ORIGINAL ARTICLE

Ameliorative Effects of Grape Seed Proanthocyanidin Extract on Histomorphological Changes in Rat Mandible Induced by Intramuscular Injection of Botox in Masseter

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ABSTRACT

Objective: This study was aimed to assess the histomorphological changes in rat mandible induced by injection botox in masseter muscle and to assess the possible remedial effects of grape seed proanthocyanidin extract (GSPE) on histomorphology of the rat mandibular condylar cartilage.

Study Design: Laboratory based experimental study.

Place and Duration of Study: This study was conducted at the Department of Anatomy and Pathology, Army Medical College Rawalpindi, Pakistan from 16th June 2020 to 07th August 2020, with the collaboration of the Military Hospital, Rawalpindi and the National Institute of Health (NIH), Islamabad.

Methods: Forty, healthy, female (non-pregnant) "Sprague Dawley rats", 10 weeks of age with weight of 200-250gm were selected and divided into four groups. Group A control, Groups B, C and D were experimental groups. Group B was sham injected whilst group C and D were injected by botox in left masseter muscle. Group D was treated with Grape seed proanthocyanidin extract for 1 month whereas rats from group B and C were given saline solution as a vehicle. H&E and Toluidine Blue after decalcification of the mandible were used for staining. Data was analyzed using the SPSS 23. The *P*-value of ≤0.05 was considered significant. One-way analysis of variance was applied followed by Tukey's post-Hoc test to seek distinction of quantitative variables between the groups.

Result: Botox injected group C developed changes when compared with the control group A and sham group B, including the discontinuation of the condylar cartilage surface and chondrocyte hypertrophy, cluster formation and disorientation of chondron columns. The experiment did not show any improvement in experimental group D after treatment with grape seed proanthocyanidin extract.

Conclusion: The administration of grape seed proanthocyanidin extract did not yield the expected improvements in the histomorphology of the mandibular condylar cartilage.

Key words: *Bone, Botox, Grape Seed Extract, Mandibular Condyle.*

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Introduction

GSPE (Grape Seed Proanthocyanidin Extract) has been examined for its function in reducing oxidative stress, including vasodilation, protection against allergens, viruses, bacteria and carcinogens. It also acts as immunostimulator, anti-inflammatory and cardioprotective agent. GSPE is also believed to play role in bone homeostasis. GSPE has been found to possess preventive as well as therapeutic properties to improve bone density and strength in osteoporosis, osteonecrosis, and many other bone pathologies.[1] The association between bone loss and its prevention through grape seed proanthocyanidin extract (GSPE) is related to the fact that it has strong

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antioxidant properties, which have made it a focal point of research for some time now. The modernization of time, captivating marketing of so called beauty standards and easy access of affordable non-invasive esthetic procedures has directed the masses towards the use of these procedures. Today, this market is rapidly flourishing in the third world countries as well. Our community is not aware of the uninvited side effects and the potential remedies for combating these side effects. Regardless of the vast contribution of scholarly work of the researchers there are quite few facts known about the role of GSPE in ameliorating the osteoclastic activity of bone secondary to muscle paralysis. In aesthetic medicine, the recent use of botulinum toxin type A to treat wrinkles, caused by dynamic use of muscles of facial expression is on the rise. It functions as a muscle relaxant by preventing acetylcholine release at the end of the cholinergic nerve. The trade name for botulinum toxin type A used worldwide is Botox. The side effects are not specific to the therapeutic or esthetic use but to the active ingredient, its mechanism of action, higher than prescribed dose and repetition of the injection within one month of the previous injection.[2] The musculoskeletal system works in perfect harmony to maintain homeostasis, and when the muscle is paralyzed by the botulinum toxin type A injection consequently it is destined to alter the bone morphology. The study of effect and side effects of this therapeutic procedure has widely been done on muscle but much less of the exact side effects of this procedure have been studied on the bone. Temporomandibular joint requires the muscles of mastication to develop, keeping that fact in mind it is of relevant importance to study the effect of muscle paralysis on the mandible.[3,4] GSPE alleviates arthritis through its anti-inflammatory and joint-protective effects.[5] Recent studies have shown that the procyanidin can help prevent cartilage degradation in osteoarthritis. Additionally, researchers observed that GSPE exhibits anti-arthritic properties by decreasing the production of specific IgG2a and reducing cytokines like TNF-α and IL-17.[6] Experiment revealed considerable bone deterioration in the alveolar and condylar regions of rats' mandibles after a four-week treatment involving injections of botulinum toxin type A into both the masseter and temporalis muscles and the effect was evident immediately following the treatment.[7-9] There remains a lack of precise understanding regarding the cellular level changes in bone and mandibular condylar cartilage, as well as effective strategies for mitigating these undesired consequences.

