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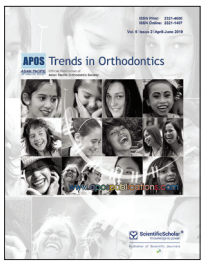

Original Article

Genetic expression of vascular endothelial growth factor, decorin, and matrix gla protein as function of anterior mandibular repositioning appliance with and without administration of insulin-like growth factor-1 and transforming growth factor–β on the growth of mandibular condyle

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ABSTRACT

Objectives: New Zealand (NZ) young rabbits with the administration of insulin-like growth factor-1 and transforming growth factor-β with and without mandibular anterior repositioning appliances are explored for the growth of the mandibular condylar cartilage (MCC). Genetic expression of vascular endothelial growth factor (VEGF), decorin (DCN), and matrix gla protein (MGP) in MCC has been studied which is confirmed by histomorphometry.

Material and Methods: Twenty-four growing NZ rabbits were divided into three groups; a group with saline injection in temporomandibular joints, a group that received anterior positioning appliance, and a group that received growth factors injection as well as mandibular repositioning appliance.

Results: Administration of growth factors along with mandibular repositioning appliances has induced (i) 4.59-fold expression of DCN ($P < 0.0005$), (ii) 3.22-fold expression of MGP ($P < 0.0005$), and (iii) 2.80-fold expression of VEGF gene ($P < 0.0005$). In contrast, the administration only of mandibular repositioning appliances has induced (i) 2.78-fold expression of DCN ($P < 0.0005$), (ii) 2.58-fold expression of MGP ($P < 0.0005$), and (iii) 1.23-fold expression of VEGF gene ($P < 0.0005$). Histomorphometry confirmed an increase in the length of the condylar cartilage.

Conclusion: Administration of growth factors along with mandibular advancement appliance has increased genetic expression of markers of condylar growth. Thus, the injection of growth factors along with functional appliances could be an excellent treatment modality for the treatment of mandibular retrognathism.

Keywords: Functional appliances, Insulin-like growth factor-1, Mandibular condylar cartilage, Mandibular repositioning appliances, Transforming growth factor-β

INTRODUCTION

The mandibular condyle plays a pivotal role in the development of the oro-facial complex since it provides endochondral bone growth to function as a regional adaptive growth site and is known to adapt to different functional factors. Growth of the mandibular condylar cartilage (MCC) is a function of genetic and epigenetic factors. The growth centers around the differential spatial

concentration of the chondrocyte's influence on growth factors such as transforming growth factor-β (TGF-β) and insulin-like growth factor (IGF). While IGF-1 promotes proteoglycan synthesis and survival of the chondrocytes to maintain cartilage homeostasis, TGF-β synergistically catalyzed the effect of IGF-1.[1,2] Accordingly, besides growth factors, condylar growth modification is induced by mandibular advancement.[3-7] Most of the studies in this regard have used either histological, histomorphometric, immuno-histomorphometric, biochemical, or auto-radiographic methods as a diagnostic tool to evaluate the growth at the condyle[8-11] or detected increased expression of some growth factors/biomarkers of MCC growth. Although these studies have dealt with condylar growth by providing valuable leads at a cellular level, several questions have remained unanswered and could be answered only on a genetic level, elucidated by cellular studies, and quantified by molecular markers. Expression of VEGF, a potent regulator of neo-vascularization, was observed in the condyles and glenoid fossa of the growing rats.[12-15] DCN plays an essential role in the development, texture, integrity, maintenance, and functions of virtually all tissues, including MCC.[16] It is postulated that MGP has a regulatory role in chondrocytes, cartilage, and mineralization of skeletal as well as dental tissues.[17-20] Thus, the present study has precisely attempted to evaluate the genetic expression of VEGF, DCN, and MGP as markers of condylar growth in young rabbits as a function of mandibular anterior repositioning appliances with and without the administration of growth factors (TGF-β and IGF-1).

MATERIAL AND METHODS

In this study, 60-day-old (pubertal age group) 32 male/female New Zealand (NZ) albino rabbits were used as approved by the Animal Ethical Committee (CPCSEA-01-2009). The rabbits were randomly divided into the following three groups of 8 each (four males and four females).

