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ORIGINAL ARTICLE

Investigation of the efficacy of epidermal growth factor, boric acid and their combination in cartilage injury in rats: An experimental study

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Joint cartilage injury brought on by osteoarthritis (OA) or traumatic injuries can result in joint deformity, motion restriction, and severe pain. Due to its avascular nature and restricted metabolic activity of mature chondrocytes, cartilage tissue is unable to self-repair after injury.[1] In the absence of treatment, cartilage damage may initiate degenerative processes in the adjacent cartilage tissue and subchondral bone, which may eventually result in OA.[2] Treatments for chondral damage include arthroscopic debridement,[3] artificial joint replacement,[4] autologous chondrocyte transplantation,[5] chondral and osteochondral grafts,[6] and bone marrow stimulation.[7] The main

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ABSTRACT

Objectives: In this study, we aimed to determine the bioefficacy of epidermal growth factor (EGF), boric acid (BA), and their combination on cartilage injury in rats.

Materials and methods: In *in vitro* setting, the cytotoxic effects of BA, EGF, and their combinations using mouse fibroblast cell (L929), human bone osteosarcoma cell (Saos-2), and human adipose derived mesenchymal stem cells (hAD-MSCs) were determined by applying MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] test. In *in vivo* setting, 72 rats were randomly divided into four groups. A standard chondral defect was created and microfracture was performed in all groups. Group A was determined as the control group. In addition to the standard procedure, Group B received 100 ng/mL of EGF, Group C received a combination of 100 ng/mL of EGF and 10 µg/mL of BA combination, and Group D 20 µg/mL of BA.

Results: The cytotoxic effect of the combinations of EGF dilutions (1, 5, 10, 25, 50, 100, 200 ng/mL) with BA (100, 300, 500 µg/mL) was observed only in the 72-h application period and in Saos-2. The cytotoxic effect of BA was reduced when combined with EGF. There was no significant difference in the histopathological scores among the groups (p=0.13).

Conclusion: Our study showed that EGF and low-dose BA application had a positive effect on cartilage healing in rats. Significant decreases in recovery scores were observed in the other groups. The combination of EGF and BA promoted osteoblast growth. Detection of lytic lesions in the group treated with 20 µg/mL of BA indicates that BA may have a cytotoxic effect.

Keywords: Boric acid, cartilage regeneration, dose dependent, epidermal growth factor, *in vitro*, *in vivo*.

disadvantages of current clinical treatments include donor site morbidity, anatomical incompatibility, pain, and infection.[8]

To date, various treatment modalities such as arthroscopic debridement, artificial joint replacement, autologous chondrocyte transplantation, chondral and osteochondral grafts, bone marrow stimulation, hydrogel systems, and tissue engineering have been developed.[9] However, current clinical treatments may have disadvantages such as donor site morbidity, anatomical incompatibility, pain, and infection. As they predominantly form fibrous or fibrocartilage healing tissue, these options have not been clearly shown to be successful in restoring the original hyaline-type articular cartilage structure in humans.[10] In recent years, intra-articular injections have been utilized extensively for the treatment of chondral injuries. The primary objective of intra-articular injections is to slow the progression of cartilage damage and rate of progression of OA.

Boric acid (BA) is a metalloid which plays a role in the metabolism of steroid hormones, the development of healthy bones, and the maintenance of cell membranes. It has been shown that BA can reduce arthritic symptoms by inhibiting specific enzyme activities involved in the inflammatory response in diseases characterized by joint swelling and inflammation.[11] In the light of current studies on BA, there are few and relatively recent studies on fracture healing, tendon healing, and nerve healing in orthopedic practice.[12]

Epidermal growth factor (EGF) is a growth factor that plays a crucial role in cell growth, proliferation, and differentiation. The EGF-ligand stimulation of EGF receptors activates intracellular signaling pathways via PI3 kinase, mitogen-activated protein (MAP) kinase, and signal transducer and activator of transcription (STAT) pathways.[13] These signaling pathways, when activated, result in increased cell proliferation and decreased apoptosis. It has been reported that activation of the MAP kinase pathway is particularly effective on osteoblast metabolism.[14] In a rat study, it was found that inhibiting EGF receptors (EGFRs) in osteoprogenitor cells and osteoblasts reduced bone production.[15] In addition to its efficacy in wound healing, EGF is angiogenic by increasing the production of vascular endothelial growth factor (VEGF) in mesenchymal cells of bone marrow.[16]

In this experimental study, we aimed to investigate the effectiveness of injections, which can be an alternative to intra-articular injections, which are widely used currently, in the early stages of OA. We hypothesized that EGF and BA would change cartilage healing in a dose- and time-dependent manner. Therefore, we aimed to examine the *in vivo* effects of BA and EGF on articular cartilage regeneration, as well as their *in vitro* pharmacosafety effects on fibroblast, osteosarcoma, and mesenchymal stem cell lines.

MATERIALS AND METHODS

A total of 72 male Wistar Albino rats, aged older than 12 weeks and weighing 300 to 350 g on average, were obtained from Afyon Kocatepe University Experimental Animals Application and Research Center, and the experimental study was performed in this center. Before and after the surgical procedures, room temperature, room light condition, water, food supply, and care of the animals were carried out daily by the procedure.

In the experimental *in vivo* study, the rats were randomized and divided into four different groups with 18 rats in each group. Group A was the control group, Group B was the group in which 100 ng/mL of EGF was applied, Group C was the group that was treated with a combination of 100 ng/mL of EGF and 10 μg/mL of BA, and Group D was the group that was administered 20 μg/mL of BA.

Cell viability tests - *in vitro* cytotoxicity test

To determine the therapeutic dose ranges of EGF and BA for *in vivo* study, first of all, *in vitro* assay was carried out to evaluate non-toxic dose ranges. The cell viability, MTT assay was used to determine the cytotoxic effects of BA and EGF using mouse fibroblast cell line (L929) (CCL1, ATCC, USA), human bone osteosarcoma cell line (Saos-2) (HTB-85, ATCC, USA), and human adipose derived mesenchymal stem cells (hAD-MSCs). The hAD-MSC was used but not differentiated from the chondrocytes in this study, as it was not used to evaluate the bioefficacy of EGF and BA *in vitro* study. It was used to set up the non-toxic dose ranges of EGF and BA. In one of our other studies, MSC was successfully differentiated into chondrocytes.[17] The reason for the used cell lines, for mouse fibroblast cell (L929) was suggested to be used for testing the biocompatibility of medical devices; Saos-2 selected due to one of the main exposed targets upon application of BA and EGF for testing toxicity.

Cell culture conditions

The L929, Saos-2, and hAD-MSCs were cultured in 10% fetal bovine serum (FBS, heat-inactivated,

Gibco, #10500-064), 1% antibiotic-antimycotic solution (10,000 U/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B, Gibco, #15240-062), 1% Minimal Essential Medium (MEM) non-essential amino acid solution, (NEAA, Gibco, #11140050), 1% GlutaMAX™ (Gibco, #35050061) and Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, #11960044) containing 1% sodium pyruvate (Gibco, #11360070), 5% CO_2, and 37°C culture conditions. Fresh medium was added every two days, until the cells reached 70 to 80% density.