The association between the loss of bone and its treatment through use of grape seed proanthocyanidin extract (GSPE) is based on its proven role as a potent anti-oxidant and has been the subject of interest for studies for quite some time now. For its properties to reduce oxidative stress, GSPE has been studied for its role as vasodilator, immunostimulator, anti-inflammatory, cardioprotective, as well as its protective role against various allergens, viruses, bacteria, microbes, and carcinogens. GSPE is also considered to play a role in bone homeostasis. Beyond its preventive and therapeutic benefits, GSPE has been demonstrated to enhance bone density and strength in conditions such as osteoporosis, osteonecrosis, and various other bone disorders.[1] Grape seed proanthocyanidins have demonstrated a wide range of pharmacological and therapeutic benefits related to oxidative stress and inflammation, addressing conditions such as cardiovascular disease, diabetes mellitus, obesity, and cancer.[10] Proanthocyanidin, the active component in grape seed extract, can neutralize free radicals, which are essential for calcium absorption.[11] There is an evidence of relationship between other flavonoids and bone health in studies.[12] The research highlighted how proanthocyanidin grape seed extract impacts inflammation and the breakdown of bone linked to free radicals. In mice, it improved arthritic changes in collagen induced arthritis and suppressed osteoclastic activity while encouraging dose dependent osteoblastic activity. In the treatment of bone loss, it can be a valuable parallel therapy. In addition, grape seed proanthocyanidin extract inhibits the expression of osteoclast related genes.[13,14] Previous studies have indicated that proanthocyanidin inhibits enzymes that are crucial in the initial stages of bone resorption.[15] Procyanidins support cartilage protection and repair by enhancing cartilage synthesis, reducing degeneration, and

offering anti-inflammatory benefits to alleviate damage. Procyanidins aid in cartilage repair by preventing collagen degradation by collagenase and promoting the production of key components like the cartilage matrix, essential for maintaining cartilage structure and function.[16]

Our community is not aware of the uninvited side effects and the potential remedies for combating these side effects. Regardless of the vast contribution of scholarly work of the researchers there are quite few facts known about the role of GSPE in ameliorating the osteoclastic activity of bone secondary to muscle paralysis. Despite considerable literature there is still a need for studies, which should investigate the histomorphological changes in mandibular condylar cartilage and bone. The role of GSPE in botulinum toxin type A affected mandible should also be assessed. Keeping all these facts in mind we have designed a study whose rationale is to analyze the effect of GSPE in botox induced mandibular condylar cartilage and subchondral bone changes. If GSPE is found to have ameliorating effect, it may become a useful guide for patients using botulinum toxin type A either for therapeutic or esthetic purposes to avoid undesired effects on the bones. Natural extracts like grape seed proanthocyanidin extract may offer a viable and safe alternative or adjunct therapy for treating bone loss associated with inflammatory conditions, potentially complementing conventional drug treatments. Studies have shown that Grape seed extract containing proanthocyanidin helps maintain the balance between osteoblastic and osteoclastic action, which is crucial for sustaining bone integrity and controls the two way mode of action of bone homeostasis and can be a possible therapeutic agent for bone destruction treatment. Enzyme trials have shown that the proteolytic enzyme is also inhibited by proanthocyanidin.[17] There is a possibility for grape seed proanthocyanidin extract to improve bone quality.[18] In animals treated with grape seed proanthocyanidin extract, bone consistency and bone strength have also improved. While various studies have demonstrated the effectiveness of grape seed proanthocyanidin extract on bone health and have showed the chondroprotective effects in human chondrocytes, with GS proanthocyanidins reducing perichondrial inflammation and alveolar bone loss by lowering levels of MMP-13, MMP-8, HIF-1α, TNF-α, and IL-17.[19,6]

