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Research Article

# The Effects of in Ovo Insulin-Like Growth Factor-1 on Embryonic Development of Musculus Longus Colli Dorsalis in Japanese Quail*

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**Abstract:** The objective of the present study was to evaluate the effects of insulin-like growth factor-1 (IGF-1) on the histological development of the thoracic part of the m. longus colli dorsalis (m. spinalis thoracis) in Japanese quail embryos. A single in ovo dose of recombinant human IGF-1 (rhIGF-1) (100 ng embryo$^{-1}$) was administered through the blunt end of eggs via a single hole made with a dental drill bit, without penetrating the chorioallantoic membrane. For histological evaluation, the embryos were collected daily from days 7 to 16 of embryonic development (E). In ovo administration of rhIGF-1 increased the diameter of muscle fibers on E7, 9, 10, 11, and 13. Additionally, in ovo rhIGF-1 also increased the number of muscle fibers ($P < 0.001$). It was concluded that rhIGF-1 accelerated skeletal muscle development in the quail embryos.

**Key Words:** Quail, skeletal muscle development, rhIGF-1, in ovo

## Japon Bıldırcınlarında Musculus Longus Colli Dorsalis'in Embriyonik Gelişimi Üzerinde in Ovo İnsulin-Benzeri Büyüme Faktörü-1'in Etkileri

**Özet:** Bu çalışmanın amacı; Japon bıldırcını embriyolarının m. longus colli dorsalis (m. spinalis thoracis) kasının thoracic kısmının histolojik gelişiminde, insulin-benzeri büyüme faktörü-1'in etkilerini değerlendirmekti. Rekombinant insan IGF-1'i (rhIGF-1), yumurtanın küt ucunda, dental drill bit ile açılan delikten, bir defa olmak üzere 100 ng/her bir embriyo oranında chorio-allantoik membrana girmeksizin enjekte edildi. Histolojik değerlendirmeler için dokular, embriyonik gelişimin 7 ile 16. günleri arasında günlük olarak toplandı. RhIGF-1 uygulamasının embriyonik gelişimin 7, 9, 10, 11 ve 13. günlerinde kas tellerinin sayı ve çapında anlamlı bir artışa neden olduğu gözlendi ($P < 0,001$). Sonuç olarak; rhIGF-1'in Japon bıldırcını embriyolarının iskelet kası gelişimini hızlandırdığı sonucuna varıldı.

**Anahtar Sözcükler:** Bıldırcın, iskelet kası gelişimi, rhIGF-1, in ovo

## Introduction

Skeletal muscle cells (myoblasts) develop from somites. Premyoblasts give rise to myoblasts that fuse to form muscle fiber precursors called myotubes. Myotubes then fuse with each other to form multinucleated cells that give rise to multinucleated skeletal muscle fibers (1).

The insulin-like growth factor (IGF) family of peptides plays a significant role in embryonic/fetal growth and development (2,3). Growth and the effects of growth hormone on tissue and organs are instigated by insulin-like growth factor-1 (IGF-1), which contains 70 amino acids (4). It was reported that IGF-1 has a considerable influence on muscle growth and development (3,4), and that many of its effects occur via type 1 IGF receptors (3).

Although the main source of IGF-1 synthesis and secretion in adult animals is the liver, IGF-1 is synthesized in almost all tissues (5). Although IGF-1 secretion is typically growth hormone-dependent, its secretion during chicken embryonic development is not (6).

Most of the effects produced by IGFs on muscle cells occur at normal or near-normal circulating concentrations of these growth factors (7). Physiological concentrations of IGF-1 stimulate differentiation in a variety of in vitro

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muscle cell types, including rat L6 myoblasts (8), primary chick embryo myoblasts (9), and rat satellite cells (10). It has been demonstrated that in vitro IGF-1 enhances the differentiation of myoblasts by inducing myogenin gene expression (4,9). Additionally, it stimulates proliferation by promoting nutrient uptake and inhibiting protein breakdown in myoblasts (8). In ovo injection of IGF-1 (10-100 ng embryo$^{-1}$) into 2-day-old chicken embryos was reported to increase the general development, growth, and metabolic parameters of the embryos at day 4 (11).

Kocamış et al. (12) found that in ovo injection of recombinant human IGF-1 (rhIGF-1) (100 ng embryo$^{-1}$) into 3-day-old chicken embryos increased the development of skeletal muscle during both the embryonic and post-hatch stages. The objective of the present study was to assess the effects of in ovo injection of rhIGF-1 (100 ng embryo$^{-1}$) on skeletal muscle development of Coturnix japonica embryos.

