotube organization is delayed and transverse banding is weak in the MSG groups. Degenerating muscle fibers (black arrows), edema (stars), Zenker's necrosis in the muscle fibers (yellow arrows), and mononuclear cell infiltrations (red arrows) between the fibers in the MSG groups. H & E.

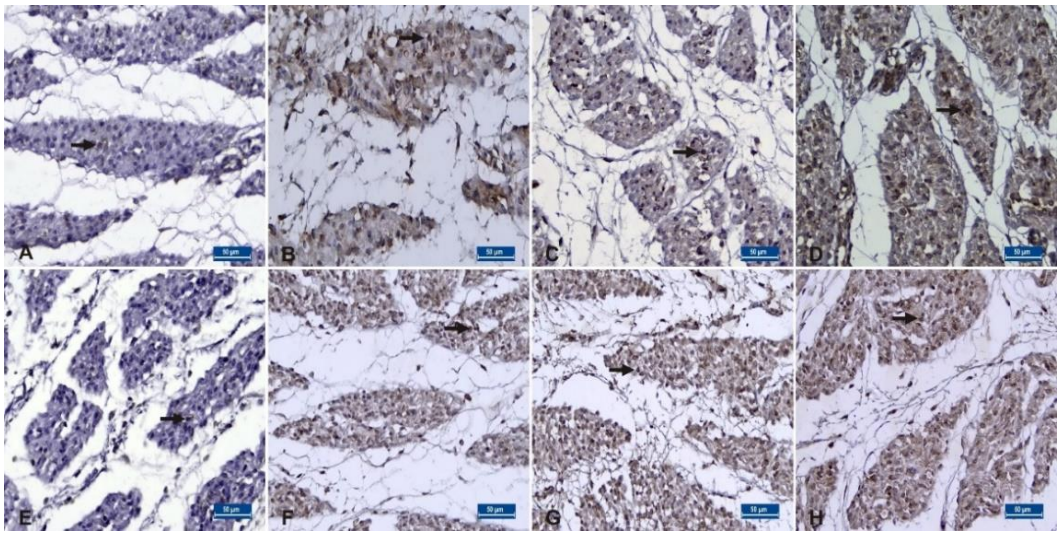

Figure 3. Leg muscle sections of chick embryos from the control (A), low-dose MSG group (B), medium-dose MSG group (C), and high-dose MSG group (D) on the 18th day of incubation. Pectoral muscle sections of chick embryos from the control (E), low-dose MSG group (F), medium-dose MSG group (G), and high-dose MSG group (H) on the 18th day of incubation. Compared to the control group (A, E), caspase-3 immunopositivity was more intense in the MSG groups. Anti-caspase-3 positive immunoreactions (arrows). Streptavidin-biotin-peroxidase method; bar: 50 μm.