Methods

An experimental study was conducted in collaboration with the National Institute of Health (NIH), Islamabad, involving the Department of Anatomy and the Department of Pathology at Army Medical College Rawalpindi, Pakistan. The research, which took place from 16th June 2020 to 07th August 2020 was approved by the Ethics Review Committee of Army Medical College Rawalpindi vide letter no: ERC/ID/103 held on dated: 13th March 2020.

The investigation employed a non-probability consecutive sampling technique, utilizing 40 healthy adult female Sprague Dawley rats. These non-pregnant subjects, aged 10 weeks and weighing between 200-250 grams, were housed in individually marked cages within a standardized environment at the NIH animal house in Islamabad. Environmental conditions were meticulously controlled, with filtered air maintained at 22°C ± 2°C and 50% ± 10% relative humidity, along with a 12-hour dark-light cycle throughout the experiment's duration. Rats were given standardized laboratory feed and water ad libitum. They were given one week to acclimate before the study started.[8] The experimental design divided the rats into four equal groups having 10 rats in each. In groups C and D, a dose of 2.5 U of botulinum toxin (Botox®), supplied by Barrett Hodgson Pakistan Pvt. Ltd. Karachi, was administered via injection into the left masseter muscle. This intervention was performed under general anesthesia using Isoflurane (Restane Solution 100ml, manufactured by Primal Critical Care, Inc. USA) during the first and third weeks of the study. To establish a control, the sham group received an equivalent volume of normal saline, also injected into the left masseter muscle.[8,20] In experimental group D, one week after the second Botox injection, 100 mg/kg of Grape Seed Extract Powder (95% Proanthocyanidin, procured from Xi'an Natural Field Bio-Technique Co., Ltd, Xi'an, China) was given daily at a dose of 0.5 mL by gastric feeding, in the form of red brown powder for 30 days.[8,21]

All animals were weighed and euthanized with the over dose of anesthetic agent, at the completion of

the experiment. The mandibular condyles were extracted from each rat following a standardized dissection procedure. The animals were placed in supine position on a dissecting board and were pinned. The V-shaped incision was made under the chin of the rat using forceps and surgical blade. The incision was extended toward left cheek up to the temporomandibular joint. Gentle manipulation with forceps and surgical scissors exposed the underlying facial muscles, which were then carefully dissected to reveal the left mandible. The bone was isolated from the temporal bone by dissecting ligaments, ensuring to safe guard the anatomical integrity of the articular cartilage of the mandibular condyle. The left mandible was separated from the right mandible using blunt dissection, washed with normal saline and were fixed in 10% formalin for 24 hours.[8]

Formic acid was employed for decalcifying the bone. Tissue samples of the mandibular condyles were collected from each specimen of the mandible. All processing and staining procedures were conducted in the histopathology section of the Pathology department at Army Medical College Rawalpindi. From each block, three sections were prepared. Hematoxylin and Eosin (H&E) was used to stain the first section for routine histological examination. Toluidine blue staining was performed at the Pathology department of Armed Forces Institute of Pathology. Light microscopy was used with 10X and 40X objectives to observe qualitative parameters. Histological analysis involved collecting three coronal sections from the widest part of each condyle, staining them with hematoxylin and eosin, and assessing them for atypical morphology, such as irregular shapes or surface defects, as well as signs of resorption, indicated by multinucleated osteoclasts in Howship's lacunae.[22] Microscopic sections were assessed at low power magnification i.e. 10X and 40X. To assess histopathology grade of depth of cartilage damage following modified parameters were used as a guide adapted from Pritzker et al. (Table-1). High-quality images were acquired using CellSens entry micro imaging software, a sophisticated tool designed for microscopic visualization.[23] Subsequently, these images underwent meticulous refinement using PhotoScape software, where crucial parameters such as contrast, brightness, sharpness, and color balance were carefully adjusted to enhance clarity and detail. To ensure accurate scale representation, the magnification factor was precisely calculated using a standardized formula, allowing for reliable interpretation of the microscopic structures observed.