## Materials and Methods

### Injection procedure

In this study, fertilized eggs obtained from the poultry facility of the Kafkas University Veterinary Faculty were used. The injection procedure was carried out as previously described by Kocamış et al. (12). Briefly, in ovo rhIGF-1 (100 ng embryo$^{-1}$) was administered through the blunt end of eggs. Before injection, the blunt end of each egg was sterilized with 70% ethanol. A single hole was made using a dental drill bit, without penetrating the chorioallantoic membrane. rhIGF-1 was injected into the albumen with a 22-gauge needle 3 days after the eggs were laid. The rhIGF-1 was diluted (10 mM acetic acid, and 0.1 g of BSA in 100 ml) and stored at –20 °C. After injection, the hole was sealed with a sticker and the eggs were placed in an incubator (temperature: 37 ± 0.5 °C; humidity: 86%-87%).

### Collection of Embryos and Histological Preparation

The time at which the eggs were placed into the incubator was set as "0" time. Between days 7 and 16, eggs were removed daily from both the rhIGF-1 treatment (5-6 eggs each day) and control groups (5-6 eggs each day). The thoracic parts of the m. longus colli dorsalis (m. spinalis thoracis) of the embryos were removed. The collected embryos were fixed in 10% formol-alcohol and Bouin's solution. The fixed tissue samples were passed through alcohol, methyl benzoate, and benzole, blocked in paraplast, and then tissue samples were cut into 6-µm thick sections on a microtome. Ten sections were taken from each embryo. Histometric values were represented at micrometer levels. To calculate the number of muscle cells, an ocular grid of 100 (10 × 10) squares was used at 10-100× magnification accounting for a total area of 0.01 mm$^2$. At least 10 adjacent areas were scanned. All muscle cells from those areas were counted.

For routine tissue examination, the sections were stained with hematoxylin-eosin and Crossmon's method. For the determination of glycogen, the periodic acid-Schiff (PAS) technique was used (13). Statistical analysis was performed with the Minitab program (14). A t-test was used for the determination of statistical differences between the control and rhIGF-1-injected groups. ANOVA was used for the determination of the significant differences of the days within groups (15).

## Results

On day 7 of incubation, development of the thoracic part of the m. longus colli dorsalis (m. spinalis thoracis) of the embryos was observed in the rhIGF-1-injected and control groups. It was observed that a total of 3 or 5 muscle fibers had come together; nevertheless, a complete bundle formation was not seen. Additionally, the proportion of intercellular connective tissue was excessive in comparison to the numbers of cells. The structure of the nuclei in the muscle cells appeared to be oval in most; however, some were round. In longitudinal cross section it was observed that myoblasts had fused together to form myotubes (Figure 1). In the myotubes, the nuclei of the cells were oval and had formed in the center of the myotubes. In the embryos injected with rhIGF-1, the development of muscle tissue on day 7 of incubation was more advanced than that of the control group (Figure 2a and b). Moreover, PAS staining of the tissue revealed that the level of glucose in the muscle cells of the IGF-1-injected group was more advanced than that of the control group.

On day 8 of incubation it was observed that the length and diameter of the myotubes, and the number of nuclei in the control and rhIGF-1 groups compared to day 7 had increased, whereas the density of glucose had decreased. On day 9 of incubation, we observed that the amount of connective tissue had decreased, the number of cells in

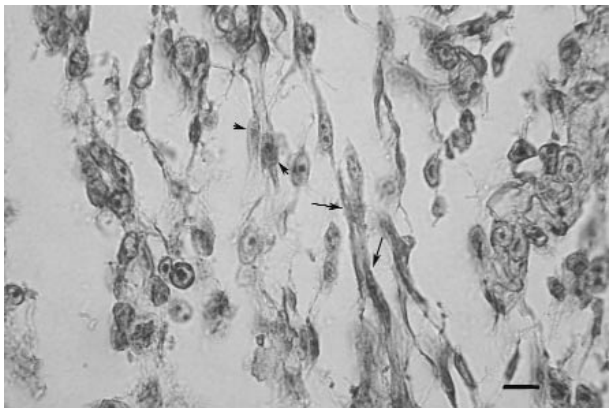

Figure 1. Myotube formation on day 7 of incubation. Arrows: myotube formation; Arrow head: nucleus. Triple staining. Scale bar = 20 µm.

