Original article

Analysis of proteoglycan content in combined intermediate and deep zones of degenerating patellar articular cartilage in adult male mice after administration of diazepam

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ABSTRACT

Abstract: Objective: The basic purpose of this study was to analyze the effect of diazepam on the proteoglycan content of combined intermediate and deep zones of patellar articular cartilage in adult male mice. Study design: An experimental study. Place and duration of study: Department of Anatomy, College of Physicians and Surgeons CPSP, Regional center, Islamabad from June 2015 - June 2016. Methodology: Animals were categorized into two main groups A control (mice not treated with diazepam) and B experimental groups (mice treated with diazepam) with further division of Group A into three subgroups A1 (mice without any intervention), A2 (mice kept immobilized for 30 days), A3 (mice kept immobilized for 30 days and then remobilized for next 30 days, treated with normal saline during remobilization period). Similarly, experimental group B mice were immobilized for initial 30 days and then remobilized for next 30 days and treated with diazepam during remobilization period. Group A1 was compared with group A2 to assess any change in proteoglycan content of combined zones of patellar articular cartilage due to immobilization. Group A2 was then compared with group A3 to analyze the effect of remobilization on proteoglycan content of combined zone. Finally, comparison between group A3 and group B was done to evaluate the effect of diazepam on proteoglycan content of combined zone remobilized mice. Qualitative analysis of staining grades of proteoglycan content in combined zones was done by chi square test using SPSS version 16. Conclusion: Diazepam administration does not have any impact on the proteoglycan content of combined zones of patellar articular cartilage in adult male mice.

1. Introduction

Diazepam, member of benzodiazepine class, is widely prescribed in developing countries for management of neuropsychological conditions like insomnia, epileptic convulsions, anxiety-related disorders etc. Decreased inhibitory signaling of Gamma-aminobutyric acid (GABA) is the prime cause of anxiety related disorders. GABA, a major inhibitory neurotransmitter in brain, shows its neurological effects by acting on GABA A receptors between their alpha and beta subunits. GABA A receptors act as chloride gated channels which are regulated by GABA. Binding of GABA to GABA A receptors increases the chloride conductance which causes hyperpolarization of neuronal membrane and results in increased firing threshold. Diazepam cannot open the chloride channels by itself rather it intensifies the effect of GABA on these receptors by activating the allosteric site of GABAergic system which is located at interface of gamma and beta subunits. Frequency of chloride channels opening is triggered by diazepam. In addition to brain, other body organs/tissues like liver, pancreas, kidneys etc. also express GABA A receptors. The autocrine/paracrine effects of GABA were demonstrated on human pancreatic cells by Braun et al. in 2010 which proved that GABA can have physiological actions other than neurotransmission. In 2001, Tamayama and colleagues demonstrated the expression of GABA A receptors on chondrocytes of rat growth plate. By their studies on ATDC5 mouse chondrogenic cell lineage, they proved that GABA can cause proliferation of chondrocytes by activating GABA A and GABA B receptors.
Chondrocytes are highly specialized cells which are responsible for the formation and maintenance of extracellular matrix in articular cartilage. Extracellular matrix (ECM) is mainly composed of collagen fibers type I, proteoglycans, glycoproteins, water and inorganic salts. The ability of articular cartilage to sustain the compressive forces is attributed to its proteoglycan content which generate the large osmotic pressure. Percentage of proteoglycans varies in different zones of articular cartilage with distance from articular surface. Aggrecan is the largest proteoglycan molecule in articular cartilage which is present in aggregates. These aggregates form by non-covalent bonding of proteoglycan monomers with the hyaluronic acid. In addition to aggrecan, non-aggregating proteoglycans like decorin and biglycan are also found in articular cartilage. Different studies have proved that degradation of aggrecan and collagen occurs due to long immobilization and chronic inflammation as in osteoarthritis. Many studies on animal models have postulated remarkable changes in composition of articular cartilage including reduction in number of chondrocytes and decreased proteoglycan content of articular cartilage after particular immobilization period which are then recovered during remobilization. Reversibility of these changes depends on the duration of remobilization. Long immobilization period leads to irreversible damage to articular cartilage. Pathological changes may become apparent in articular cartilage after the immobilization period of 4-6 weeks. Recovery of proteoglycan content and collagen is mainly dependent on the number of chondrocytes and their anaerobic activity. Chondrocytes express GABA A receptors. The activation to these receptors results in proliferation of these chondrogenic cells. Being positive allosteric modulator of GABA A receptors, diazepam may activate these receptors resulting in proliferation of chondrocytes in degenerating articular cartilage. Whole of this process is expected to end up in increased production of proteoglycans, thus repair of ECM.