Magnification Power of eye piece x Power of objective x Camera zoom

Data analysis used IBM-SPSS version 23. Quantitative variables were analyzed using one-way ANOVA and expressed as mean ± SD. Post-hoc Tukey test was used for intergroup comparisons. Qualitative variables were presented as frequencies and percentages, with Fisher's exact test for comparisons. Statistical significance was set at $P \leq 0.05$.[8]

Results

This study aimed to investigate the impact of grape seed proanthocyanidin extract on the histomorphology of the mandibular condylar cartilage following intramuscular botox injection in the rat masseter muscle. Each group of animals were housed individually in cages at a comfortable room temperature, allowing free mobility. Through the period of the study, all animals maintained excellent health with a survival rate of 100%. The light microscopic evaluation showed the normal articular cartilage and subchondral bone, with distinct layers of articular cartilage and perpendicular bony trabeculae. The articular cartilage and subchondral exhibited normal structure showing layers of the articular cartilage and perpendicular to the articular cartilage were the boney trabeculae. The histological examination of H & E stained preparation of mandibular condyle in group C showed changes in the articular cartilage. The surface fibrous layer of cartilage appeared discontinuous in most of the regions. The chondrocyte columns exhibited change in their orientation. Hypertrophy of the chondrocytes and clusters were observed depicting the cell death in most of the regions. Matrix depletion and small vertical fissures were also observed in a specimen of group C. (Figure.1). Grape seed proanthocyanidin extract was given to the animals of group D which showed no significant improvement in the condylar cartilage structure, but

Table-1: Grading Criteria[23]

Grade (key feature)	Associated criteria	
Grade 0	Surface intact, morphology of the cartilage intact	
	Extracellular Matrix (ECM)	Normal composition and organization
	Chondrocytes	Viable with appropriate spatial orientation and density
Grade 1	Surface intact	
	Extracellular Matrix (ECM)	Preserved integrity in superficial zone
	Chondrocytes	Evidence of apoptosis, proliferation, cluster formation, or hypertrophy
Grade 2	Surface discontinuity	
	Extracellular Matrix (ECM)	Disruption in superficial zone
	Chondrocytes	Disorientation of columnar arrangement
	Staining	Reduced Toluidine Blue staining in upper 1/3 of cartilage and or focal increase in peri-cellular matrix staining (mid-zone)
Grade 3	Vertical fissures or clefts	
	Extracellular Matrix (ECM)	Fissures extending into the mid-zone, with branching patterns
	Chondrocytes	Persistent abnormalities as in Grades 1 and 2
	Staining	Reduced Toluidine Blue staining in lower 2/3 of cartilage (deep zone)
Grade 4	Erosion	
	Extracellular Matrix (ECM)	Delamination of superficial layer, cyst formation in middle layer
	Excavation	Loss of ECM in upper two zones of articular cartilage
Grade 5	Denudation	
	Articular Surface	Exposed sclerotic subchondral bone or presence of restorative fibrocartilage
	Subchondral region	Evidence of micro-fractures with regions of bone repair
Grade 6	Deformation	
	Articular surface	Significant deformation of surface contour
	Subchondral region	Extensive subchondral bone changes including micro-fracture sites

the findings in the cartilage were almost coinciding with that of group C. In group D, the condylar cartilage displayed disruptions in the cartilage surface, loss of cartilage layers, hypertrophic chondrocytes in the matrix, and numerous specimens showed clusters of chondrocytes but there were not any vertical fissures observed in group D (Figure. 1). Microscopic analysis was conducted by examining H&E stained slides under a light microscope at 10X and 40X magnification to identify atypical morphology. Cartilage damage was then graded on a semi quantitative scale from Grade 0 (surface and cartilage morphology intact) to Grade 6 (deformation). (Table-1). A and B Mandibular condyle of control group A and sham group B showing intact surface, layers of articular cartilage (red line) and chondrocyte columns (green line). C and D Mandibular condyle of experimental group C and D showed surface discontinuation (blue arrow head), layers of articular cartilage (red line) and

