Effects of hyaluronic acid applied by different methods on intervertebral disc degeneration: Reduction of inflammation and histological damage via CD44 and RHAMM receptors

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Abstract
This study investigated the effects of epidural (ED) and intradiscal (ID) hyaluronic acid (HA) injections on intervertebral disc degeneration (IDD) in rats. IDD, a common cause of spinal pain and disability, lacks effective treatment options. HA, known for its anti-inflammatory and regenerative properties, has shown potential in managing disc degeneration. The aim was to compare the efficacy of ED and ID HA injections in reducing IDD and promoting disc health. Sixty male Sprague-Dawley rats (10–12 weeks old) were randomly assigned to six different groups. Control (CG) and intervertebral disc degeneration (IDDG); sham (S)+ID, S+ED, IDDG+ED, and IDDG+ID HA injection (0.1 mL, single dose on the day of the procedure) were established in six groups. Histological changes and blood levels of IL-6 and TNF-α were measured. No significant changes in body weight, infection, or mortality were observed. Histologically, the Sham+ID group showed mild disc tears, vacuoles, and serpentine appearance, while the IDDG+ID group displayed indistinct nucleus pulposus(NP) and annulus fibrosus(AF) borders, necrosis, and vacuoles. The IDDG+ED group exhibited regular collagen fibers, reduced vacuoles, and edema, with a complete absence of necrosis. IL-6 and TNF-alpha were elevated in IDDG and Sham+ID groups. However, both cytokines were significantly reduced in the IDDG+ED and IDDG+ID groups. These findings suggest that HA injection reduces inflammation and histological damage, with the epidural method proving more effective than the intradiscal method. HA likely acts by interacting with membrane receptors such as CD44 and RHAMM. No weight change or mortality was observed after HA treatment.

Keywords: *CD44 receptor, epidural injection, histological damage, hyaluronic acid, intervertebral disc degeneration, RHAMM receptor*

INTRODUCTION

Intervertebral disc degeneration (IDD) has become a significant health-related and economic issue for many countries, due to the costs associated with diagnosis and treatment, loss of productivity, lumbar pain, and increased insurance costs (Samanta et al., 2023; Yong et al., 2022). Although several studies have been conducted on the subject in recent years, only temporary solutions have been proposed, and the issue remains unresolved (Navone et al., 2017).

IDD refers to progressive changes that affect the spine as part of the natural aging process, resulting from the combined impact of multiple factors (Navone et al., 2017). Various factors, such as age, can induce IDD, including increased mechanical load, genetic transmission, obesity, incorrect exercise, and smoking (Yong et al., 2022). Proinflammatory cytokines TNF, IL-1β, IL-1α, IL-6, and IL-17 are secreted by disc cells, and an increase in these cytokines (inflammation) is observed during IDD (Risbud & Shapiro, 2014). Inflammation plays a crucial role in the onset and progression of IDD by promoting the continuous loss of extracellular matrix, apoptosis, and oxidative stress (Kang et al., 2023). Reactive oxygen species (ROS) play a crucial role in the development and progression of IDD (Feng et al., 2017). Excessive ROS production induces oxidative stress, leading to mitochondrial dysfunction and activation of endoplasmic reticulum stress. As a result, it contributes to triggering apoptosis and cell death (Wang et al., 2018). Apoptosis, cell death, inflammation, and endoplasmic reticulum stress have essential roles in IDD disorders (Feng et al., 2017; Wang et al., 2018; Zhao et al., 2010).

Although several studies have been conducted on the subject in recent years, only temporary solutions have been proposed, and the issue remains unresolved (Yong et al., 2022). One of the treatment methods is the application of hyaluronic acid (HA). HA is a polysaccharide consisting of D-glucuronic acid and N-acetyl-glucosamine. HA applied in IDD plays a role in anti-inflammation by interacting with membrane receptors, such as CD44 and the hyaluronan-mediated motility receptor (RHAMM), thereby preventing contact with pathways activated by inflammatory cytokines, including IL-1β, IFNα, and TNFα (Kazezian et al., 2020; Marinho et al., 2021) (Figure 1).