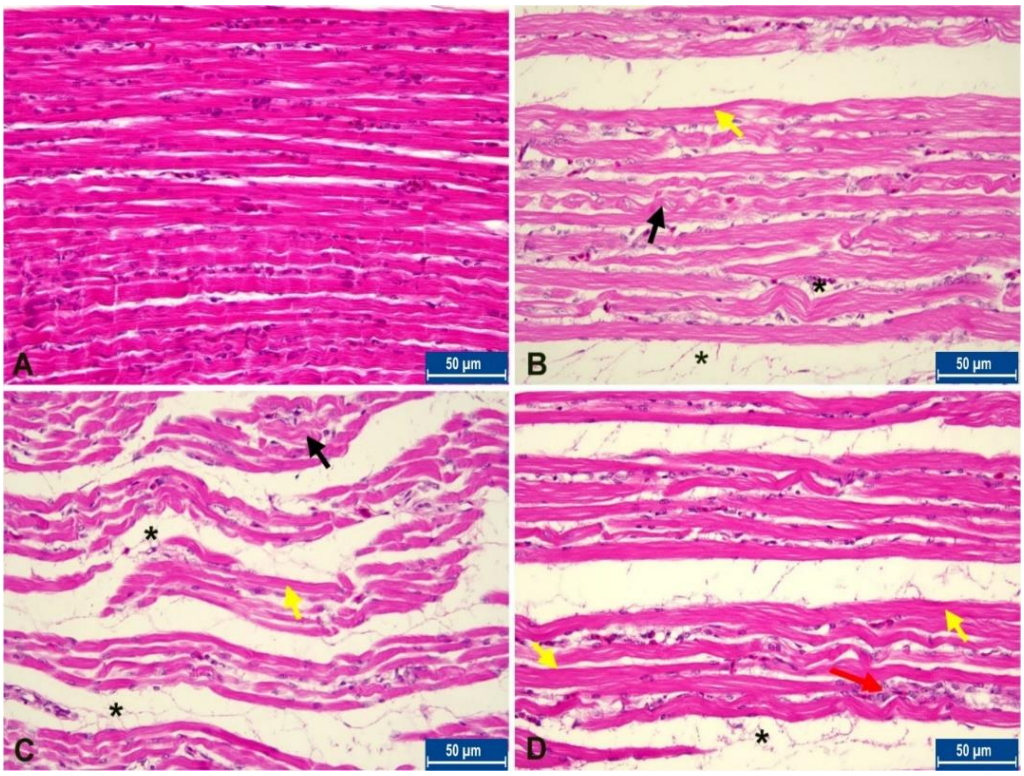

Figure 4. Sections of leg muscle in the control (A), low-dose MSG group (B), medium-dose MSG group (C), and high-dose MSG group (D) on the 21st day of incubation. Compared to the control groups, the MSG groups showed irregular formation of myotubes and weak transverse banding. Degenerating muscle fibers (black arrows), edema (stars), Zenker's necrosis in the muscle fibers (yellow arrows), and mononuclear cell infiltrations (red arrows) between the fibers in the MSG groups. H & E.