Table-2: Frequency and percentages cartialge damage in control group A and experimental groups B, C and D

Parameter	Findings	Group A n=10	Group B n=10	Group C n=10	Group D n=10
Histological grade of affected articular surface of mandibular condyle	Grade 0	10 (100%)	10 (100%)	__	__
	Grade 1	__	__	2 (20%)	2 (20%)
	Grade 2	__	__	7 (70%)	8 (80%)
	Grade 3	__	__	1 (10%)	__
	Grade 4	__	__	__	__
	Grade 5	__	__	__	__
	Grade 6	__	__	__	__

P-values showing intergroup comparison of Histological Grading

Parameter	C vs A	D vs A	C vs B	D vs B	C vs D	B vs A
Grading	0.000**	0.000**	0.000**	0.000**	0.587	_

P-value ≤0.05 Significant* *P*-value <0.001 Highly significant**

cluster formation (black arrow head). The observation were made under the light microscope at 10X and 40X objectives for toluidine blue stained specimens in group A and B, they showed no matrix depletion by exhibiting darker staining of the specimens while in the experimental group C and D the toluidine blue staining revealed reduced proteoglycan secretion by showing less affinity for matrix of the specimens in experimental groups. (Figure.2) (Table 2). Groups A (control) and B (sham) exhibited no alterations in the condylar cartilage or subchondral bone. Observations of experimental group C showed that 10% of the specimens have vertical fissures in the cartilage and 20% exhibited cell death with intact surface, whereas 70% showed surface discontinuation and matrix depletion in addition to cell death. While in experiment group D 80% of the specimens have surface discontinuation, matrix depletion and cell death and 20% of the specimens revealed cell death with intact surface. (Figure.2). Statistical significance was assessed by applying Fisher's exact test. The intergroup analysis revealed highly significant differences between experimental groups C and D and control groups A and B, with a *P*-value of 0.000, while comparisons between groups C and D showed no significant difference, with a *P*-value of 0.587. (Table-2).

A and B Mandibular condyle of control group A and sham group B showing intact surface of articular cartilage and chondrocyte C and D Mandibular condyle of experimental group C and D showed surface discontinuation (red arrow head), stain depletion (black arrow head) when compared with control.

Discussion

Grape seed proanthocyanidin, derived from Vitis vinifera seeds, has demonstrated beneficial biological effects against various pathologies by enhancing the activity of free radicals required for calcium absorption. Epidemiological studies have linked grapes and grape polyphenols to improved bone health.[24] By positively affecting osteoblast differentiation, GSPE significantly inhibits osteoclast differentiation, reduces its activity, and promotes bone formation. Maintaining bone quality relies on balancing osteoblast and osteoclast activity. Proanthocyanidin from grape seeds regulates bone homeostasis by inhibiting osteoclastic activity and promoting osteoblastic function, positioning it as a potential therapeutic agent for treating bone destruction.[15] grape seed proanthocyanidin extract influenced the mechanical properties related to bone loss in the mandibular condyle of rats.[19] Grape seed proanthocyanidin extract alleviates ankle joint destruction by significantly reducing joint inflammation, synovial proliferation, cellular

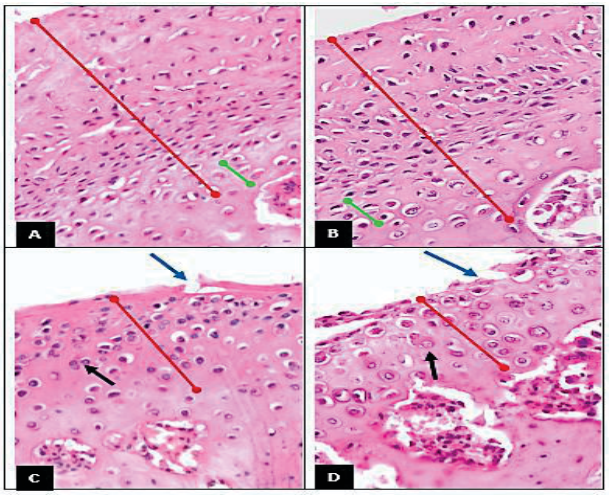

Fig.1: Photomicrograph showing comparison of histological changes in condylar cartilage and subchondral bone tissue of control group A and experimental group B, C and D. H & E stain. Approximately X300