**OBJECTIVE:**

To analyze the effect of diazepam on proteoglycan content of combined (intermediate and deep) zones of degenerating patellar articular cartilage in adult male mice.

**HYPOTHESIS:**

Proteoglycan content in combined zones of degenerating articular cartilage is increased after the administration of diazepam.

**MATERIAL AND METHODS:**

Balb cadut male mice of age 8 weeks or weight 25-30 grams were selected as experimental animals in this study. For this experimental research work, mice were first categorized in two main groups A and B. Group A, which is taken as control, was further subdivided in three subgroups designated as A1, A2 and A3. Each subgroup contains 30 mice. Group B also comprising of 30 mice was labelled as experimental group. Mice of subgroup A1 were normal adult mice that have not undergone any intervention and sacrificed after 30 days. Mice were first anesthetized with chloroform and then sacrificed. Mice of subgroup A2 underwent the immobilization procedure of 30 days and then sacrificed. In this procedure, the right legs of mice were kept immobilized by applying plaster of Paris cast. Mice of subgroup A3 also passed through the same procedure but after 30 days of immobilization period, the casts were removed and they were remobilized for next 30 days and then sacrificed. During this remobilization period, they have been injected with normal saline intraperitoneally. The same procedure of immobilization and remobilization was also performed on mice of Group B for same time periods and then sacrificed but these animals were injected with diazepam intraperitoneally at the rate of 4mg/kg/day and then sacrificed. After skin removal, joints were separated from legs by using bone cutter. Portions of tibia, femur with quadriceps tendons were also included in the specimen. Knee joints were then cut into two halves longitudinally and fixed in Neutral buffered Formalin for 72 hours. Decalcification was done with ethylene diaminotetraacetic acid (EDTA). Sections of 7 micrometers thickness were then cut and stained with alcian blue for grading the proteoglycan content in patellar articular cartilage. Staining pattern for proteoglycan content in combined intermediate and deep zones of patellar articular cartilage was graded on scale of 0 to 3 where 0 = absence of stain in interterritorial matrix (faint staining), 1 = light blue stain in interterritorial matrix (mild staining), 2 = blue bands in pericellular matrix (moderate staining), 3 = intense blue bands in interterritorial matrix (intense staining). Statistical analysis of this staining pattern was done by chi square test using SPSS version 16. P value ≤ 0.05 will be considered as significant.

**RESULTS**

Combined intermediate and deep zones of 10% (3 out of 30) of subgroup A1 specimens revealed moderate (grade 2) staining while 90% specimens (27 out of 30) showed intense (grade 3) staining character. In subgroup A2, alcian blue sections revealed mild (grade 1) staining with alcian blue in combined zone of 73.3% (22 out of 30) specimen and moderate (grade 2) staining in 3.3% (1 out of 30) specimen while no stain (grade 0) was taken by 23.3% (7 out of 30) specimen. Comparison of staining pattern of combined zones between subgroup A1 and A2 showed significant decrease in staining grades of combined zones in subgroup A2 (Table 1) which was indicating decreased proteoglycan content in combined zones of subgroup A2 as a result of immobilization.

In subgroup A3, 71.4% of specimens (20 out of 28) lied in category of moderate (grade 2) staining while 28.6% (8 out of 28) specimen showed mild (grade 1) staining in combined zones. Analysis of staining grades of combined zones between subgroup A2 and subgroup A3 showed significant increase in staining grade in combined zones of subgroup A3 (Table 2) which represented increased proteoglycan content in articular cartilage during remobilization period.