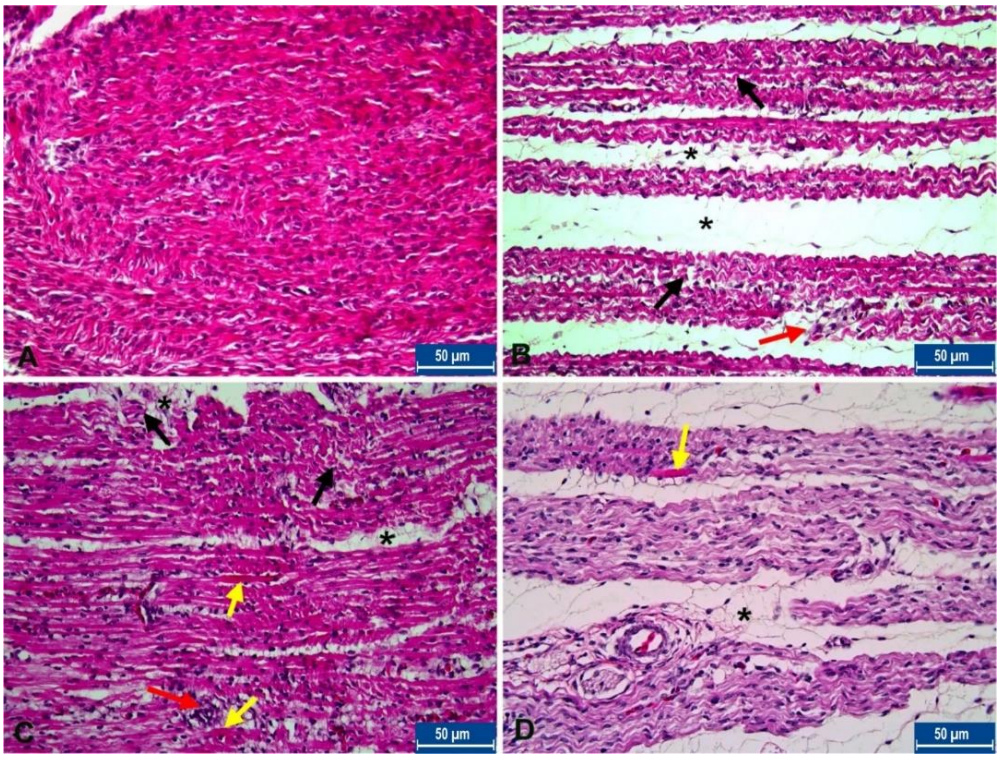

Figure 5. Sections of pectoral muscle in the control (A), low-dose MSG group (B), medium-dose MSG group (C), and high-dose MSG group (D) on the 21st day of incubation. Compared to the control groups, the MSG groups exhibited irregular formation of myotubes and weak transverse banding. Degenerating muscle fibers (black arrows), edema (stars), Zenker's necrosis in the muscle fibers (yellow arrows), and mononuclear cell infiltrations (red arrows) between the fibers in the MSG groups. H & E.

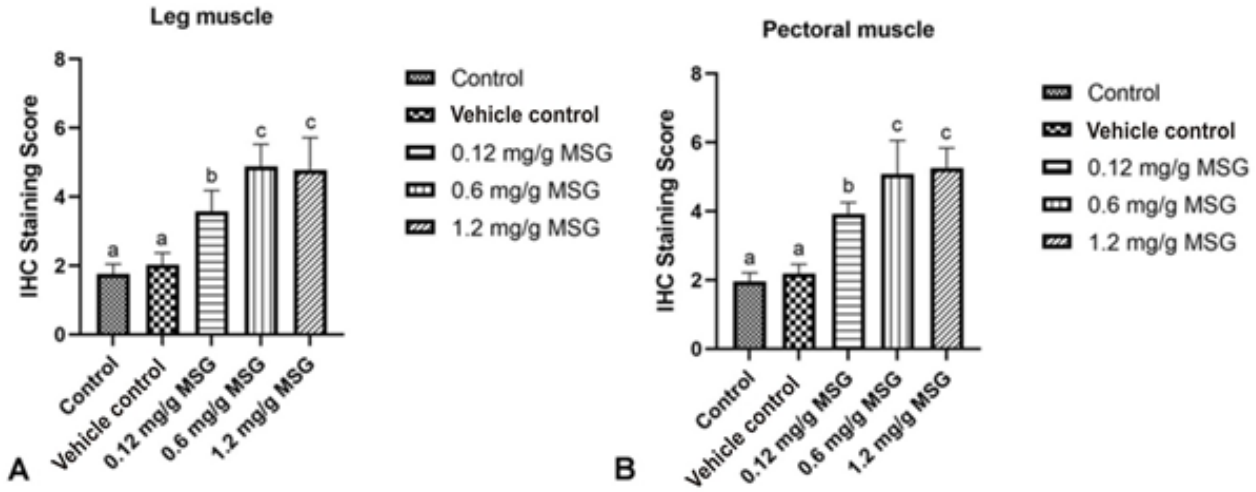

Figure 6. Immunohistochemical staining rates of caspase-3 in the leg and pectoral muscles at day 21 of incubation. Different superscript letters in the columns indicate statistical differences (mean ± SD, $p < 0.05$).

