Isolation and culture of chicken embryonic synovial fibroblasts

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Summary
Cells obtained from chicken embryos are often preferred for *in vitro* studies. These cells, which easily adapt to rapid and continuous growth in the appropriate cell culture environment, are thought to be one of the effective methods in the investigation of leg diseases that are frequently observed in poultry. Leg diseases, especially affecting the joints in chickens, cause locomotor problems and adversely affect animal welfare. In addition, they cause significant health problems and increase mortality. It is known that synovial fibroblasts play an important role in joint diseases. In this study, chicken embryonic synovial fibroblasts were isolated from tissue explants taken from the tibio-metatarsal joint region of brown layer chicken embryos. Characterization of cells was evaluated by immunocytochemistry and hemacolor staining. chicken embryonic synovial fibroblasts showed a strong positive reaction to the vimentin antibody. As a result of hemacolor staining, it was noted that the cell morphology was spindle-shaped. The absence of macrophages in chicken embryonic synovial fibroblast culture was confirmed by the carbon powder uptake. In this present study, we aim to present a useful cell culture protocol such as primary culture, passage, and characterization suitable for chicken embryonic synovial fibroblast to be used in the new scientific research.

Introduction

Animal experiments play an important role in scientific studies investigating the pathogenesis of diseases, but they are also a subject of ongoing ethical debate. *In vitro* methods are a good alternative minimizing ethical concerns (Freshney 2010). Besides being used in studies investigating the pathogenesis of various diseases in domestic animals, *in vitro* methods are also widely used in studies involving chickens (Akkoc and Kahraman 2011). Chicken embryonic cells are particularly preferred for *in vitro* models as they are easy to use, economical (Urja *et al.* 2018), and can easily adapt to rapid and continuous growth (Rubin 1966).

Leg diseases cause significant health problems, increase mortality, and thus have major economical impact in poultry industry (Butterworth 1999, Wideman 2016). They can lead to clinical problems such as lameness, growth depression, and decreased egg production in chickens (Landman *et al.*, 1994). Infectious agents such as reovirus (Choi *et al.* 2022), *Enterococcus faecalis* (Landman *et al.* 1999, Sevimli *et al.* 2004), *Staphylococcus aureus*, and *Escherichia coli* cause have been reported as cause of leg problems in poultry (Ekesi *et al.* 2021). In addition, it plays an important role in leg problems in poultry in diseases such as amyloid arthropathy induced by intra-articular injection of noninfectious agents such as Freund's adjuvant (Sevimli *et al.* 2008) or vitamin A (Sevimli *et al.* 2005).

Leg diseases can involve bones, joints, ligaments, and tendons (Bradshaw *et al.* 2002). Amyloid arthropathy, arthritis, synovitis, and tenosynovitis, which affect joints and cause locomotor problems in chickens, adversely affect animal welfare (Braga *et al.* 2016). Amyloid arthropathy, which is associated

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with many diseases, especially chronic infections, and is characterized by the accumulation of amyloid A protein in the joints, is one of the most important causes of lameness, especially in brown layer chicken breeds (Landman *et al.* 1998, Upragarin *et al.* 2005). Infectious agents such as *Mycoplasma synoviae* (Liu *et al.* 2021, Sun *et al.* 2022) and *Salmonella pullorum* (Li *et al.* 2022) cause lameness by causing synovitis and arthritis in chickens.

Synovial fibroblasts (SFs), are mesenchymal cells originating from fibroblasts and they share many features of fibroblasts including vimentin, type IV collagen, and CD90 expression. These cells, which account for about 2/3 of the synovial cells, are also known as type B synoviocytes (Ghadially and Roy 1967, Upragarin *et al.* 2005). It is known that SFs located in the intima layer of the synovium play a role in the production of cytokines and the production of proteases that cause cartilage destruction (Bartok and Firestein 2010), and are also involved in acute phase response (APR) and expression of acute phase proteins (APPs) (Upragarin *et al.* 2005, Blanco *et al.* 2016).

The SF cell culture model is an efficient method for investigating the physiology and pathology of synovial tissues (Zhao *et al.* 2016). Besides determining the role of these cells in diseases, cell culture is also useful in the development of therapeutic approaches for various joint diseases (Zhao *et al.* 2016). To the best of our knowledge, a detailed protocol for the isolation and primary culture of chicken embryonic synovial fibroblasts (CESFs) has not been established so far. In this study, we attempted to adapt and validate a useful cell culture protocol for CESFs obtained from synovial tissue of brown layer chicken embryos. The aim of the present study is to contribute to *in vitro* research on leg diseases caused by joint problems in chickens. Also, it is to present a suitable cell culture system for CESFs with primary culture, passage, characterization, and evaluation of the effect of different culture media on cells to be used in new scientific research.