In *in vitro* study, the applied dose ranges of BA and EGF were determined according to the previous studies. The dose ranges of BA and EGF used in *in vitro* studies were very wide. The Panel on Food Additives and Nutrient Sources added to Food reported 0.16 mg boron/kg body weight/day ADI (amount allowed per day) concluded.[18] Gizer et al.[19] used BA in a dose range of 0.006 to 6 µg/mL. Klooster and Bernier[20] used 10 ng/mL of EGF to investigate the control of chondrocytes through the integration of multiple extracellular signals using for primary chondrocytes isolated from rats.

MTT assay

The L929, Saos-2, and hAD-MSCs were seeded at 7×10^3 cells/well in 96-well plates and incubated at 37°C and 5% CO_2 for 24 h. One day later, BA dilutions (10, 20, 50, 100, 200, 300, 500, 1,000, 3,000, 10,000, and 30,000 µg/mL), EGF dilutions (1, 5, 10, 25, 50, 100, and 200 ng/mL) were prepared in the DMEM and each dilution was added into the six wells of the 96-well plate. On the other hand, to evaluate synergistic, agonistic, antagonistic, or/and additive effects, each EGF dilution (1, 5, 10, 25, 50, 100, and 200 ng/mL) was combined with: *(i)* 100 µg/mL BA; *(ii)* 300 µg/mL BA; and *(iii)* 500 µg/mL BA) and added six wells of the 96-well plates. Then, 96-well plates were incubated at 37°C and 5% CO_2 for 24, 48, and 72 h. After each incubation period, DMEM containing 10% of MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] solution (5 mg/mL in DPBS, Sigma-Aldrich #M5655) was added and the cells were incubated for 4 h. Then, 100 µL of dimethyl sulfoxide (DMSO) was added to each well of the plate on a shaker for 2 h at room temperature to dissolve the purple formazan crystals formed due to the metabolic activity of living cells. The absorbance of the dissolved formazan was measured at 540 nm with a microplate reader (Biotek Instruments Inc., Winooski, Vermont, USA). The absorbance from treated cells was normalized to control cells and then cell viability was expressed in the mean percentage (%) of control values.

Surgical procedure

Xylazinbio (xylazine active ingredient, Bioveta, Czech Republic) 50 mL of 2% solution (0.1 mL per 400 g) and Alfamine (ketamine active ingredient, Atafen, Izmir, Türkiye) 50 mL of 10% solution (0.3 mL per 400 g) were prepared by applying appropriate anesthesia procedures. An anesthetic solution was injected into the quadriceps muscle of the rats for induction of anesthesia. An additional dose of 0.1 mL was administered, if needed during the surgical period. A surgical operation was planned on the left knee of all rats for standardization. After induction of anesthesia, the left knees of the experimental animals were shaved, washed, and cleaned. Batticon (Adeka, Istanbul, Türkiye) solution containing 10% povidone-iodine was used as an antiseptic. The knees of the animals were cleaned with povidone-iodine. The covering was provided by using a surgical sterile drape. A paramedian incision was used to open the left knee of the test animals. Through a single incision, the subcutaneous soft tissue connections and blood supply were preserved, while the skin was removed. The patella laterally deviated with a medial S-shaped incision in the parapatellar tissue. Utilizing appropriate retractors, the trochlear region of the femur was exposed. Using a 2-mm thick drill bit, a 3-mm deep cartilage defect covering 20% of the total joint surface area was, then, created. Up until this point, the procedures were carried out uniformly across all four groups. After that, injection solutions (at doses determined to be non-toxic in the *in vitro* cytotoxicity test) specific to the group in which each animal was implanted were prepared. After controlling the bleeding and repairing the parapatellar tissue, intra-articular injections were administered. The subcutaneous and dermal layers were repaired using 5/0 Vicryl sutures and dressed (Figure 1). As a preventative antibiotherapy, 0.1 mL of sulfamethazine and 100 mL were infused intravenously. After the operation, the rats were placed in cages that permitted free movement and received daily care. Six rats from each group were sacrificed for early, middle, and late evaluations of cartilage tissue healing at 4, 8, and 12 weeks after surgery. For induction, the same surgical and anesthesia procedure was utilized. Using the previous incision, the knee joint was accessed following anesthesia. For histopathologic preparation, the femoral condyles were completely excised while paying close attention to the distal articular surface of the femur and separated from the soft tissues.

Macroscopic evaluation

During the evaluation process, cartilage formation in the defective cartilage area and repair quality

were evaluated with the classical cartilage tissue repair criteria of modified Mankin[21] and O'Driscoll[22] classifications, and the macroscopic appearance of the materials belonging to each group was evaluated.

Histopathological evaluation

After the removal of the distal left femur of the rats, the soft tissues of the materials obtained were cleaned and placed in 10% buffered formaldehyde solution and fixed for seven days. After fixation, the entire material was decalcified in a solution composed of 50% formic acid and 20% sodium citrate solution in equal parts. Following the completion of the decalcification procedure, the bone tissue specimens were washed, passed through increasing levels of ethanol series (50 to 99%) and xylene series, and then embedded in paraffin blocks following paraffin infiltration melted at 62°C. From the paraffin blocks, 5 to 7-µm thick sections were taken on slides using a microtome (Leica RM2245). These sections were stained with hematoxylin-eosin (Figure 2) and Masson's Trichrome (Figure 3) staining methods and evaluated according to the modified Mankin and O'Driscoll scales. Using the modified Mankin scoring system, each sample is scored in five different categories by evaluating articular cartilage morphology, surface regularity, tidemark zone characteristics, and whether there is pannus formation on the cartilage surface. In this scoring system, scoring varies between 0 and 14; low scores indicate mild changes in cartilage tissue and high scores indicate severe degeneration.[21] The O'Driscoll score system includes parameters such as cellular morphology, safranin-O staining of the matrix, surface regularity, structural integrity, thickness, bonding to the adjacent cartilage, freedom from cellular changes of degeneration (hypocellularity), chondrocyte clustering, and freedom from degenerative changes in adjacent cartilage. The score value in this complex system ranges from