Control Group (S) – Saline injection in temporomandibular joints (TMJ)

Experimental-I Group (A) – Mandibular anterior repositioning appliances

Experimental-II Group (I) – Growth factors injection

Experimental-III Group (AI) – Mandibular anterior repositioning appliances with growth factor injection

Appliance fabrication and cementation

Impressions were taken using alginate impression material for all rabbits, and appliances were accordingly fabricated using heat-cured acrylic material (Ivoclar Vivadent-SR Triplex Hot).

The rabbits fitted with the above bite-jumping devices were closely observed daily at 0600, 0930, 1230, 1400, 1630, 2130, and 2400 hours for retention of the appliance, its wear or damage, tissue irritation, somatic growth status (if any), and their tolerance as judged from the quantum of food intake and body weight [Figure 1].

Injection of growth factors

IGF-1, lyophilized powder from mouse recombinant expressed in *Escherichia coli;* Sigma 25 ng/25μL, and TGF-β, lyophilized powder from human platelets, 1×10^6 units/mg; Sigma 20 ng/25 μL were injected in the inferior joint space. The needle was directed at 45° in reference to the mid-sagittal plane, in the fossa behind the posterior orbital ridge until it contacted the condyle. Digital radiographs were taken to confirm the precise location of the needle before injecting both growth factors at the same time [Figure 2]. The control group was injected with an equal volume of phosphate-buffered saline instead of growth factors. The injections were administered on day 7, day 14, and day 21 and were euthanized on day 30. The condyles were immediately recovered, snap-frozen in liquid nitrogen, and stored at −80°C until the isolation of RNA.

Real-time reverse transcription-polymerase chain reaction (PCR) technique

The condylar tissue isolated from both groups was treated with a Ribopure kit (Ambion), for rapid purification of total high-quality RNA as per its protocol and stored at −20°C. Subsequently, RNA from each control and experimental sample was converted into complementary DNA (c-DNA) using an equal quantity of 2× RT master mix in PCR tubes and thermo-cycled as per the protocol by c-DNA reverse transcription kit (Applied Biosystems). The reaction was set in 48 well plates, with each well containing 10 μL Master mix TaqMan 2×, 1 μL primer (gene-specific), 2 μL c-DNA (sample specific), and 7 μL sterile H_2O, making a total 20 μL reaction mixture, with glyceraldehyde-3-phosphate dehydrogenase as the endogenous control. The primers used for the corresponding genes are shown in [Table 1].

Table 1: Profile of primer sequence used for the corresponding gene for TaqMan technique in real-time RT-PCR.

Gene	Primer sequence (FAM)
VEGF	CCCGAGCTAACACTTC
DCN	GATTGTCATAGAACTGGGCACCAAC
MGP	TCGCCATCCATCTCTG

RT-PCR: Reverse transcription-polymerase chain reaction, VEGF: Vascular endothelial growth factor, DCN: Decorin, MGP: Matrix gla protein

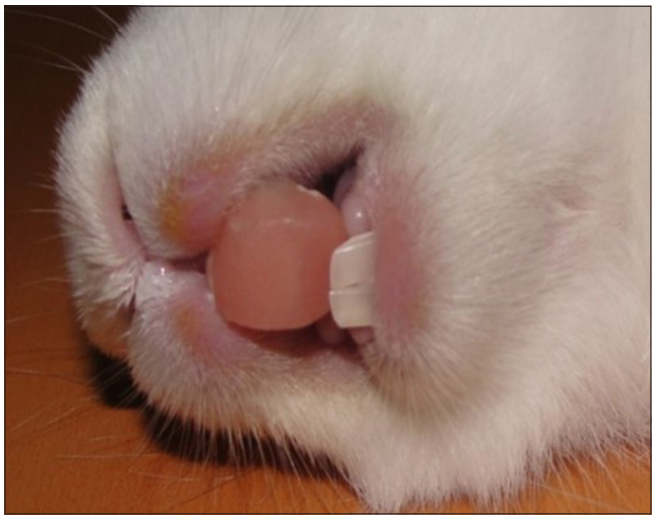

Figure 1: Bite jumping device fitted in the oral cavity.

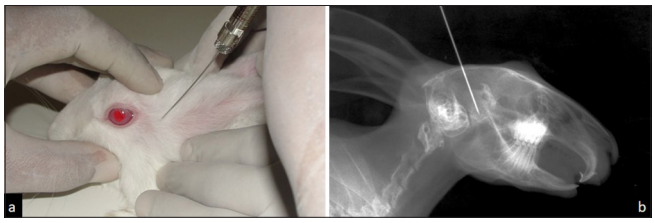

Figure 2: (a) Angulation of the Injection and Insertion; and (b) radiograph confirming the same.