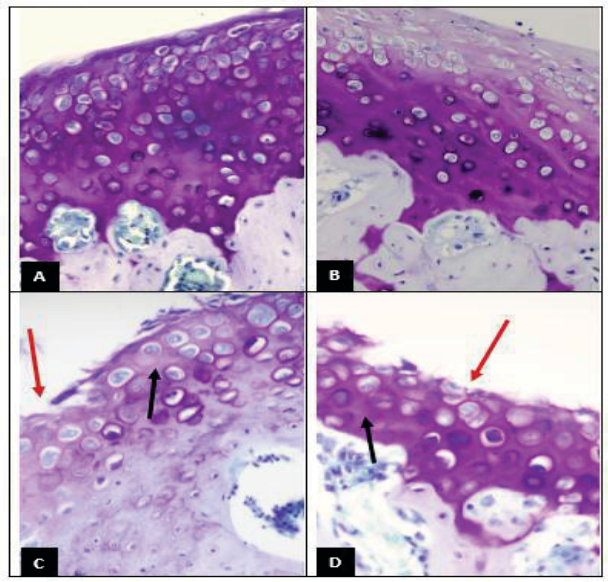

Fig.2: Photomicrograph showing comparison of histological changes in condylar cartilage and subchondral bone tissue of control group A and experimental group B, C and D Toluidine blue stain. Approximately X300

infiltration, and cartilage degradation when administered at the dose of 100 mg/kg.[25]

In experimental group C, the histomorphology of the mandibular condyle revealed discontinuities in the condylar cartilage surface, along with hypertrophic chondrocytes and clusters that indicated cell death within the cartilage. The articular cartilage comprises of three layers, the top fibrous layer, proliferative layer and hypertrophic chondrocyte layer covering the calcified subchondral bone. There was depletion of toluidine blue stain in the cartilage matrix, the results when compared with control group A and sham group B were highly significant (*P* value < 0.001). Toluidine blue is a cationic dye that stains both proteoglycans and glycosaminoglycan. Due to the fact that it has a higher affinity for sulphur in cartilage, Toluidine blue has been documented to provide more intensive staining. The effect of staining is relative and must be contrasted with normal internal controls, as the severity of staining of articular cartilage tissue can differ from one species to another.[26] These results were aligned with the study by Dutra EH et al., which showed that a decreased toluidine blue staining area indicated reduced proteoglycan secretion on the side injected with Botox.[27,28] The histological study of group D exhibited the same level of changes as were observed in group C that are surface discontinuity, chondrocyte hypertrophy and clusters formation as well as decrease matrix staining with toluidine blue. When compared to control group A and sham group B, the results were highly significant (*P*-value < 0.001). However, experimental group D did not show statistically significant differences compared to experimental group C (*P*-value > 0.05), which was inconsistent with the findings of Cho M-L et al., who reported that grape seed proanthocyanidin extract at a concentration of 100 mg/kg significantly reduced cartilage loss.[28,29]

Conclusion

The grape seed proanthocyanidin extract did not result in the expected improvements in the histomorphology of the mandibular condylar cartilage. The literature might be in favor of remedial effects of GSPE on cartilage and bone but present studies failed to yield the chondron-protective effect of GSPE on Botox induced changes in mandibular condylar cartilage. Future research could investigate higher dosages and longer treatment durations to better assess the extract's potential benefits for bone and articular cartilage. Furthermore, initiating grape seed proanthocyanidin extract treatment at the start of the experiment might reveal its protective effects on bone, cartilage, and muscle tissues.

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