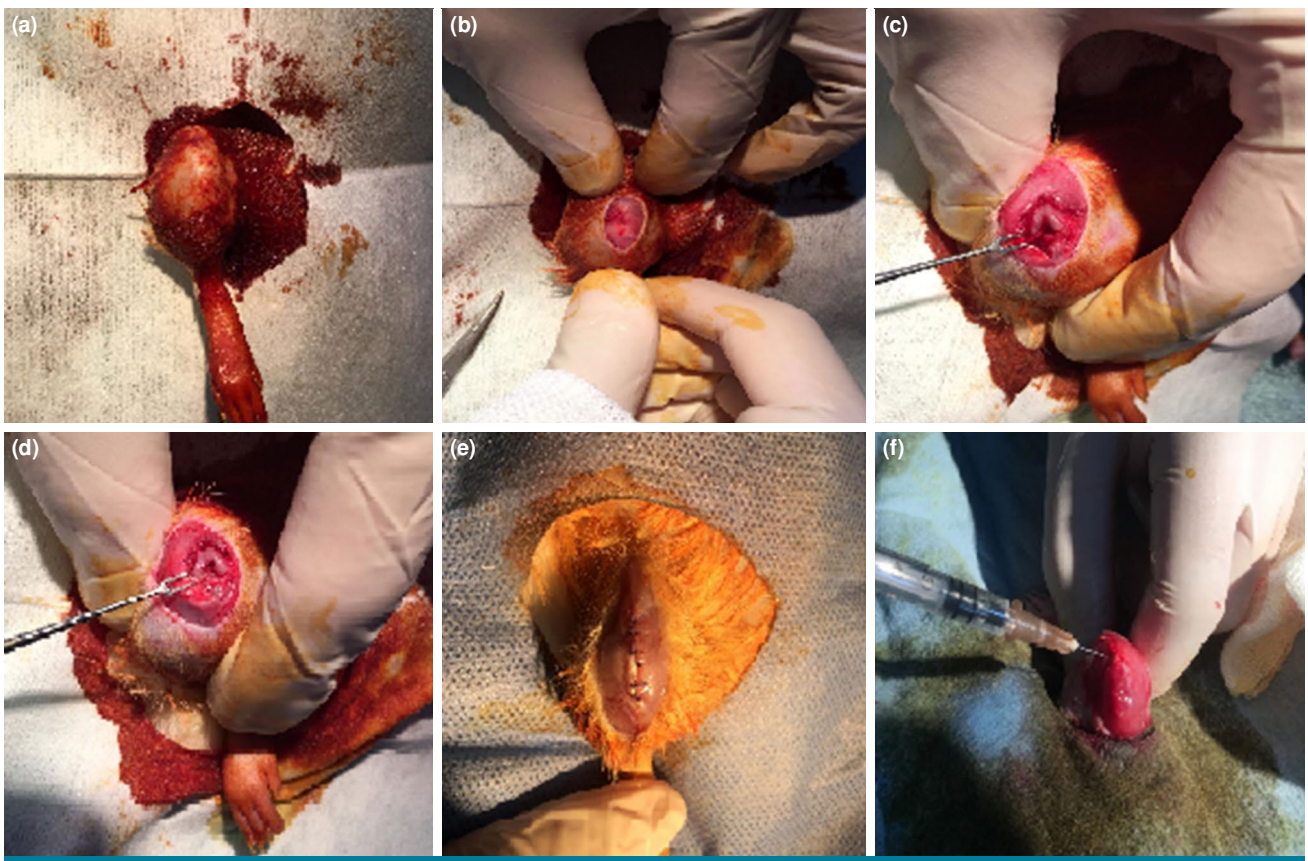

FIGURE 1. Stages of surgical experimentation. **(a)** Image of the experimental animal after general cleaning covering with a sterile drape. **(b)** Paramedian incision image of the left knee with skin and subcutaneous incision. **(c)** Image of lateral deviation of the patella and subsequent distal condyles of the femur. **(d)** Image of the distal condyles of the femur and the defective area. **(e)** Image after patellar tendon repair. **(f)** Intra-articular administration of a group-specific active substance.

0-24 points where 0 is no sign of cartilage repair and 24 is complete regeneration, the maximum points value for one parameter range between 2 and 4.[22] The stained sections were photographed using a Nikon Ci-S light microscope, Nikon DS-Fi3 camera, and NIS-Elements D image analysis system (Nikon Corp., Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 23.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were expressed in mean ± standard deviation (SD), median (min-max) or number and frequency, where applicable. The normality assumptions of the variables were examined by skewness and kurtosis coefficients, Kolmogorov-Smirnov test and histogram, while the Kruskal-Wallis test was used to compare continuous variables that did not show normal distribution among multiple groups. If a significant difference was found as a result of the Kruskal-Wallis test, the Mann-Whitney U test with the Bonferroni correction was performed to determine between which groups the difference originated. A p value of <0.05 was considered statistically significant.

RESULTS

Effects of EGF and BA on cell viability

The cytotoxic effect of BA on L929, Saos-2, and hAD-MSCs in the studied concentration range increased proportionally with the exposure time (Figure 4a). However, the cytotoxic effect on Saos-2 was greater than on L929, followed by hAD-MSCs. The EGF at a dose of 200 ng/mL caused less than a 30% decrease in cell viability against L929 and hAD-MSCs at exposure times, while it showed 50% and 70% cytotoxic effects on Saos-2 at