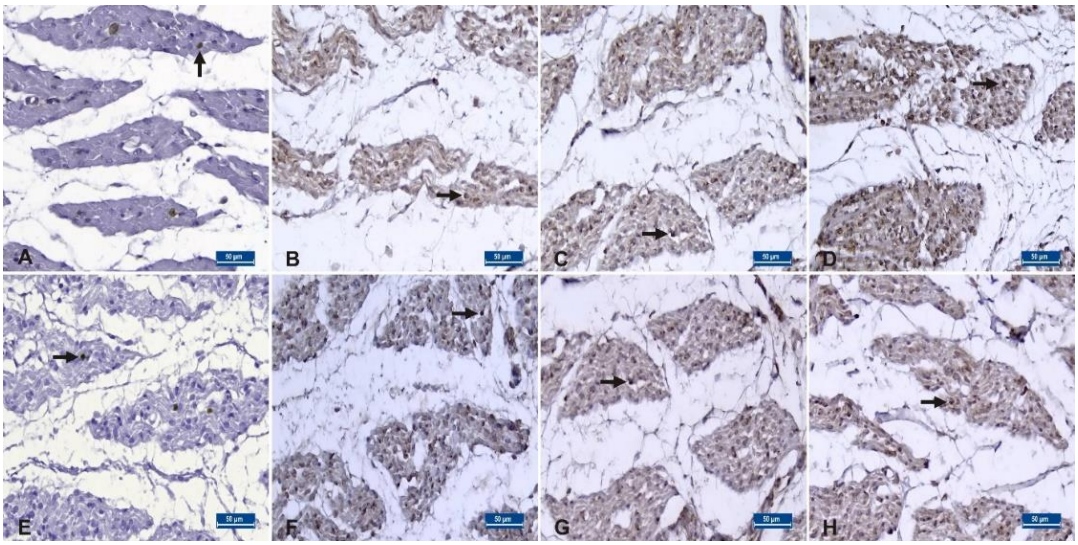

Figure 7. Leg muscle sections of chicks from the control (A), low-dose MSG group (B), medium-dose MSG group (C), and high-dose MSG group (D) on the 21st day of incubation. Pectoral muscle sections of chicks from the control (E), low-dose MSG group (F), medium-dose MSG group (G), and high-dose MSG group (H) on the 21st day of incubation. Compared to the control group (A, E), caspase-3 immunopositivity was more intense in the MSG groups. Caspase-3 positive cells (arrows). Streptavidin-biotin-peroxidase method; bar: 50 µm.

DISCUSSION

MSG has the appearance of a crystalline powder and is the most widely used additive as a flavor enhancer in processed foods (Chakraborty 2019). The acceptable daily intake level (ADI) for glutamic acid and its salts was determined by the FAO/WHO in 1970 as 120 mg/kg body weight. However, the safety of MSG was reevaluated by the European Food Safety Authority (EFSA) in August 2017, and the maximum daily intake of MSG was revised as 30 mg/kg body weight. Excessive consumption of foods high in MSG may have side effects for all individuals in the community and, particularly, for susceptible groups (children and adolescents) (Mortensen et al., 2017). MSG consumption has increased all over the world with the increase in ready-to-eat food consumption, with reported daily average intakes of 1 g in European countries and 4 g in Asian countries (Park et al. 2014). Recent studies have shown that MSG may also have
negative effects on many organs and tissues such as the digestive, reproductive, nervous, and immune systems (Jubaidi et al. 2019, Zanfirescu et al. 2019, Banerjee et al. 2021, Bölükbaş and Öznurlu 2022, Bölükbaş and Öznurlu 2023a, Bölükbaş and Öznurlu 2023b).

Chicken embryos are one of the most frequently used models to determine the embryotoxic, genotoxic, and teratogenic effects of agents such as drugs, toxins (Stoloff et al. 1972, Berg et al. 1999). Studies using fertilized chicken eggs do not express a unanimous opinion on the ways active substances can be administered to the egg for testing (Stoloff et al. 1972, Kemper and Luepke 1986, Öznurlu et al. 2012). The egg yolk serves as the counterpart of the placenta in mammals and serves as the main food source for the offspring (Bauer et al. 2013). Generally, test solutions are injected into the egg at a volume of 20–100 µL (Berg 2000, Öznurlu et al. 2022).