The 48-well plate was set in an Applied Biosystems StepOne Real-Time PCR machine with the TaqMan technique. The results were analyzed for gene expression using Step One (Version 2.1) software. Alician Blue–Periodic acid–Schiff staining and hematoxylin and eosin staining were carried out for histologic sections, and the slides were observed under the Leica DM 5000B microscope under 10× magnification. The images were captured with a Leica DFC 320 camera and were analyzed by Leica Application Suite 3.70 Image analysis software.

Statistical analysis

The data were statistically analyzed using the Statistical Package for the Social Sciences ver 11.5, Inc. Chicago, USA. $P < 0.05$ is statistically significant. All the hypotheses were formulated using two-tailed alternatives against each null hypothesis (hypothesis of no difference). All results are presented as mean ± standard deviation across the study groups and statistical significance of difference for histology tested using the Mann–Whitney U-test.

RESULTS

Effects of VEGF gene expression

As depicted in [Figure 3], VEGF gene expression in a group with mandibular repositioning appliance was 1.23-fold ($P <$ 0.0005); with an injection of growth factors, only was 1.65 fold ($P < 0.0005$) whereas, in the group with mandibular repositioning appliance and growth factors, the injection was 2.80-fold ($P < 0.0005$), as depicted in [Figure 3].

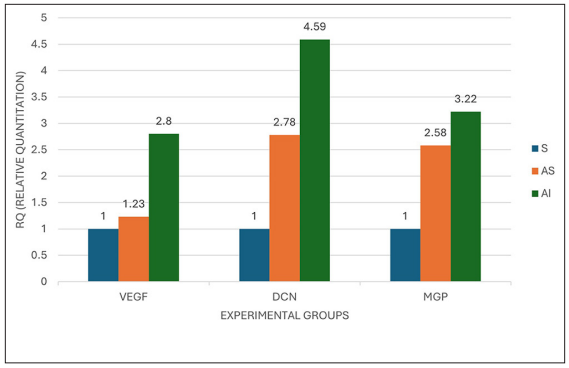

Figure 3: Histogram representing RQ of VEGF, DCN, and MGP gene expression in control and experimental groups. RQ: Relative quantitation, VEGF: Vascular endothelial growth factor, DCN: Decorin, MGP: Matrix gla protein. S: Injection of growth factors only, AS: mandibular repositioning appliance, AI: mandibular repositioning appliance and growth factors.

Quantitative assessment of the prostaglandin content in the condylar cartilage and histomorphometry

For quantitative assessment, a representative measurement frame of 300 × 300 μm was selected with a focus on an area of 4760.992 μm^2. Figure 4 illustrates that the area indicating prostaglandin presence, as represented by the magenta color, was the smallest in the control group [Figure 4a], increased in the appliance-only treatment group [Figure 4b], and was approximately twice as large as the control group in the combined treatment group [Figure 4c]. The histochemical profile [Figure 5] showed an increase in the size and length of the proliferative layer of the cartilage. The length of the condylar cartilage from the fibrous layer to the hypertrophic layer was synergistically greater in the group treated by appliances and growth factors than in the growth factor alone or appliance-only groups [Figure 5]. The reading by the image analysis software and statistical analysis are summarized in [Tables 2 and 3].

Effects of DCN gene expression

The effect of mandibular repositioning appliances along with the administration of IGF-1 and TGF-β on the DCN gene expression is depicted in [Figure 3]. Accordingly, DCN gene expression was (i) 4.59-fold ($P < 0.0005$) in the mandibular repositioning appliances along with the administration of IGF-1 and TGF-β group; (ii) 4.15 fold ($P < 0.0005$) in the injection of growth factors only group; and (iii) 2.78-fold ($P < 0.0005$) in the mandibular repositioning appliances