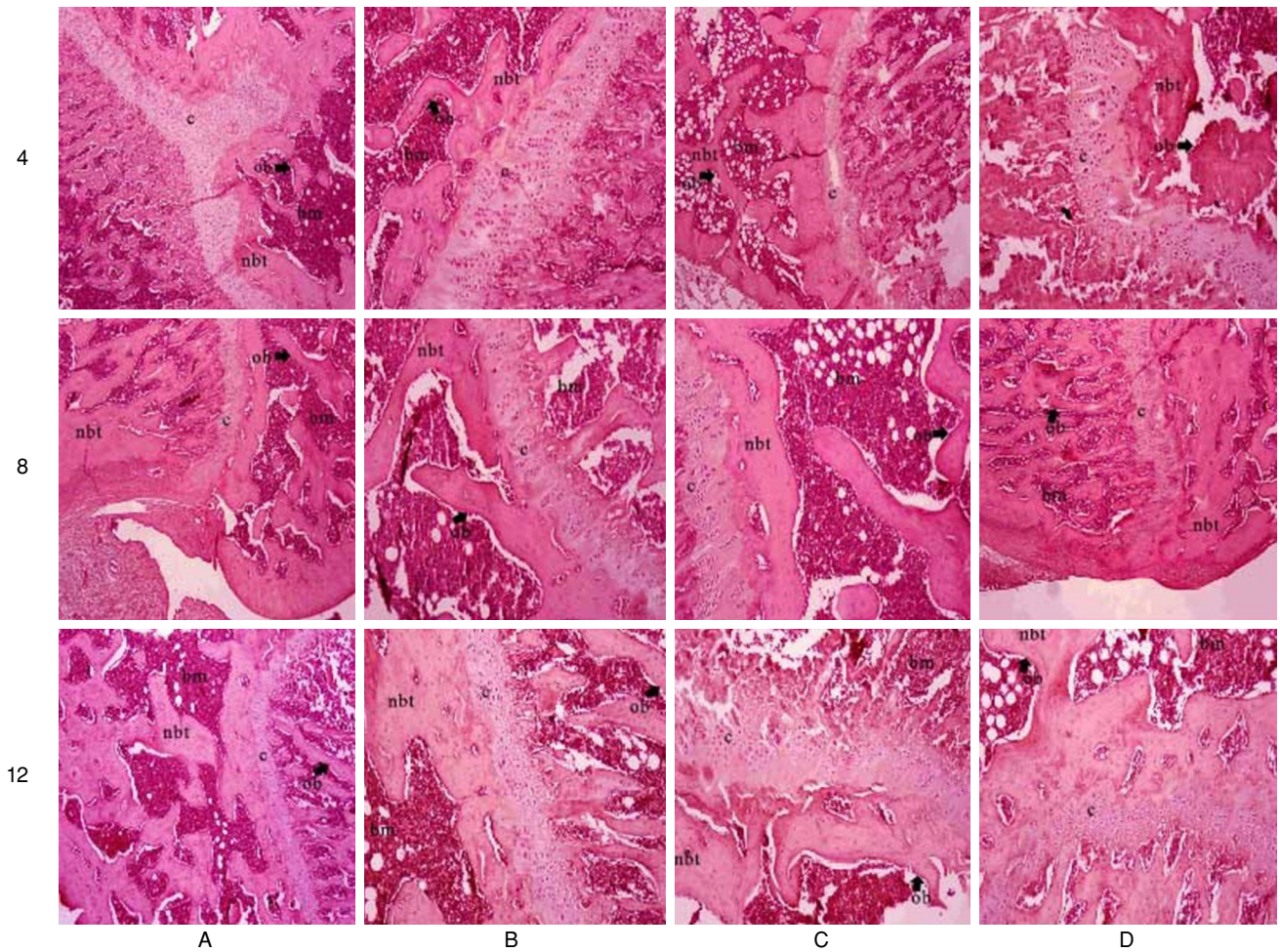

FIGURE 2. Microscopic imaging with hematoxylin-eosin (H&E, ×10). A-E: Group names, Evaluation weeks 4, 8, and 12.

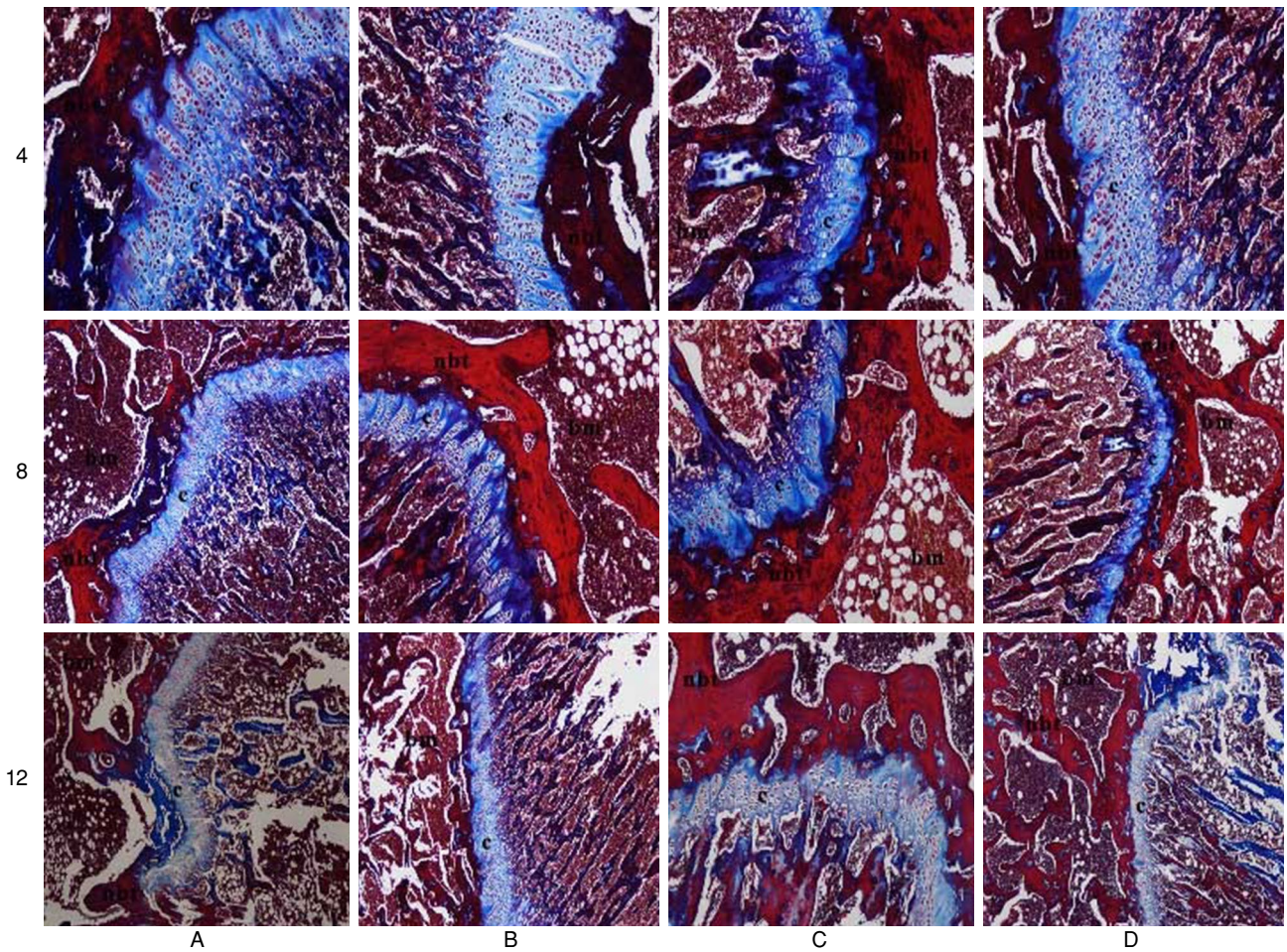

FIGURE 3 Microscopic imaging with Masson's Trichrome (×10). A-E: Group names, Evaluation Weeks 4, 8, and 12.

48 and 72 h of exposure (Figure 4b). The 50% cell viability inhibition concentrations (IC50) of BA and EGF applied separately and in combinations against L929, Saos-2, and hAD-MSCs were calculated from the dose-response curves obtained from the MTT assay (Figure 4a, b). The IC50 values of BA and EGF are given in Table I. In general, BA had lower IC50 values against Saos-2 than L929 and hAD-MSCs. On the other hand, EGF induced a cytotoxic effect on Saos-2 only at 48 and 72 h of exposure in the tested concentration range (Table I).

The cytotoxic effects of EGF and BA combinations on L929, Saos-2, and hAD-MSCs are presented in Figure 4. The combination of EGF dilutions with 300 and 500 µg/mL doses of BA significantly increased cell viability when applied to L929 for 24 h, compared to the effect when applied alone. However, there was no significant change when the tested combinations were applied for 48 h. However, combinations of EGF dilutions with 100 µg/mL BA caused a significant increase in L929 cell viability when applied for 72 h (Figure 5a). The combination of EGF dilutions with 100 µg/mL BA significantly induced cell viability in Saos-2 after 48 and 72 h of treatment (Figure 5b). While there was no significant difference in cell viability when combinations of EGF dilutions and BA were applied to hAD-MSCs for 24 and 48 h, combinations of EGF dilutions with 300 and 500 µg/mL doses of BA caused a significant decrease in cell viability when applied for 72 h (Figure 5c).