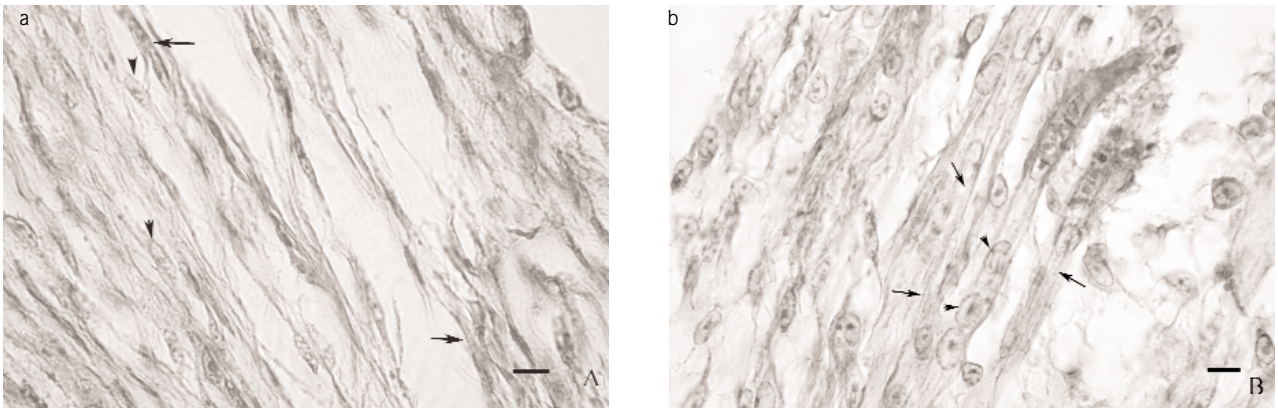


Figure 2. Striation in the striated muscle fibrils on day 7 of incubation.
a: control group; b: rhIGF-1-injected group; Arrows: striation; Arrow head: nucleus. Triple staining. Scale bar = 20 µm.

the muscle tissue had increased, the muscle cells had come together to form muscle bundles, and the length and diameter of the myotubes had increased proportionally to each other. On day 10, primary and secondary muscle bundles were observed to have formed in both groups. Additionally, the amount of connective tissue had decreased, the numbers of cells had increased, and in some of the muscle cells oval nuclei had formed at the periphery. The proportion of glucose had decreased in both groups.

On day 11 of incubation, the number of muscle cells and blood vessels had again increased, and the amount of connective tissue had decreased in both groups; however, PAS staining of horizontal sections of embryos revealed that glucose in certain muscle cells, such as those of the breast muscle, was not observed in the thoracic part of the m. longus colli dorsalis (m. spinalis thoracis) of the Japanese quail embryos.

On day 12 of incubation, the findings for both groups of embryos were similar to those of day 11; however, striation of muscle fibers and muscle spindles were now also observed in the embryos. On day 13 of incubation, in both the control and rhIGF-1–injected groups, it was observed that the number of muscle cells had increased, that the nuclei were generally oval and located at the cell periphery, and that blood vessels were present in the muscle tissue. In the horizontal sections, the striation, which was first observed on day 7, was now clearly evident in the developed embryos (Figure 3). Furthermore, the presence of glucose was determined in both groups, though its level was slightly higher in the rhIGF-1 group.

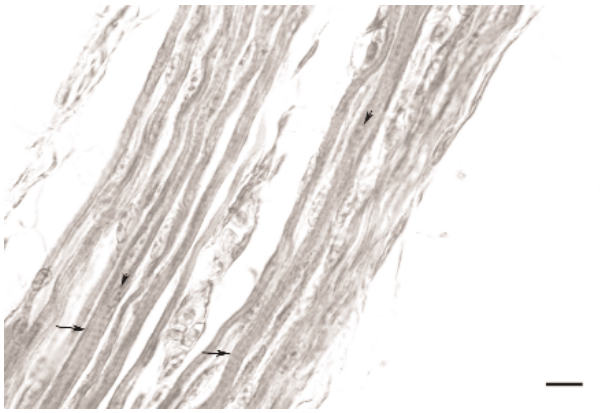

Figure 3. Striation in the striated muscle fibrils on day 13 of incubation.
Arrows: striation; Arrow head: nucleus. Triple staining. Scale bar = 20 µm.