Materials and methods

Ethics and animals used in the study

The experimental protocol was approved by Afyon Kocatepe University Animal Experiments Ethics Committee. For the study, specific pathogen-free eggs of the brown layer chicken breed (ISA Brown) were obtained from a commercial hatchery flock (Güres Yumurta A.S., Turkey). After the eggs were transferred to the laboratory, they were incubated at 37°C in a humidified 5% CO2 atmosphere (Cimuka, PD60, Turkey) and embryo development was ensured for 11 days. At the end of the incubation period, the eggs were opened and embryos were taken into sterile petri dishes with a diameter of 100 mm (Corning, USA). Embryos were then decapitated using sterile surgical instruments as indicated by the Institutional Animal Care and Use Committee (IACUC) Policy for Use of Avian Embryos (2016).

Isolation of CESFs

After euthanasia, 0.5 cm x 0.5 cm tissue samples including the tibio-metatarsal joints of the embryos were excised with surgical scissors (Figure 1). Tissues were placed in petri dishes filled with 50 ml Dulbecco's phosphate buffered saline (DPBS) solution (Sigma Aldrich, D5662, USA) containing 2X penicillin-streptomycin (Sigma Aldrich, P4333, USA). The skin and all soft tissues were dissected and the synovial membranes were divided into explants of approximately 1 mm x 1 mm.

To allow adherence of the tissue explants to the floors of the 6-well plates (TPP, Switzerland), one tissue explant was placed in each well after creating superficial scratches on the floor of the well with scalpel blades. Then 6-well plates were incubated for 10 minutes in a humidified 5% CO2 atmosphere at 37°C (Jouan, France). After incubation, fetal bovine serum (FBS) (Sigma Aldrich, F2442, USA) was added to each well to cover the tissue explants and plates were incubated in a humidified 5% CO2 atmosphere at 37°C overnight.

The next day, FBS in 6-well plates was removed and Dulbecco's modified Eagle medium (DMEM) containing 20% FBS and 2X penicillin-streptomycin was added to the explants. The medium was changed every other day. The morphology and growth of the cell population were observed regularly using an inverted microscope (Olympus, CKX41, Japan).

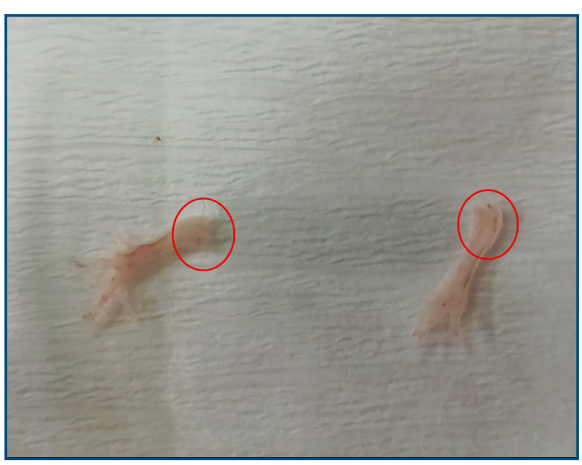

Figure 1. *Tibio-metatarsal joint region of brown layered chicken embryos (circle).*

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Passage

The cells were subcultured when they reached confluency. The culture medium in the 6-well plates was removed and each well was rinsed for five minutes with preheated DPBS. Then, DPBS was removed, 0.5 ml of preheated trypsin-EDTA solution (Biological Industries, 1109199, Israel) was added, and the 6-well plates were incubated for two minutes in a humidified 5% CO2 atmosphere at 37°C. Trypsin activity was stopped by the addition of 2 ml of DMEM after incubation. The cell suspension was transferred to 25 cm2 flasks and approximately 3 ml DMEM was added. The medium was changed every other day. Then cells were subcultured with RPMI 1640 and M199 media, respectively, and their adaptation to culture medium and growth conditions were assessed. Table I summarizes the media used for the isolation and subculture of CESFs.