The IC50 values of the effects of EGF dilutions (1, 5, 10, 25, 50, 100, 200 ng/mL) in combination with BA (100, 300, 500 µg/mL) on L929, Saos-2, and hAD-MSCs were calculated from the dose-response graph obtained by MTT cell viability colorimetric assay (Table II). The cytotoxic effect of the combination of EGF dilutions with BA was observed only at 72 h of treatment time and in Saos-2. The most important result is the reduction of the cytotoxic effect of BA due to its use in combination

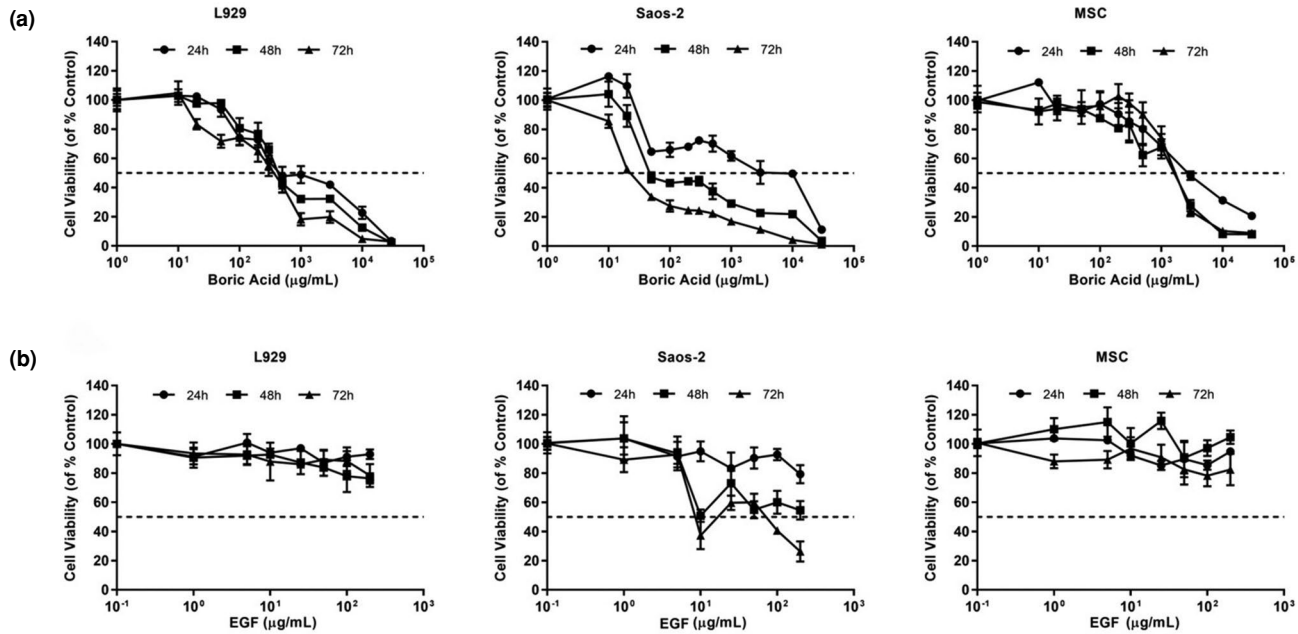

FIGURE 4. After **(a)** boric acid and **(b)** EGF compounds were applied on L929, Saos-2, and hAD-MSCs at the tested concentration range for 24, 48, and 72 h, the dose-response relationship was determined by MTT cell viability assay (mean ± standard deviation, n=3).
EGF: Epidermal growth factor; hAD-MSC: Human amnion-derived mesenchymal stem cell; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

with EGF, although it is highly cytotoxic alone for the cells used.

Macroscopic evaluation

A total of 72 rats were studied and no wound infection or death was observed. Mild edema and swelling were observed in the knee joint areas of all subjects for the first seven days. At the time of sacrifice, all defect areas were macroscopically distinguishable from the surrounding cartilage tissues and no synovitis or infective tissue was detected in the surgical field (Figure 6).

Histopathological evaluation

The cartilage repair tissue surface was regular in the control group and irregular in the other groups. The difference among the group scores was not significant (p=0.085) (Table III).

When the matrix was examined, the repair tissue was fibrocartilage in the control group and hyaline and fibrocartilage in the other groups. The mean score in the control group was the highest at 3.06 and the mean score in Group C was the lowest at 1.8. The difference was not significant (p=0.088) (Table III).

In terms of cell distribution, the control group had the highest scores during the early recovery period, and Group C had the highest scores over the long term. In Group C, the healing of cartilage repair was observed to improve over the subsequent weeks. The increase in Group C score was statistically significant (p=0.014) (Table III).

TABLE I
IC50 values (mean ± standard deviation, n=3) calculated from the dose-response curve determined by MTT cell viability assay after boric acid and EGF compounds were applied on L929, Saos-2, and hAD-MSCs for 24, 48, and 72 hours

	Boric acid (µg/mL)			EGF (ng/mL)		
Exposure time (h)	L929	Saos-2	hAD-MSC	L929	Saos-2	hAD-MSC
24	949.63±110.79	8988.06±282.6	3905.20±375.54	>200	>200	>200
48	762.05±48.46	52.29±1.45	1803.10±52.24	>200	>200	>200
72	316.24±24.57	29.72±0.55	1762.88±51.47	>200	77.90±20.89	>200

EGF: Epidermal growth factor.