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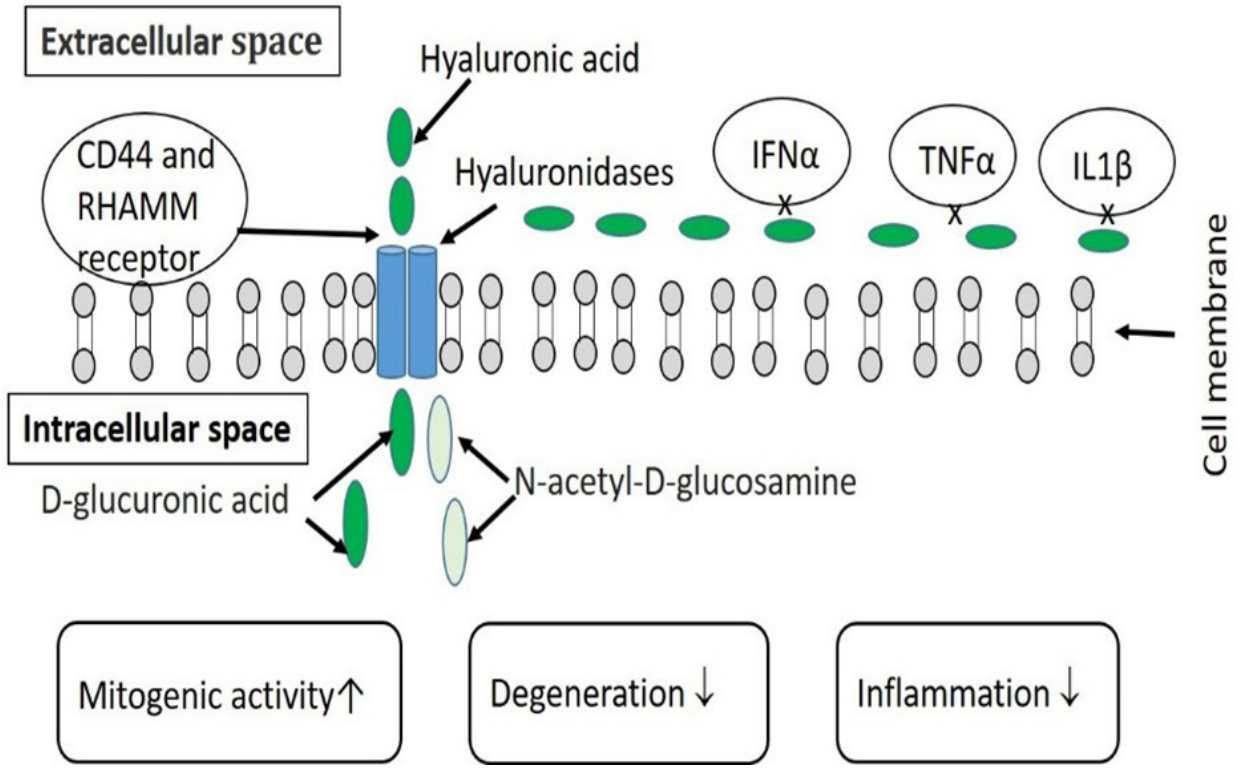


Figure 1. *The role of hyaluronic acid (HA) in mitigating inflammation in intervertebral disc degeneration. RHAMM: hyaluronan-mediated motility.*

Due to its outstanding viscoelastic properties, high biocompatibility, and ability to retain moisture, HA is commonly used in the treatment of various conditions, particularly osteoarthritis and IDD (Vasvani et al., 2020). Moreover, it is preferred in the treatment of joint degenerative diseases due to its pharmacological effects, including anti-inflammatory, antioxidant, anti-apoptotic, analgesic properties, as well as its ability to promote macrophage activation and inhibit extracellular matrix degradation (Cooper et al., 2017; Zhang et al., 2022). Our examination of the literature revealed no extensive studies comparing the effects of HA and different methods of application thereof on IDD. The objective of this study was to assess the influence of epidural (ED) and intradiscal (ID) HA injections on IDD in an experimentally induced rat model.

MATERIALS AND METHODS

Ethics and laboratory conditions

Rats were purchased from Aksaray University Experimental Animal Application Research Center. Ethical approval for this study was granted by the ethics committee of Niğde Ömer Halisdemir University, Türkiye (protocol number 2019/19 dated 08.05.2019). All procedures followed the European Directive 2010/63/EU for animal research, and all animal-related practices were conducted in line with the ARRIVE guidelines.