Table I. *Media used for the isolation and subculture of chicken embryonic synovial fibroblasts.*

Day	Medium	Brand	Added Content	Flask
7th	DMEM	Sigma Aldrich, D6429	2X penicillin-streptomycin +%20 FBS	25 cm^2
9th	RPMI 1640	HyClone, SH30255.01	2X penicillin-streptomycin +%20 FBS + 100 µl bFGF	25 cm^2 + 75 cm^2
15th	*M199*	Sigma Aldrich, M3769	2X penicillin-streptomycin +%20 FBS + 100 µl bFGF	75 cm^2

bFGF: Basic fibroblast growth factor (Sigma Aldrich, F0291)

Cell counting

After trypsinization, the cell suspension was centrifuged in a 15 ml centrifuge tube at 3000 rpm for one minute at room temperature. The pellet was resuspended with 9 ml of complete medium. 100 µl of the cell suspension prepared as 10 ml was taken and transferred to an Eppendorf tube. The same amount of trypan blue was added and mixed. It was loaded between the Thoma slide and the coverslip. The viable cells on the four outer squares on the Thoma slide and one central square in the middle were counted. The final cell count per 1 ml was determined as $98x10^4$.

Characterization of cells

The morphology of the cells was examined under an inverted microscope. Additionally, immunocytochemistry, hemacolor staining and carbon powder uptake tests were performed. Before the eighth passage, 100 µl of cell suspension (98x103 cell count) was added to round coverslips placed in 24-well plates and incubated in a humidified atmosphere of 5% CO2 at 37°C overnight. When reached 80% confluency, cells were stained with monoclonal anti-vimentin and anti-cytokeratin antibodies. In addition, carbon powder uptake was performed to show that there were no macrophages except CESFs in the tissue samples taken by adding 100 µl of cell suspension into 24-well plates.

Immunocytochemistry

The cells were seeded onto coverslips placed in 24-well plates and were stained immunocytochemically using a commercial kit (Thermo Fisher Scientific, UltraVision ONE Detection System, HRP&DAB, anti-polyvalent, TP-015-HDJ, USA). After the wells of the culture plates were rinsed with PBS for five minutes, 0.5 ml of cold (-20 °C) methanol was added to the wells and incubated for five minutes. Then, the methanol in the wells was decanted and cells were rinsed with PBS for twice for five minutes. The protein blocking solution was applied for 10 minutes. Anti-vimentin (mouse anti-human, 1:500, Dako/Agilent Technology, M0725, USA) and anti-cytokeratin (mouse anti-human, 1:200, Santa Cruz Biotechnology, Sc-57004, USA) primary antibodies were applied to cover the coverslips and the coverslips were incubated for one hour. Then, the cells were rinsed twice with PBS for five minutes and incubated with a secondary antibody for 30 minutes. Later, 3,3-diaminobenzidine (DAB) (40 µl DAB plus chromogen solution+2 ml DAB plus substrate solution) was added for two minutes as the chromogen. After removal of the DAB, the cells were finally counterstained with Harris hematoxylin (Sigma Aldrich, SLCC1848, USA) for a few minutes. Then, the coverslips were removed from the wells and adhered with Entellan. All steps were carried out at room temperature.

Hemacolor staining

The cells seeded onto coverslips in culture plates were rinsed with PBS for five minutes, and then incubated in approximately 0.5 ml of cold methanol (-20 °C). After the removal of methanol, cells were rinsed twice with PBS for five minutes. They were stained with reagent red (Merck, 1.11956.2500, Germany) and then reagent blue (Merck, 1.11957.2500, Germany), each for five minutes. Coverslips were removed from the wells, adhered with Entellan, and examined under a light microscope (Nikon Eclipse 80i).

Carbon powder uptake

The phagocytic capability of CESFs and the absence of macrophages were evaluated by the uptake of carbon powder (Upragarin *et al*. 2005). The carbon powder (Sigma Aldrich, 05105, USA) was suspended in M199 culture medium at a 50 µg/ml concentration.

Then the carbon powder suspension was added to the cells grown in 24-well plates and the plates were incubated at 40°C for 30 minutes.

The 24-well plates were rinsed five times with PBS and examined under an inverted microscope. Rat peritoneal macrophage cells isolated primarily from tissue were used as the positive control.

Results

Cell culture results

The tissue explants placed in 6-well plates were examined under an inverted microscope, and proliferation of spindle-shaped cells was observed in the first three days. These cells were morphologically homogeneous and resembled fibroblasts.

After reaching confluency, the cells were seeded into 25 cm^2 tissue culture flasks (Figure 2) and DMEM was used as the culture medium.

Vacuolar degeneration was observed in the cells (Figure 3) and the cells were not confluent on day 9.