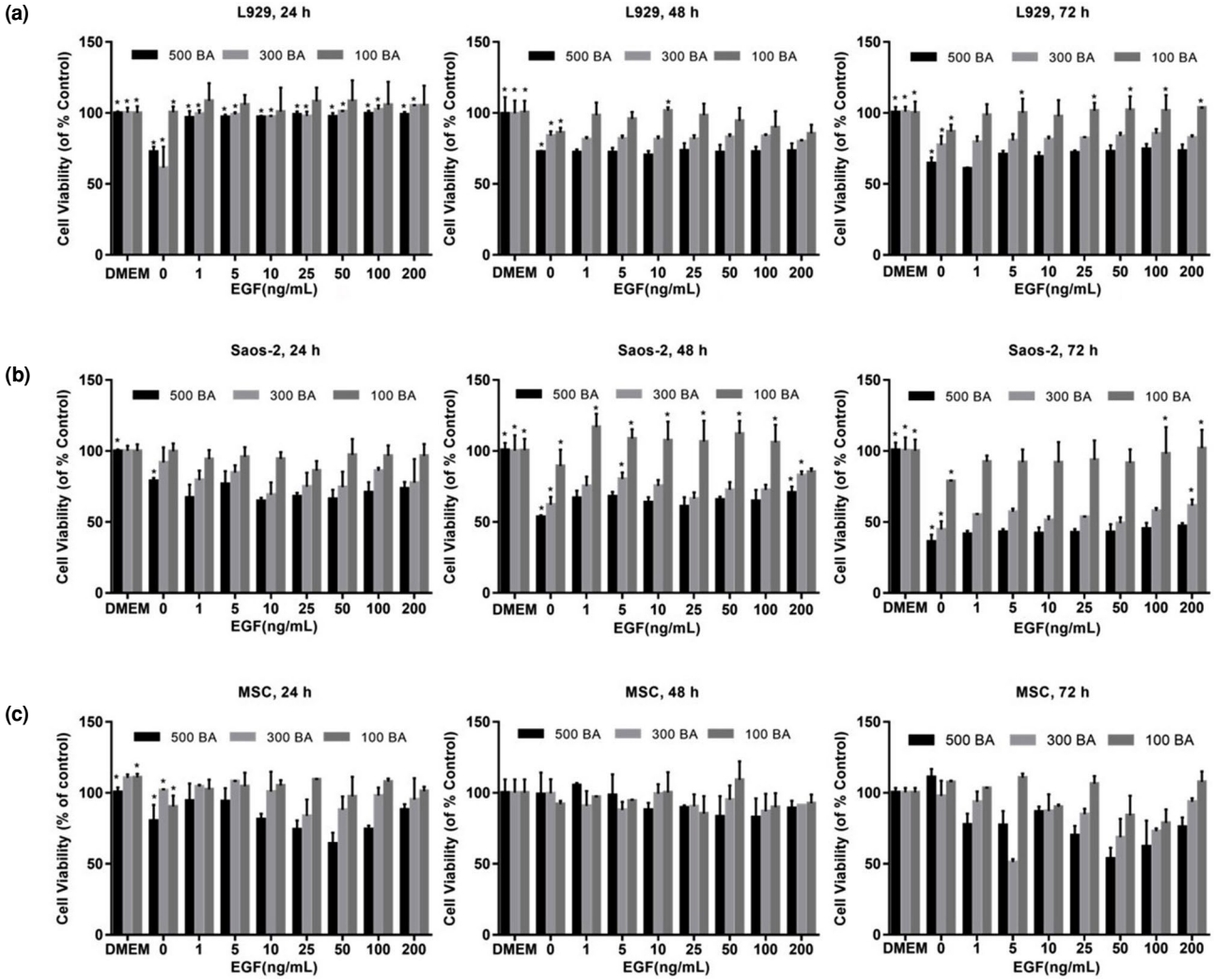

FIGURE 5. The effects of EGF dilutions (1, 5, 10, 25, 50, 100, 200 ng/mL) in combination with BA (100, 300, 500 µg/mL) on cell viability were analyzed by MTT assay after 24, 48 and 72 h of treatment on **(a)** L929, **(c)** Saos-2 and **(c)** hAD-MSCs (mean ± standard deviation, n=3). The DMEM medium containing no compounds was used as a control.
EGF: Epidermal growth factor; BA: Boric acid.

TABLE II

IC50 values (mean ± standard deviation, n=3) of EGF dilutions (1, 5, 10, 25, 50, 100, 100, 200 ng/mL) in combination with Boric Acid (100, 300, 500 µg/mL) concentrations calculated from the dose-response curve determined by MTT cell viability assay after 24, 48 and 72 h on L929, Saos-2 and hAD-MSCs

	EGF (ng/mL) with 500 µg/mL Boric acid, IC50 values			EGF (ng/mL) with 300 µg/mL Boric acid, IC50 values			EGF (ng/mL) with 100 µg/mL Boric acid, IC50 values		
Exposure time (h)	L929	Saos-2	hAD-MSC	L929	Saos-2	hAD-MSC	L929	Saos-2	hAD-MSC
24	>200	>200	>200	>200	>200	>200	>200	>200	>200
48	>200	>200	>200	>200	>200	>200	>200	>200	>200
72	>200	7,35±2,14	>200	>200	>200	>200	>200	>200	>200

EGF: Epidermal growth factor.

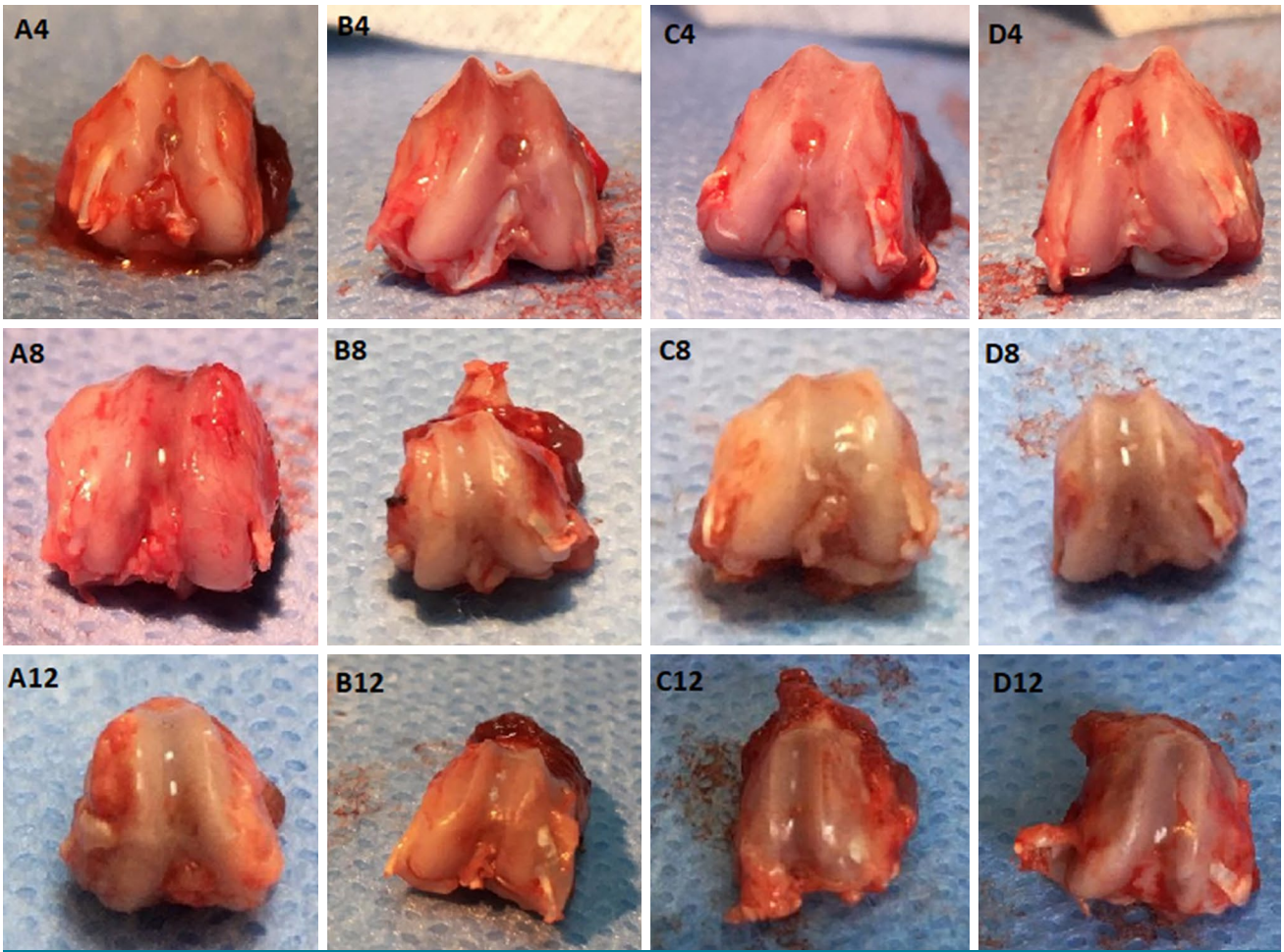

FIGURE 6 Macroscopic Imaging. A-E: Group names, Evaluation Weeks: 4, 8, and 12.