Rats were housed in Standard Type III cages equipped with an auto-adjusting system, maintaining a laboratory environment with an average temperature of 22 ± 2 ºC, 50% ± 5% humidity, and a 12-hour light/dark cycle. Standard rat pellet chow and drinking water were used. All animal daily checks were conducted throughout the experiment.

Animals and experimental procedures

Sixty male Sprague-Dawley rats (10-12 weeks) were randomly divided into six distinct groups. Examination of previous surgical procedures revealed deaths occurring due to infection, and larger numbers of animals were therefore included in the surgical group Fernández-Susavila et al. 2018).

1. Control group (CG)(n=8): The rats in this group were not exposed to any procedure.

2. Sham plus intradiscal HA injection group (S+ID) (n=8): The rats in this group underwent skin incision, and suturing was performed on the intervertebral disc tissues between regions Co6-Co10. HA was applied to the disk in area Co 8-9 using the ID injection method (a single dose of 0.1 mL on the day of the procedure).

3. Sham plus epidural HA injection group (S+ED) (n=8): The rats in this group underwent skin incision, and suturing was performed to the intervertebral disc tissues between regions Co6-Co10. HA was applied to the Co 8-9 area using the ED injection method (a single dose of 0.1 mL on the day of the procedure).

4. Intervertebral disc degeneration group (IDDG) (n=12): The rats in this group underwent degeneration between the Co 8-9 region of the intervertebral disc tissues.

5. IDD plus epidural HA injection group (IDDG+ED) (n=12): HA was applied to the (Co 8-9 area) by ED injection method in this IDD group of rats (a single dose of 0.1 mL on the day of the procedure).

6. IDD plus intradiscal HA injection group (IDDG+ID) (n=12): HA was applied into the disk in the Co 8-9 area by ID injection method in this IDD group of rats (a single dose of 0.1 mL on the day of the procedure).

Rat intervertebral disc degeneration model

Before the study commenced, 10 mg/kg of xylazine hydrochloride (Rompun) and 100 mg/kg of ketamine hydrochloride (Ketalar, Parke-Davis, Eczacıbaşı) were applied via the intraperitoneal route. The tail skin was first sterilized with 70% alcohol and Betadine. A 4-cm dorsal midline incision was made between caudal vertebrates Co6 and Co10, which were identified by palpation of the tail at the annulus fibrosus (AF) and nucleus pulposus (NP). The tail skin was retracted, and the muscles and tendons of the back were separated to visualize the intervertebral discs. The coccygeal intervertebral spaces Co 8-9 were selected for the study. After penetration, a 20-gauge needle was inserted into the disc, rotated 3600, and held in place for 30 sec. The depth of needle penetration was checked. The incision was closed with 4-0 non-absorbable surgical thread (Sönmez & Keleş, 2023; Mert et al., 2025) (Figure 2). HA (Truvisc sodium hyaluronate, 32 mg/2.0 ml, VSY Biotechnology, Germany) was applied in a single dose of 0.1 ml on the day of the procedure (Işık et al., 2015) (Figure 2d). The rats in all groups were weighed before and at the end of the experiment using sensitive digital scales (KERN & SOHN GmbH, D-72336 Balingen, Germany), and the results were recorded.

Inflammatory cytokine analysis by ELISA

In this study, the levels of serum TNF (Cat No: ELK1396, ELK Biotechnology, USA) and IL-6 (Cat No: ELK1158, ELK Biotechnology, USA) were quantified using commercially available ELISA kits. Calibration curves were generated using the provided standard solutions, and the concentrations of these cytokines were expressed in picograms per milliliter (pg/mL).

Histological procedures

Examination of the literature revealed that degeneration occurred in the first and fourth weeks following the IDD procedure (Mert et al., 2025) and that the effects of HA on tissue were observed at the fourth and eighth weeks (Işık et al., 2015; Salamanna et al., 2019). At the end of the fifth week following the HA injection, the animals in this study were euthanized via cervical dislocation. Intervertebral disc tissues between Co8 and Co9 were harvested from all groups. The samples were then fixed overnight in 10% formaldehyde and subsequently decalcified (Keleş et al., 2025; Mert et al., 2025).

Following decalcification, the tissues underwent routine histological procedures and were stained with Verhoeff-Van Gieson (VVG) and hematoxylin and eosin (H&E) stains (Mert et al., 2025). Five-micron sections were prepared from all tissue samples, and histological analysis was conducted using a light microscope (Olympus BX53) with a DP80 digital camera and CellSens Standard software version 1.17 (Japan). Histopathologic evaluations are performed by a histologist and a neurosurgeon with blind evaluation (using slides with coded labels).