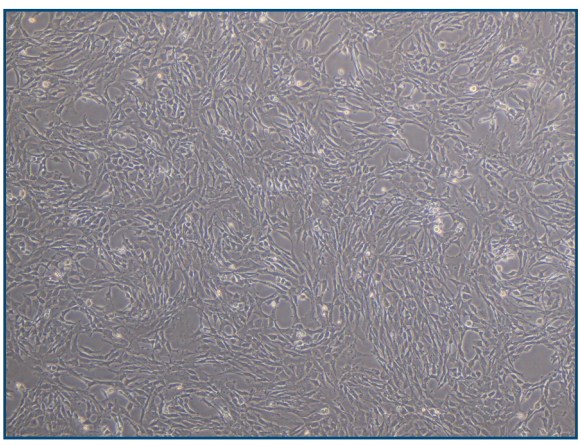

Figure 2. *The isolated and cultured chicken embryonic synovial fibroblasts with spindle-shaped morphology, first passage (100x).*

When the culture medium was changed to RPMI 1640, vacuolar degeneration continued. Also were used for the following passage 75 cm^2 tissue culture flasks and RPMI 1640 culture medium was continued to be used. However, it was noted that the cells examined under an inverted microscope every other day were not reached confluent. Also, it was determined that vacuolar degeneration continued. Therefore, the culture medium was changed to M199, and the cells were examined every other day. At this stage, no degenerated cells were observed and the passage was continued until the cells reached confluence.

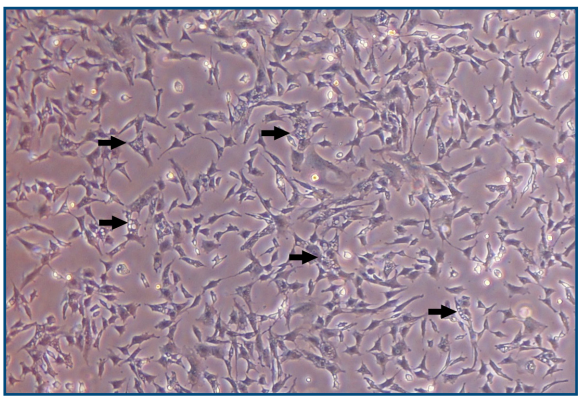

Figure 3. *Vacuolar degeneration of chicken embryonic synovial fibroblasts at day 9 (arrows), (200x).*

Immunocytochemistry, hemacolor staining, and carbon powder uptake results

CESFs displayed a strong positive reaction against vimentin antibody (Figure 4A) and a negative reaction against to the cytokeratin antibody (Figure 4B). Numerous spindle-shaped cells with fibroblastic morphology were observed on hemacolor staining (Figure 5). These results suggested the fibroblastic nature of the CESFs. The CESFs did not phagocytose the carbon particles, ruling out that the cells be macrophages.

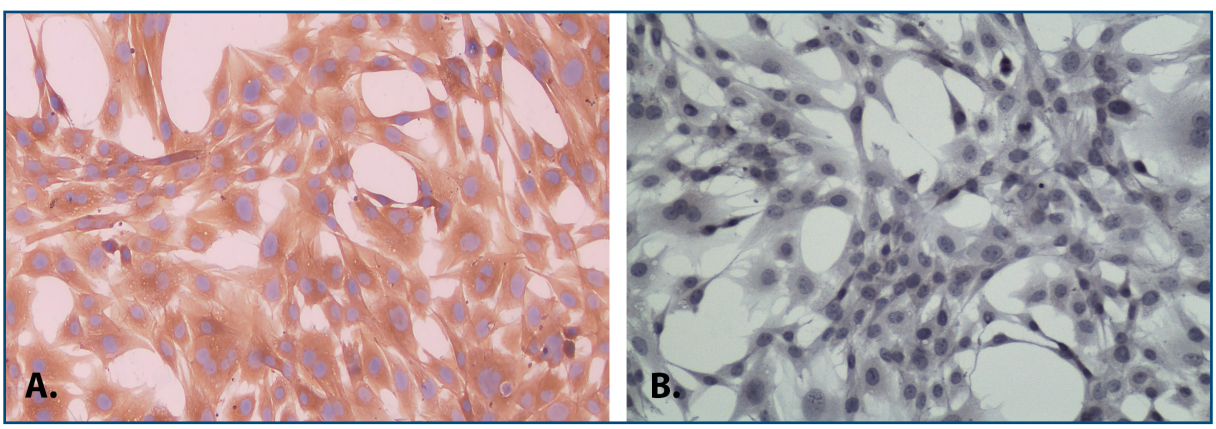

Figure 4. *Immunocytochemical staining of chicken embryonic synovial fibroblasts. (A) The cells demonstrated of strong positive reaction against the vimentin antibody and (B) a negative reaction against the cytokeratin antibody (streptavidin-biotin-peroxidase, DAB chromogen, 200x).*