On day 14 of incubation, in the horizontal sections, the muscle cells of both groups were observed to be more closely packed, and, in some areas of the embryos, red and white muscle fibers could be differentiated. Furthermore, in both groups the proportion of glucose had increased as compared to day 13. On day 15, in the horizontal sections of both groups, primary bundles, muscle spindles, space between the bundles, and blood vessels were clearly determined. Moreover, a more distinct differentiation between red and white muscle fibers was evident. On day 16 of incubation, in both the control and rhIGF-1 groups, muscle tissue was seen to be fully developed and myofibrils could be distinguished. Furthermore, complete striation of myofibrils could be seen in the longitudinal sections of embryos.

During development, the diameters of the muscle fibers decreased per unit area in inverse proportion to the numbers of muscle cells (Figure 4a). Embryonic development was more marked in the rhIGF-1-injected group than in the control group.

The diameters of the muscle cells were measured and cell counting was undertaken for each day (days 7-16). The diameters of the muscle cells and cell counting were significantly different for the rhIGF-1-injected and control groups on the same days ($P < 0.001$). In addition, the increase in the diameter of the muscle cells and in the cell

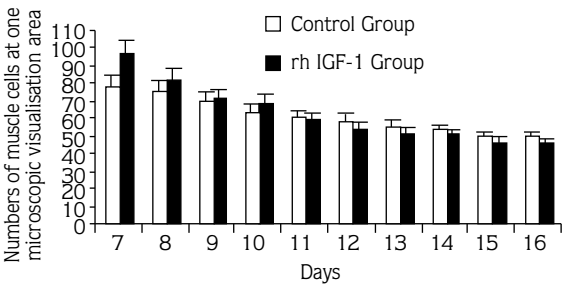


Figure 4a.

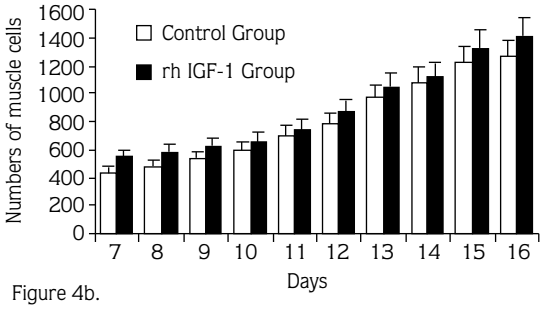


Figure 4b.

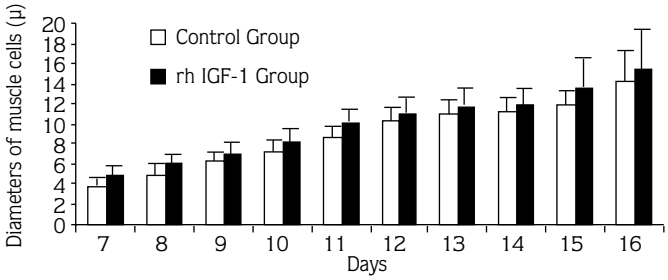


Figure 4c.

Figure 4a. In the rhIGF-1 treatment and control groups embryo alteration with respect to number of muscle fibers in unit area of m. longus colli dorsalis, pars thoracica (m. spinalis thoracis).

Figure 4b. In the rhIGF-1 treatment and control groups embryo alteration with respect to number of muscle fibers of m. longus colli dorsalis, pars thoracica (m. spinalis thoracis). Every day, rhIGF-1 treatment and control groups were different ($P < 0.001$).

Figure 4c. In the rhIGF-1 treatment and control groups embryo alteration with respect to age of m. longus colli dorsalis, pars thoracica (m. spinalis thoracis). Every day, IGF-1 treatment and control groups were different ($P < 0.001$).

count was greater in the rhIGF-1 embryos than in the control group (Tables 1 and 2). The diameters of the muscle cells and the cell count increased gradually in both groups (Tables 1 and 2, and Figure 4b and c).

## Discussion

Embryonic growth and fetal growth are versatile processes that consist of proliferation, migration, and differentiation of selected cells, and apoptosis (16). Hamburger and Hamilton (17) proposed that myogenic cells first separate from somite 15 (in embryos that have 20-22 somites), which corresponds to stage 14 (approximately day 2.5 of incubation). Grim (18) reported that this process continues until somite stage 36. Sweeney et al. (19) suggested that differentiation does not begin until stage 25. Michael et al. (20) reported that myogenesis starts in chickens in stage 26 and in quails on day 6. In another study (21), it has been claimed that the main myogenic period in chick embryonic breast and leg muscle occurs from approximately days 7 to 17 of incubation, and that in this period the ratio of differentiated cells in both breasts and legs increases by 80%. In the light of these studies, we removed the thoracic part of m. longus colli dorsalis (m. spinalis thoracis) of Japanese quail embryos from day 7 to 16 of incubation. We observed that on day 7 the muscle cells were very small, with a central oval nucleus. The findings in our experiment are in accordance with those reported by Sweeney et al. (19), Michael et al. (20), and O'Neill (21).