Grading of intervertebral discs

Evaluations were performed on ten samples (five H&E and VVG sections) from each animal for histological rating scales. The intervertebral disc cross-sections were assessed using histological evaluation scales developed for H&E staining (except for the cellularity of the NP) and VVG staining (Mert et al., 2025).

Statistical analyses

Statistical analyses were performed on SPSS (Statistical Package for the Social Sciences, version 22.0, SPSS Inc., IBM [International Business Machines, USA]) software. Statistical significance was assessed using two-way ANOVA with Tukey's post hoc test. p-values <0.05 were regarded as significant for all comparisons.

RESULTS

Evaluation in disc tissues

Intervertebral disc areas from CG and IDDG were evaluated at the end of the experiment. CG discs exhibited a normal appearance. When the incision regions were opened, edema was present in the IDDG discs. The intervertebral discs were removed and cleaned, and marked color changes (black) were observed in the IDDG disc region (Figure 2).

Body weight results

No significant differences in rat weights were determined at the commencement of the study between CG and the S+ID (p=0.567), S+ED (p=0.533), IDDG (p=0.192), IDDG+ID (p=0.390), or IDDG+ED (p=0.400) groups. No significant differences were also observed at the end of the experiment between CG and the S+ID (p=0.835), S+ED (p=0.823), IDDG (p=0.816), IDDG+ D (p=0.149), or IDDG+ED (p=0.163) groups (Figure 3).

Inflammatory cytokines

In our study, the levels of TNF-α and IL-6, two inflammatory cytokines, were determined (Figure 4). A statistically significant decrease in TNF-α levels was detected when the IDDG group was compared with the IDDG+ED and IDDG+ID groups (respectively, p=0.038 and p=0.006). TNF-α showed no statistically significant differences between the CG and the S+ED, S+ID, IDDG+ED, and IDD+ID groups (respectively, $p = 0.961$, $p = 0.146$, $p = 0.232$, and $p = 0.061$); however, the mean value of S+ID was higher. TNF-α was significantly higher in the IDDG (p=0.038) than in the CG (Figure 4a). IL-6 levels were significantly higher in the IDDG (p=0.001) than in the CG. A statistically significant decrease in IL-6 levels was observed when the IDDG group was compared with the IDDG+ED and IDDG+ID groups (respectively, $p = 0.005$ and $p = 0.005$). IL-6 showed no statistically significant differences between the CG and the S+ED, IDDG+ED, and IDD+ID groups (respectively, $p = 0.447$, $p = 0.064$, and $p = 0.197$). IL-6 was significantly higher in the IDDG (p=0.001) and S+ID (p=0.001) than in the CG (Figure 4b).

Histopathological results

The intervertebral discs from all groups were subjected to histological examination following H&E staining. The intervertebral discs in the CG and S+ED treatment groups exhibit a generally normal appearance. S+ID group discs exhibited vacuoles in AF collagen fibers,