In group B, 40.7% of specimens revealed mild (grade 1) staining but 59.3% (16 out of 27) showed moderate (grade 2) staining grade. (Figure 2) Comparison of staining grades of combined zones of subgroup A3 and group B showed insignificant difference between these two groups (Table 3).
Table 1: Grades of alcian blue stained proteoglycan content in combined zones of patellar articular cartilage of subgroups A1 and A2

<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>Grades of staining</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A1= 30m</td>
<td>Faint</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A2= 30m</td>
<td>Mild</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>Moderate</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>Intense</td>
<td>27</td>
</tr>
</tbody>
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Table 2: Grades of alcian blue stained proteoglycan content in combined zones of patellar articular cartilage of subgroups A2 and A3

<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>Grades of staining</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A2= 30m</td>
<td>Faint</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A3= 30m</td>
<td>Mild</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>Moderate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>Intense</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3: Grades of alcian blue stained proteoglycan content in combined zones of patellar articular cartilage of group A3 and B

<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>Grades of staining</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A3= 30m</td>
<td>Faint</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B= 30m</td>
<td>Mild</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>Moderate</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Intense</td>
<td>0</td>
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Diazepam, a member of benzodiazepines, is the most prescribed drug in developing countries for the management of neurological and psychiatric disorders. But now the scientists are also working on its extra neurological effects after the discovery of expression of its receptors on other body organs and tissues like pancreas, liver, chondrocytes of articular cartilage etc. Articular cartilage refers to hyaline cartilage which covers the articular surfaces of bones. Degenerative diseases like osteoarthritis and surgical procedures requiring prolonged immobilization for recovery results in disastrous changes in articular cartilage that can hinder one’s daily life activities. Regeneration of articular cartilage is still a question for researchers due to its avascularity and low cellularity. New techniques and medicines are being explored to recover the degenerative changes in articular cartilage. This study has been conducted to determine the effects of an agent that has receptors on chondrocytes of the degenerating articular cartilage. The results of our study revealed that diazepam does not significantly increase the proteoglycan content of degenerating articular cartilage. Subgroup A2 were immobilized to induce the degenerative changes in their patellar articular cartilage. Decreased content of proteoglycans in combined zones of articular cartilage of immobilized mice (subgroup A2), which is basically hallmark of degenerative changes, as compared to normal mice (subgroup A1) may result due to oxidative stress. This oxidative stress causes death of number of chondrocytes and reduced functioning of rest of chondrocytes. This factor can reduce the proteoglycan content in articular cartilage as proteoglycans production is the prime function of the chondrocytic cells. Our result coincides with other studies in which authors have declared that immobilization causes reduction in number of chondrocytes and proteoglycan content. This decreased proteoglycan content can be imputed to the free radical nitric oxide (NO) which is reported to be secreted by chondrocytes of degenerating articular cartilage. NO may inhibit the synthesis of proteoglycans by chondrocytes and can also play a role in programmed cell death of chondrocytes. Production of matrix-degrading enzymes particularly the ones which attack the aggrecans further exacerbates this condition.

Comparison of staining grades of combined zones of articular cartilages of immobilized (subgroup A2) and remobilized (subgroup A3) mice showed significant increase in proteoglycan content in remobilized mice (subgroup A3). This increase in proteoglycan content can be attributed to the regained functioning of remaining chondrocytes due to removal of stressful immobilization. These viable chondrocytes may then increase their activity to compensate the loss. On the basis of results of this experimental study, we can say that remobilization provides the chondrocytes with the environment in which they can regenerate and increase their functional activity.

Analysis of staining grades in combined zones of patellar articular cartilage of subgroup A3 and group B mice showed insignificant
increase in proteoglycan content in articular cartilage of group B mice treated with diazepam. The imbalance between catabolic and anabolic activities of chondrocytes is the hallmark of degenerating articular cartilage. Diazepam may have increased the proliferation of chondrocytes, thus tends to increase the proteoglycan content but it fails to increase the activity of degenerating chondrocytes up to the mark at which they can compensate the loss of matrix especially proteoglycans.

LIMITATIONS OF STUDY

In our study, we have restricted the dose of diazepam as it also has central effects i.e. sedation that can reduce the mobility of mice during remobilization period. Diazepam may enhance the proteoglycan content of degenerating articular cartilage by increasing the rate of proliferation and anabolic activity of chondrocytes in high doses but one has to co-administer the central GABA A antagonist with diazepam.

CONFLICT OF INTEREST

No conflict of interest was declared by anyone of the authors.

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