In terms of cell population viability, the highest scores in all periods were in the control group, and the lowest scores were in Group D. A time-dependent increase in Group C score was significant ($p<0.05$) (Table III).

When the subchondral bone was examined, the highest scores in all periods were in Group C, and the lowest scores were in Group D. Time-dependent scores increased in Group C. This increase was significant ($p=0.05$) (Table III).

The total score was 18.81 in the control group, 18.65 in Group B, 16.75 in Group C and 14.87 in Group D (Figure 7). The difference between the groups was not significant ($p=0.13$). Contrary to the other groups, the total scores increased in Group C in a time-dependent manner.

According to the modified Mankin scoring, the best cartilage healing scores in all weeks were in Group A. In the late period results, Group D scores were closest to Group A scores. In the late period, a significant decrease was observed in Group D scores and the lowest score was obtained. According to the O'Driscoll scoring, the best scores in early period cartilage healing were observed in Group A, while the lowest scores were observed in Group C. In the middle period, the lowest scores were observed in Group D. While the lowest scores in the late period were observed in Group D, the scores were equally and significantly higher in the other groups. In the long term, a significant decrease was observed in Group D scores and the lowest score was obtained (Figure 7).

When the groups were evaluated in a time-dependent manner according to both scoring systems, a significant increase was observed in Group C, while a significant decrease was found in the cartilage healing scores of the other groups in the following weeks (Figure 7).

TABLE III

Histopathological evaluation was made according to the MMS and ODS, and the scores are given in a single table. While scoring, average values are taken as basis. (A, B, C, D, E indicate group names, values of 4, 8, 12 indicate the week of examination)

		Groups and Score											
Evaluation	Parameters	A4	A8	A12	B4	B8	B12	C4	C8	C12	D4	D8	D12
MMS	Articular cartilage morphology	1.99	2.59	4.02	2.62	2.80	3.15	5.25	5.95	6.65	4.90	5.39	5.95
	Surface regularity	2.27	3.43	3.78	2.27	2.62	3.15	5.60	6.30	5.95	5.25	5.77	6.65
	Tidemark zone	2.20	2.06	1.92	4.97	5.25	5.42	4.02	2.80	2.45	3.32	3.53	3.67
	Pannus formation	2.06	2.41	2.80	5.60	5.95	6.30	4.90	4.20	2.80	2.45	3.81	5.25
	Total	2.15	2.52	2.89	4.10	4.37	4.69	4.76	4.41	4.06	3.85	4.41	5.04
ODS	Cellular morphology	19.20	18.00	17.40	19.20	18.66	18.18	15.96	15.42	14.58	18.80	17.76	15.00
	Staining of the matrix	18.80	18.00	17.40	19.20	17.40	16.80	11.58	10.62	10.20	16.20	13.20	12.60
	Surface regularity	19.80	18.60	18.00	19.20	18.60	18.00	13.32	17.10	18.60	15.60	13.20	12.00
	Structural integrity	21.00	20.40	18.00	19.8	19.20	18.60	14.46	16.5	18.60	16.80	13.20	10.8
	Thickness	20.40	19.80	18.60	19.20	18.66	18.36	15.66	17.70	20.40	19.20	12.00	9.60
	Bonding	19.50	18.60	18.00	18.90	18.60	18.30	14.49	16.20	18.00	18.00	14.10	11.40
	Hypocellularity	17.40	17.40	17.40	18.00	18.30	18.60	19.14	19.26	19.56	18.60	16.20	12.66
	Chondrocyte clustering	18.60	19.20	18.60	18.00	18.60	19.20	15.66	18.24	20.40	18.00	15.00	12.00
	Degenerative changes	20.40	18.60	18.00	20.40	20.16	19.80	14.46	16.50	18.60	19.20	14.40	10.80
	Total	19.70	18.75	18.00	19.10	18.61	18.24	15.14	16.80	18.19	18.05	14.53	12.05

MMS: Modified Mankin Score; ODS: O'Driscoll Score.

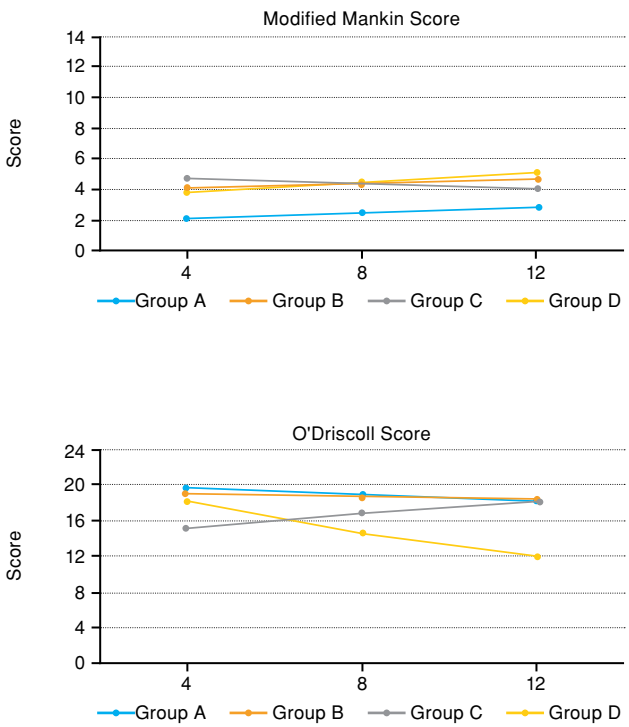

FIGURE 7. Modified Mankin and O'Driscoll Scoring.

DISCUSSION

In our study, we determined that EGF and low-dose BA application had a positive effect on cartilage healing. It has been reported that high doses of BA can decrease the viability of mouse osteoblasts and human bone marrow mesenchymal stem cells (hBMSCs),[23] while low doses do not alter the viability of the cells. It has been also reported that BA increases the mineralization of mouse osteoblasts[23] and the alkaline phosphatase (ALP) activity of hBMSCs[24] and rat bone marrow mesenchymal stem cells (rBMSCs).[25] In a study, BA treatment of Saos-2 at a dose range of 0.006 to 0.6 µg/mL did not affect the proliferation rate on Days 1 and 3, but it significantly reduced the proliferation of Saos-2 on Day 5.[19] However, BA at the same doses did not affect the proliferation of hBMSCs compared to untreated cells during all exposure periods. In our study, BA did not show any cytotoxic effect at low doses, but at high doses, it decreased the viability of cell lines.