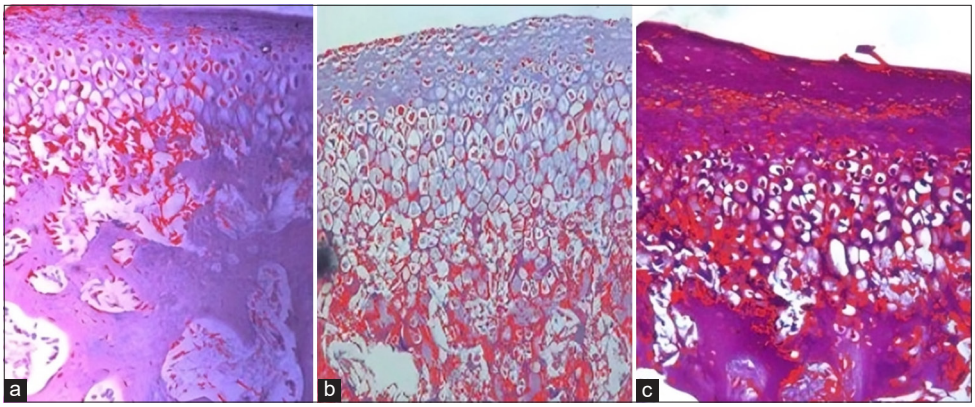

Figure 4: Assessment of prostaglandin content (a) control group treated with saline only; (b) experimental group treated with appliance only; and (c) experimental group treated with appliance and growth factor injection. (Magnification 1000X).

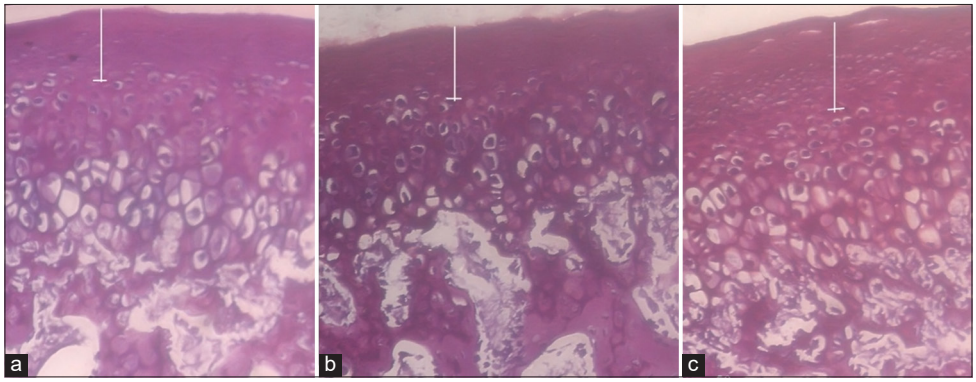

Figure 5: Increase in the proliferative layer as compared to the hypertrophic layer and a concomitant increase in thickness in the cartilage in experimental groups. (a) Control group treated with saline only; (b) experimental group treated with appliance only; and(c) experimental group treated with appliance and growth factor injection. (Magnification 1000X).

Table 2: Comparison of histologic field measurements across the study groups.

	Group I: Saline controls	Group II: Mandibular appliance	Group III: Mandibular appliance+Growth factors
Fibrous+Proliferative zone	54.7±19.1	120.7±15.7	270±37.9
Hypertrophic+maturational zone	146.3±49.3	89.4±27.4	89.5±30.9
Length of cartilage	214.9±61.7	262±24.2	359.6±58.4
Field measurements for PG area %	20.6±10	44.3±9.7	49.2±6.4

group. Its response to MCC growth at the molecular (PG) level is depicted in [Figure 3].

Effects of MGP expression

At this juncture, it was considered worthwhile to examine if an enhanced expression of the DCN gene (complimentary to VEGF) has also concomitantly enhanced the expression of the MGP gene (stimulatory to VEGF). This is depicted in [Figure 3], according to which MGP gene expression was (i) 2.58-fold ($P < 0.0005$) as a function of mandibular repositioning appliance group; (ii) 1.96 fold as a function of growth factors injection only; and (iii) 3.22-fold ($P < 0.0005$) as a function of mandibular repositioning appliances along with the administration of IGF-1 and TGF-β group.

DISCUSSION

Rationale underlying the choice of rabbits as search model

The choice of NZ rabbits as a dependable research model in the present study was made by their (i) placement as higher mammals than rodents and closer to human beings on an evolutionary scale for extrapolating observations

Table 3: Statistical comparison of histomorphometric measurements.