It has been reported that, in myogenesis, myoblasts divide through mitosis until there are a sufficient number of cells, which then fuse together to form myotubes. Actin and myosin are then synthesized in the myotubes and converted into fibra muscularis (1). In another study (22), it was suggested that early striation of muscle cells can be observed at 2 weeks more clearly from the breast muscle of 10-11-day-old white leghorn chick embryos. In our study, we also found that striation of myofibrils could be seen clearly in the embryos on days 12-13.

In general, researchers (23-25) have reported that in adult animal skeletal muscle, the muscle fibers have flat, peripheral nuclei; however, it has also been reported that (25) in some poultry the nuclei are located in the center. In our experiment, we found that the nuclei in the thoracic part of m. longus colli dorsalis (m. spinalis thoracis) were flat and oval, and located under the sarcolemma. This finding was consistent with the literature in general (23-25). We also determined that in some muscle fibers the nuclei were central at the beginning of myogenesis, but that they changed in proportion to development, becoming flatter and settling at the periphery.

Table 1. Statistical evaluation of the diameter of skeletal muscles of the rhIGF-1-treated and control groups, from E7 to E16. abcdefg: The means represented by the same letter are not statistically different within each of the groups.

| Day | n | rhIGF-1 Treatment Group | Control group |
|---|---|---|---|
| 7 | 40 | $5.01 \pm 0.15^{g}$ | $3.72 \pm 0.12^{f}$ |
| 8 | 40 | $6.23 \pm 0.14^{fg}$ | $5.01 \pm 0.18^{e}$ |
| 9 | 40 | $6.95 \pm 0.18^{ef}$ | $6.33 \pm 0.14^{d}$ |
| 10 | 40 | $8.16 \pm 0.23^{e}$ | $7.39 \pm 0.17^{d}$ |
| 11 | 40 | $10.03 \pm 0.20^{d}$ | $8.63 \pm 0.20^{c}$ |
| 12 | 40 | $11.16 \pm 0.22^{cd}$ | $10.36 \pm 0.20^{b}$ |
| 13 | 40 | $11.81 \pm 0.25^{c}$ | $11.03 \pm 0.21^{b}$ |
| 14 | 40 | $11.89 \pm 0.28^{c}$ | $11.37 \pm 0.22^{b}$ |
| 15 | 40 | $13.57 \pm 0.46^{b}$ | $11.99 \pm 0.23^{b}$ |
| 16 | 40 | $15.63 \pm 0.62^{a}$ | $14.32 \pm 0.48^{a}$ |

n: number of counting unit area; $\bar{X}$: value mean; $S\bar{x}$ : standard error.
** $P < 0.01$ *** $P < 0.001$.

Table 2. From E7 to E16, the number of muscle cells in the rhIGF-1 treated and control groups, for statistical analysis.

| Day | n | rhIGF-1 Treatment Group | Control group |
|---|---|---|---|
| 7 | 10 | 548.6 ± 2.0 | 430.9 ± 2.3 |
| 8 | 10 | 586.2 ± 1.5 | 480.3 ± 1.8 |
| 9 | 10 | 626.4 ± 2.1 | 533.8 ± 1.9 |
| 10 | 10 | 657.3 ± 2.0 | 595.8 ± 1.8 |
| 11 | 10 | 743.6 ± 1.6 | 696.8 ± 1.3 |
| 12 | 10 | 871.0 ± 1.8 | 782.1 ± 1.3 |
| 13 | 10 | 1049.8 ± 1.7 | 967.8 ± 2.1 |
| 14 | 10 | 1116.2 ± 1.7 | 1081.8 ± 1.4 |
| 15 | 10 | 1320.5 ± 0.8 | 1241.9 ± 1.1 |
| 16 | 10 | 1403.7 ± 1.2 | 1262.7 ± 1.7 |

*** $P < 0.001$ n: number of total cells in whole area

In terms of its morphologic properties, the skeletal muscle of poultry contains white, intermediate, and red muscle fibers, as is found in mammals (23-25). In our study, morphologic differentiation of muscle fiber was not evident until day 13.