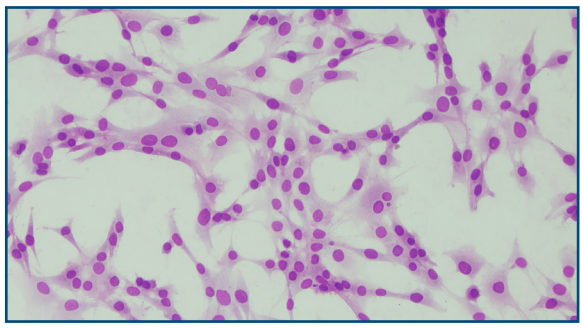

Figure 5. *Hemacolor staining of chicken embryonic synovial fibroblasts. Numerous spindle-shaped cells with fibroblastic morphology (200x).*

Discussion

SFs are an important group of cells located in the intima layer of the synovial membrane in joints (Ghadially and Roy 1967). These cells are thought to play an important role in the pathological process of joint-related diseases (Upragarin *et al.* 2005). SFs isolated from people with rheumatoid arthritis increase the release of inflammatory factors, causing inflammation and cartilage damage in the joints (Fanqi *et al.* 2022). It has been determined that SFs isolated from the knee joint in chickens play a role in the pathogenesis of amyloid arthropathy by causing an increase in serum amyloid A (SAA). (Upragarin *et al.* 2005).

The use of *in vitro* culture models of SFs obtained from the joint tissues of chickens in the embryonic period can provide important information about the pathogenesis of joint problems that cause important health problems in the poultry industry and increase our understanding on the mechanisms of inflammation. To our knowledge, no studies have been conducted on the isolation and culture of CESFs. In the present study, a suitable cell culture protocol for CESFs and the characterization of these cells are presented. The results were compared with some studies on humans and laboratory animals. Using three different media in cell culture, the most suitable medium for CESFs was investigated. For example, in some studies, while DMEM was used for SFs isolated in humans and rats (Koppikar *et al.* 2015), Upragarin et al 2005 used RPMI 1640 medium for the isolation and culture of chicken SFs. In this study, we used the DMEM until the second passage. However, we changed the cell culture medium to RPMI 1640 after we observed cease of cell proliferation and also degeneration in the cells. Therefore, we transferred CESFs to the M199 medium used in chicken lung fibroblasts in another study (Akkoc and Kahraman 2011). We observed that cell proliferation increased and degenerated cells recovered. When the contents of the cell culture media were compared, we found that there were more amino acids and vitamins in the M199 culture medium. As a result, we determined that M199 supplies the nutritional need of CESFs adequately compared to other mediums. M199 can be a more suitable culture medium for CESFs than DMEM and RPMI 1640.

We think that this may be related to the fact that the cells we isolated in our study belong to the embryonic period and need a more nutritious environment. Moreover, this idea can be explained by the fact that DMEM and RPMI 1640 were designed to sustain the growth requirements of mammalian cells (Rubin 1966). In the characterization of the CESFs in the present study, we observed a strong vimentin expression. Vimentin is the main intermediate filament protein expressed in mesenchymal cells (Yocum *et al.* 1988, Zhao *et al.* 2016). For this reason, it is often used as a marker of SFs (Zhao *et al.* 2016). Vimentin expression was observed in SF *in vitro* studies in mice (Zhao *et al.* 2016), rats (Yocum *et al.* 1988), chickens (Upragarin *et al.* 2005), and humans (Fanqi *et al.* 2022). Accordingly, the observation of vimentin expression in CESFs in the present study shows that these cells are similar to SFs of other species. In addition, the absence of macrophages as indicated by the lack of carbon powder uptake suggested that the isolated cells were type B synoviocytes only. We think that future research will evaluate the characterization of CESFs from a molecular perspective and that this present study will contribute to new scientific research.

With the findings obtained, it was confirmed that the methods used to obtain CESF cell lines were suitable and it was concluded that the M199 medium was more effective for CESFs culture. We believe that this study will contribute to *in vitro* research on leg diseases caused by joint problems in chickens.

Ethical Statement

This study was approved by Afyon Kocatepe University Experimental Animal Research and Application Center (Afyonkarahisar, Turkey) (AKUHADYEK-313).

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