	Group I vs. Group II	Group I vs. Group III	Group I vs. Group IV
Fibrous+ Proliferative zone	0.001	0.001	0.001
Hypertrophic+ maturational zone	0.001	0.001	0.001
Length of cartilage	0.001	0.003	0.001
Field measurements forprostaglandin area %	0.001	0.001	0.001

made to clinical applicability, (ii) TMJ being essentially identical to those in human beings, (iii) jaw apparatus being specialized for herbivorous diet as in human beings, and (iv) administration of growth factors in the TMJ/retro-discal tissue appeared to be precise due to larger TMJ compared to that of mice on which majority of the studies have been conducted so far.

Significance of condylar growth at genetic level

Since impaired growth of the condyles contributes to the development of mandibular asymmetries and retrognathia,[7] the role of the mandibular condyles in the development of the oro-facial complex has received focused attention from orthodontics. Accordingly, earlier studies on growing mice have thrown light on the post-natal craniofacial skeletal growth and development in its entirety by delineating biochemical processes, certainly regulated by a complex network of genes and interacting genetic/epigenetic factors[21-23] which induce secretion of enzymes, growth factors, their receptors, etc., representing integration of various physiological sub-processes.[11] As cartilage provides endochondral bone as an adaptive growth site during mandibular development,[20,24] the present study has attempted to understand the expression of genetic factors (VEGF, DCN, and MGP) in young NZ rabbit model as a function of mandibular anterior repositioning appliances with and without the administration of growth factors (TGF-β and IGF-1).

Effects of VEGF gene expression

As depicted in [Figure 1], a similar expression of VEGF, a potent regulator of neo-vascularization, was observed in the condyles and glenoid fossa of the growing rats.[12] That an effect of IGF-1 and TGF-β is expressed in the cartilage reflected their clinical significance in its genesis/repair/homeostasis. Their dose-dependent accelerated effect reported on rats in literature validates their precise role.[11] These observations were physically validated by the change in the incisor relationship of the rabbit (lower incisors were ahead of the upper incisors, indicating the growth of the mandibles). Similar growth of the condyles in the growing rats was also noted by Rabie *et al*.[3,14]

Condylar growth modulation induced by mandibular advancement has also been reported in a few experimental studies.[3,4,17] In the present studies too, adaptation of the condylar cartilage to mandibular forward positioning has constituted a biological basis for the altered osteogenic transition of chondrogenesis in the young rabbits that lead to an increased endochondral ossification.[25]

The fact that VEGF expression indicated that mechanical strain caused by the pull of the disc solicited a cellular response, in turn leading to the expression of several factors in the condyles as judged from the endochondral ossification.[13] From this discussion, it is apparent that VEGF, through its multi-functional profile, catalyzes several biochemical reactions and physiological processes.

Quantitative assessment of the PG content in the condylar cartilage

For quantitative assessment, a representative measurement frame of 300 × 300 μm was selected with a focus on an area of 4760.992 μm². [Figure 5a-c] shows that the area reflecting PG, as judged from the magenta color in the combined treatment [Figure 5c] group, was approximately two-fold greater than that of the control group [Figure 5a] and greater as compared to the group treated by appliance only [Figure 5b]. The increasing intensity of staining as a function of treatment observed in the present studies was in concurrence with the earlier observations,[26] while studying the role of mesenchymal cells in skeletal regeneration. Thus, these studies have pointed out that IGF-1, an anabolic factor, stimulated the PG synthesis observed earlier.[27] It is our estimate although more PG is synthesized, its significant accumulation is not quantifiable, being used immediately in the genesis of different ingredients of ECM (viz., glycosaminoglycan [GAG], DCN, and MGP). The above contention is independently verifiable through biochemical changes in the quantum of PG underlying the growth profile as observed in the histochemical staining pinning down the site and the precise nature of the change [Figure 5c]. Accordingly, the increase in the PG content in the cartilage is in the descending order AI>I>AS>S. In totality, these observations on the young rabbits reflected condylar growth, confirming the earlier observations on mice and rats that IGF-1 and TGF-β have statistically significant effects ($P < 0.0005$), thereby complementing the up-regulation of VEGF.[11,28,29]