It has been reported that the diameter of muscle fibers in poultry (23-25) and mammals (25) ranges from 10 to 120 μm. In our study, the average diameter of the muscle fibers on day 7 of incubation was 3.72 μm in the control group and 5.01 μm in the rhIGF-1-injected group, and rose to 14.32 μm in the control group and to 15.63 μm in the rhIGF-1-injected group on day 16. Some studies report that white muscle fibers are large in diameter, while red muscle fibers are small (23-25). In our examination of the thoracic part of m. longus colli dorsalis (m. spinalis thoracis), morphologic differentiation was made from day 14 of incubation; we observed that the diameters of the white muscle fibers were larger than those of the red muscle fibers.

Peptides of the IGF family are known to play an important role in embryonic and fetal development and growth (2,3). Murray et al. (26) reported that, while insulin was the most effective metabolic hormone, IGFs were more effective in the stimulation of development. It has been suggested that IGFs have an important effect on the development and growth of muscle (3,4), achieved mainly through IGF-1 receptors (3). It has also been reported that in myoblast cultures IGF-1 stimulates myoblast proliferation (4).

According to Van Wyk (27), IGF-1 stimulates DNA synthesis in 20 different cell lines originating from the ectoderm, endoderm, and mesoderm. The expression of IGF-1 plays an important role in chicken embryo development. In addition, Vasilatos-Younken and Scanes. (28) found that in vitro IGF-1 influenced the metabolism, differentiation, and proliferation of cells from post-hatch chicks. Some researchers (4,9) have reported that IGF-1 enhances differentiation by stimulating myogenin gene expression. Ewton and Florini. (8) found that IGF-1 influenced proliferation by promoting nutrient uptake and inhibiting protein breakdown in myoblasts. Girbau et al. (11) observed that the injection of IGF-1 (10-100 ng embryo$^{-1}$) into 2-day-old chicken embryos increased general development and growth (weight, total protein, DNA, and RNA), biochemical differentiation (total creatine kinase), and metabolic parameters (triglycerides, cholesterol, and phospholipids) of the embryos on day 4. Kocamış et al. (12) reported that the injection of rhIGF-1 (100 ng embryo$^{-1}$) into 3-day-old chicken embryos increased the development of skeletal muscle, both during the development of the embryos and the post-hatch chicks.

IGF-1 plays a significant role in the embryonic development of chicks (29). In the turkey and chick embryo, IGF-1 plasma levels peak during the mid-embryogenesis period (2,4,29). It has also been reported that serum IGF-1 increases 10-fold, from about 3 ng ml$^{-1}$ on embryonic day 10 to a pre-hatch peak of 35-45 ng ml$^{-1}$ during embryonic days 15-17, and eventually declines to the concentrations observed immediately after hatching (> ng ml$^{-1}$) at 3-5 months of age (29). Anabolic effects of IGF-1 have been observed when muscle cells are treated with physiological levels of IGFs in the absence of other hormones, serum, or any other proteins (7).

It has been reported that physiological concentrations of IGF-1 stimulate differentiation in a variety of muscle cell types, including rat L6 myoblasts (8), primary chick embryo myoblasts (9), and rat satellite cells (10). In addition, using the opposite approach in which transgenic animals over-expressed IGF-1, Mathews et al. (30) found that muscle and bone growth increased by approximately 30% when circulating levels of IGF-1 were 50% above control values. In our study, the thoracic part of m. longus colli dorsalis (m. spinalis thoracis) of the IGF-1-injected group had a greater cell diameter and more cells than the control group, consistent with other research.

We determined that after the formation of the thoracic part of m. longus colli dorsalis (m. spinalis thoracis) on day 7 of quail embryonic development, the number of muscle cells and muscle cell diameters increased. Daily increases in muscle cell diameters in the rhIGF-1-injected group were significantly different between days 7 and 9, 10 and 11, 14 and 15, and 15 and 16. In the control group, significant differences were found between days 7 and 8, and 8 and 9, and between days 10 and 11, and 11 and 12. The differences in findings between days 12 and 15 were not significant, but there was significant differentiation between days 15 and 16. Moreover, the differences found daily between the muscle cell diameters and cell quantity of the control group and the rhIGF-1-injected groups were significant ($P < 0.001$). In conclusion, an increase in muscle diameter and in the number of muscle cells, in proportion

to growth, was histologically observed in the thoracic part of m. longus colli dorsalis (m. spinalis thoracis) of Japanese quail embryos, from days 7 to 16 of the experiment. As a result, rhIGF-1 injection had a positive effect on skeletal muscle development.

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