Gok et al.[26] created a full-thickness osteochondral defect of 4 mm in diameter and 3 mm in depth in the medial condyle of the femur of rabbits. They injected

8 mg/kg of BA solution intra-articularly every week for six weeks. In the evaluation at the end of the eighth week, they found significant differences in favor of the BA group in terms of the degree of defect repair, integration of the border region, and macroscopic appearance in the macroscopic evaluation of the defect area. In our study, no significant difference was found in cartilage healing in Group D that underwent BA. The lytic lesions seen in this group suggest that BA may have a high concentration of chondrogenic effects.

In their study, Korkmaz et al.[27] compared the effect of BA and hyaluronic acid (HA) in the repair of a full-thickness defect of 1.5 mm in diameter and 2 mm in depth in the femoral condyles of rats. They administered 10 mg/kg of BA to the BA group, 0.1 mL of HA to the HA group, and 0.1 mL of physiological saline to the control group, intra-articularly every week for four weeks. In the evaluation at the end of Week 12, the total cartilage repair score of the HA group was better than the BA and control groups. As a result, they suggested that BA was not as effective as HA in repairing osteochondral defects, but its antioxidant properties were superior to HA. In our study, we found that the BA group received lower scores than the control group.

Chandra et al.[15] demonstrated that EGFR signaling promoted proliferation and inhibited apoptosis, which is essential for maintaining the number of osteoprogenitor cells. By regulating the ratio of proliferative and apoptotic osteoprogenitor cells in bone, EGF treatment was shown in the aforementioned study to significantly increase the number of osteoblasts. Laflamme et al.[28] examined the *in vitro* osteogenic activity of EGF about bone morphogenetic proteins BMP2 and BMP7. This study demonstrated that EGF might promote osteoblast growth and specific marker expression in the early phase, as opposed to the late phase of cell differentiation/mineralization. In our study, EGF reduced the cytotoxic effect of BA, which on its own was highly cytotoxic to the used cells.

There are limited recent studies on BA in the practice of orthopedics in the literature. Experimental studies on the effects of BA on fracture, tendon, and nerve healing are available in the literature. In these studies, BA had a positive effect on fracture healing, had a positive effect on tendon healing, but this effect should be supported by biomechanical tests and reduced myelin and axonal injury in nerve healing.[12,29,30] In a clinical study, intra-articular injection of 8 mg/kg of BA 10% concentration had a positive effect on cartilage healing.[26] The authors reported that the possible mechanism for this positive effect was the suppression of inflammatory and oxidative processes in addition to increased synthesis and release of extracellular matrix (ECM) content. Benderdour et al.[31] found that BA regulated ECM formation in their study. The BA decreased the synthesis of extracellular matrix macromolecules such as proteoglycans and collagen in cultured human dermal fibroblasts and increased the release of these molecules into the culture medium. Again, in a study by Benderdour et al.[32] on the effects of BA on cartilage metabolism, they found that the presence of boron in the culture medium of pelvic cartilage in chick embryos decreased the synthesis of proteoglycans, collagen, and total protein amount, but on the other hand, it increased the release of these macromolecules.

The EGF stimulates a variety of biological responses, including cell proliferation, differentiation, and migration, via the EGFR receptor, which can also be combined with the other growth factors, and the EGF/EGFR signaling regulates normal development. Additionally, it is involved in pathophysiological processes. This has been demonstrated clinically, particularly in ulcer and wound healing and tissue repair following ischemia and reperfusion injury. Exogenous administration of EGF reduces tissue damage following ischemia-reperfusion injury through antiapoptotic and antioxidant effects.[33] In a rat study, inhibiting EGFR in osteoprogenitor cells and osteoblasts reduces bone production.[15] In addition to its efficacy in wound healing, EGF is angiogenic by increasing the production of VEGF in mesenchymal cells of bone marrow.[16]

Gui et al.[34] showed that EGFR signaling was necessary to protect chondroprogenitors during articular cartilage development and homeostasis. They also found that mechanistically, activating EGFR signaling promoted the survival and lubrication of chondrocytes at the lesion site, thereby reducing the loading effect on cartilage.

Several studies have shown that EGFR gene cartilage-specific knockout mice have cartilage surface defects and may be subject to cartilage degeneration.[35] Jiang et al.[36] analyzed the development mechanism of OA and the factors and pathways that might affect it and found that EGFR was critical for tissue metabolism.

Articular cartilage is maintained by balanced anabolic and catabolic actions of growth factors. Earlier studies on the EGFR signaling pathway have demonstrated its dual regulatory actions

on chondrocytes, emphasizing the importance of analyzing this pathway *in vivo*. The EGFR signaling is critical for adult cartilage homeostasis. The EGFR signaling is also required for cartilage protection after OA initiates. Wei et al.[37] in their studies in the past years found that EGF family ligands acted on EGFR and played a critical role in cartilage development, hemostasis, and degeneration. The BA slows down the acceleration of the arthritic process by suppressing the activity of some enzymes specific to the inflammatory response.[11] In addition to being effective in wound healing, EGF increases the production of VEGF in bone marrow mesenchymal cells and exerts angiogenic activity.[16] Our study and previous studies showed that the use of EGF in combination with BA at low doses promoted osteoblast growth. In our study, the use of EGF and low-dose BA combination had a positive effect on cell lines and cartilage damage healing in the long term.

Nonetheless, there are certain limitations to our study. Among these, the sizes of cartilage defects created in experimental animals were not standardized and the surgical field was small. Standardization could not be made, as the animals were between 300 and 350 g on average and the sizes of the femoral condyles were different. In addition, the lytic lesions of the BA dose used in Group D may have caused low scores in the evaluation of cartilage healing.

In conclusion, EGF and low-dose BA application had a positive effect on cartilage healing in rats. Significant decreases in recovery scores were observed in the other groups. The combination of EGF and BA promoted osteoblast growth. Detection of lytic lesions in the group treated with 20 µg/mL of BA indicates that BA may have a cytotoxic effect. In the light of these findings, further long-term studies should be performed in animal models with larger cartilage defects to evaluate intra-articular injections.

Ethics Committee Approval: The study protocol was approved by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (AKUHADYEK) Ethics Committee (date: 01.04.2020, no: 49533702/252. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Idea/concept, control/supervision, critical review: B.K.Y., M.N.K.; Design, data collection and/or processing: B.K.Y.; Analysis and/or interpretation: H.H.D., S.İ.; Lıterature review: B.K.Y., Y.Ç., A.G.; Writing the article: B.K.Y., Y.Ç.; References and fundings: M.N.K., Y.Ç., A.G.; Materials: B.K.Y., S.İ., H.H.D.

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