Effects of DCN gene expression

Expression of DCN along with an increase in PG is in good agreement with that of Holland *et al*.[30] whose

histomorphometric measurements have unequivocally shown an increase in the thickness of the cartilaginous layer. This was further supported by the histochemical analysis [Figure 4]. Cumulatively, these observations on the young rabbits have confirmed the earlier observations on mice and rats that mandibular repositioning appliances with/without IGF-1 and TGF-β have a statistically significant effect ($P < 0.0005$) on the expression of DCN and, in turn, on the MCC growth. This has been interpreted in terms of a 100-fold increase in the modulatory effect of IGF-1 in an equine model[2]. Decorin (DCN) and biglycan are two dermatan sulfate PGs of articular cartilage that have GAG chains in their N-terminal. During the condylar development, by involving its core protein, DCN (a) regulates type I collagen fibrils, (b) interacts with type II collagen fibrils, (c) influences the process of fibrillogenesis, (d) remains bound at the surface of mature collagen fibrils, (e) controls cell-surface receptors, (f) interacts with retinoic acid and growth factors, (g) promotes mineralization during the development, and (h) regulates cell movement in numerous physiological and pathological conditions.[31]

While TGF-β-1 markedly decreased the expression of DCN in either primary or passaged chondrocytes, their transfer to alginate increased its expression. These observations cumulatively pointed to the role of DCN complementary to VEGF in MCC-treated with IGF-1 and TGF-β.

Expression of the MGP gene

Although the MGP gene is expressed widely in several tissues, its significant amplification is noted only in dentin, cartilage, and bone. Expression of the genes that affect calcification in the ECM has been observed in the trabecular meshwork.[32] As anticipated, the MGP gene is expressed in the proliferative and late hypertrophic chondrocyte zone but not in the intervening chondrocyte zone. Accordingly, in the mammalian growth plate during the endochondral bone formation, MGP gene expression is confined to proliferative, late hypertrophic chondrocytes, and vascular smooth muscle cells only.[33]

Accordingly, it is postulated that MGP has a regulatory role in chondrocytes, cartilage, and mineralization of skeletal as well as dental tissues.[33] The biosynthesis of MGP is more actively pursued in the peripheral zone (where newly differentiated chondrocytes arise) than in the central zone (where chondrocytes mature), reflecting its role in the maturation of chondrocytes in mammals.[33] Second, the requirement of MGP in early development, closely associated with the differentiation of chondrocytes/cartilage formation, reflected its likely involvement in the regulation of the rate of calcification. Third, the presence of TGF-β-1 enhances the synergistic effect of MGP, presumably by neutralizing its antibodies, showing its role concomitant with the role of IGF-1. Fourth, the reduction in the stimulatory effect of MGPs on VEGF expression is another pointer suggesting its role is concomitant with the role of multi-functional VEGF. Finally, as TGF-β-1 has been reported to stimulate the expression of both VEGF and MGP,[34] as a corollary, MGP may activate TGF-β by direct binding in the extracellular space, analogous to that of the connective tissue growth factor, another modulator of TGF-β-1, and BMP-2. It is, therefore, obvious that cumulatively, if not singularly, MGP has a finite role in the condylar development either directly through the (a) chondrocyte differentiation/maturation and (b) mineralization or indirectly through the (c) TGF-β-1 activity profile and (d) VEGF expression.

CONCLUSION

The administration of growth factors along with the mandibular advancement appliance has increased the genetic expression of growth markers. The following genes were up-regulated in decreasing order as a function of injection of IGF-1 and TGF-β and anterior positioning mandibular appliances: (i) DCN gene 4.59-fold, (ii) MGP gene 3.22-fold, and (iii) VEGF gene 2.80-fold, whereas the following genes were up-regulated as a function of injection of growth factors: (i) DCN gene 4.15-fold, (ii) MGP gene 1.96-fold, and (iii) VEGF gene 1.65-fold, whereas the following genes were up-regulated as a function of anterior repositioning mandibular appliances: (i) DCN gene 2.78-fold, (ii) MGP gene 2.58-fold; and (iii) VEGF gene 1.23-fold. This effort is superior to the *in vivo* studies in the scope and certainly educative in the observations derived from the *in vitro* studies. Thus, the injection of growth factors alone and along with functional appliances could be an excellent treatment modality for the treatment of mandibular retrognathism.

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Declaration of patient consent: Patient consent was not required as there are no patients in this study.

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Use of artificial intelligence (AI)-assisted technology for manuscript preparation: The authors confirm that there was no

use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript, and no images were manipulated using AI.

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