It has been reported in previous studies that at the 12th and 16th days of incubation, secondary myofibrils originated from myoblasts that cluster and differentiate around the existing primary myofibrils (Henry and Burke 1998, Kikuchi 1971). In this study, the transverse bands started to become evident on the 18th day of incubation and the banding was quite evident on the 21st day. Among the groups given MSG, the myotubes were irregularly shaped. In addition, degenerative and necrotic changes such as muscle fiber degeneration, Zenker's necrosis, and mononuclear cell infiltrations were observed in the muscles of the MSG groups.

MSG causes physiological and behavioral changes, such as loss of muscle strength and decreased locomotor activity (Campos-Sepúlveda et al. 2009). MSG-supplemented foods have been reported to cause muscle stiffness, redness of the skin, burning in the chest and unusual pain sensations, migraines, atypical facial pain or pressure, and needle-pricking sensations (Schaumburg et al. 1969, Rhodes et al. 1991, Yang et al. 1997). MSG administration has been reported to cause biochemical and morphological changes in the heart tissue, as well as changes in heart rhythm (Baky et al. 2009, Liu et al. 2013, Kumar and Bhandari 2013). Swamy et al. (2014) administered 2 g/kg of MSG intraperitoneally to Wistar albino rats and reported that MSG caused behavioral and physiological changes (decreased locomotor activity and loss of muscle strength).

Other studies have reported that MSG administration causes a significant increase in the concentration of interstitial glutamate in the masseter muscle (Kitamura et al. 2010) and leads to a decrease in the afferent mechanical threshold (Cairns et al. 2007).

In previous studies, it has been reported that the agents exposed during the prenatal period adversely affect muscle development (Gündüz and Öznurlu 2014, Sadighara et al. 2013). Öznurlu et al. (2022) injected different doses of bisphenol A (BPA) into the yolk and reported that the embryonic development of the skeletal muscle was delayed in the BPA-treated groups and that the number of PCNA-positive cells was higher compared to the control. Mokhtar and Sewelam (2021) showed that MSG administration in neonatal albino rats caused disruption in the cardiac muscle fibers, extravasated hemorrhage, and massive degenerative changes such as nuclear pycnosis, necrosis, and cytoplasmic vacuolation in cardiomyocytes. Elwan et al. (2021) reported that aflatoxin B1 injected into the egg at different doses increased the expression of caspase-3 in the chick embryo. El Kotb et al. (2020) reported that strong positive caspase-3 immunoreactivity was observed in the cells of the interstitial spaces and seminiferous tubules in the groups given MSG. Mohamed and Mohamed (2021) reported an increase in positive caspase-3 immunoreactivity in the testis tissue of rats as a result of oral administration of MSG for 4 weeks.

In this study, the development of skeletal muscle was delayed in the MSG groups compared to the control groups. This delay was characterized by the formation of myotubes or the prolongation of their formation process. Caspase-3 immunopositivity reactivity was also found to be higher in the MSG groups on the 21st day of incubation than in the control groups, and a statistically significant difference was observed between the MSG groups administered different doses ($p < 0.05$). In particular, caspase-3 immunopositivity in the skeletal muscle was found to be significantly ($p < 0.05$) higher in the groups administered 0.6 and 1.2 mg/g MSG than in the group administered 0.12 mg/g MSG. Higher caspase-3 immunopositivity in the MSG groups may be due to degenerative and necrotic in muscle fibers changes and the presence of incomplete myotubes.

CONCLUSION

In conclusion, it was determined that in ovo administered MSG could adversely affect embryonic development of skeletal muscle tissue along with causing may be due to degenerative and necrotic in muscle fibers changes in developing the skeletal muscle.

Conflict of interest: The authors declared that there is no conflict of interest.

Authors Contribution Rate: The authors declare that they have contributed equally to the article.

Ethical Approval: This study has received permission with Selcuk University Faculty of Veterinary Medicine Experimental Animals Production and Research Center Ethics Committee number 2022/59 and 02.06.2022.

Financial support: No support was received during this study.

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