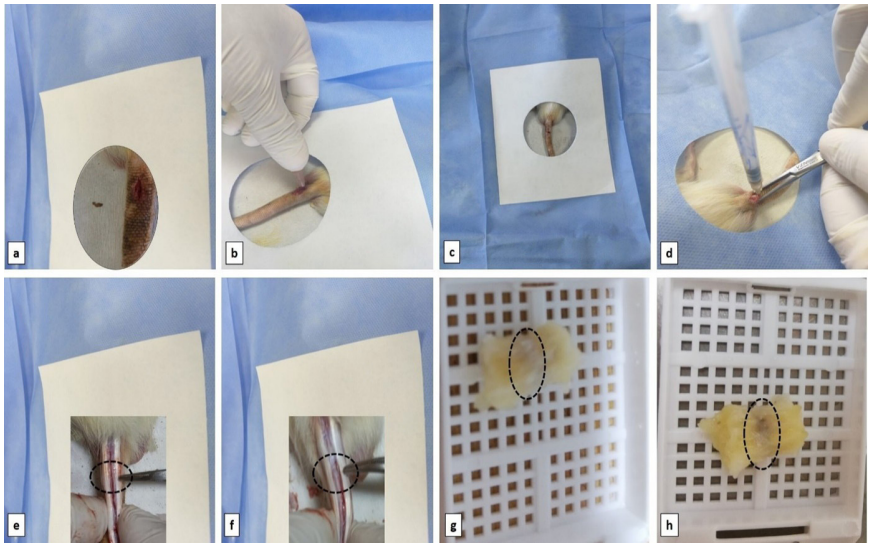


Figure 2. *The disc degeneration model. (a)The disc degeneration zone was opened; (b)After detecting the disc area, the needle was inserted into the disc and rotated 360°; (c)Injection was performed in the degeneration area; (d)The incision site was sutured; (e) Image of a disc from the control group at the end of the experiment; (f)Image of edema in the disc in the degeneration area at the end of the experiment; (g)Image of a disc from the control group at the end of the experiment; (h)Image of necrosis in the disc in the degeneration area at the end of the experiment.*

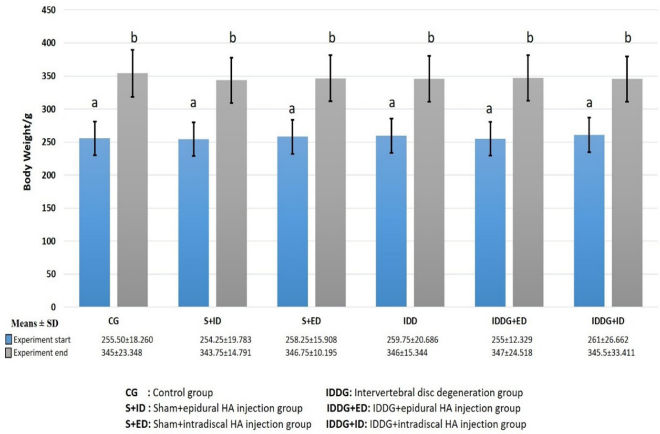


Figure 3. *The body weights (at the start and end of the experiment) of the experimental rats. The data are expressed as mean ± standard deviation (SD). Values marked with "a" and "b" indicate p > 0.05, which is not statistically significant.*

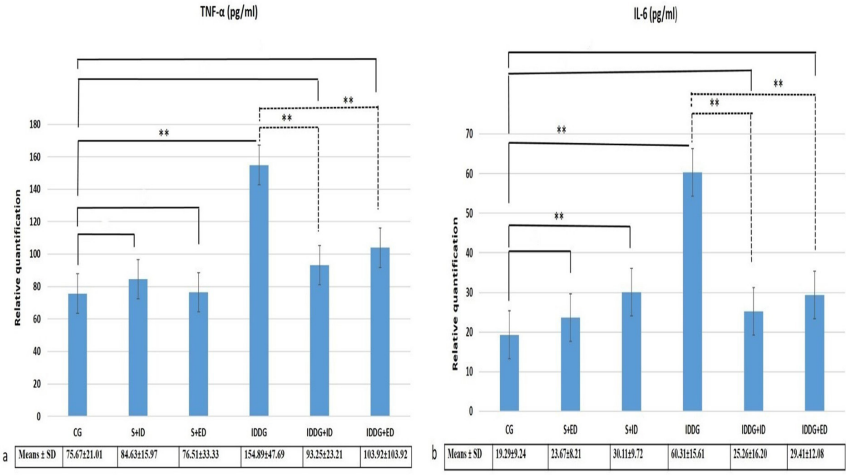


Figure 4. *The pro-inflammatory cytokine levels of all experimental groups. (a and b) TNF-α and IL-6 expression was significantly reduced in the treatment groups (IDDG+ID and IDDG+ED). IL-6 and TNF-α expressions decreased on average in the IDDG+ID group, but were not significant. IL-6 and TNF-α expressions higher in the IDDG groups. (a)TNF-α expression was higher in the S+ID groups but were not significant. (b) IL-6 was significantly higher in the S+ID. Statistical significance was determined using two-way ANOVA followed by a post-hoc Tukey test. Results are expressed as mean ± SD. **p<0.05 statistically significant. TNF, tumor necrosis factor, IL, interleukin, CG: Control, S+ED: sham+epidural, S+ID: sham+intradiscal, IDDG: intervertebral disc degeneration, IDDG+ED: IDDG+epidural, IDDG+ID: IDDG+intradiscal groups.*

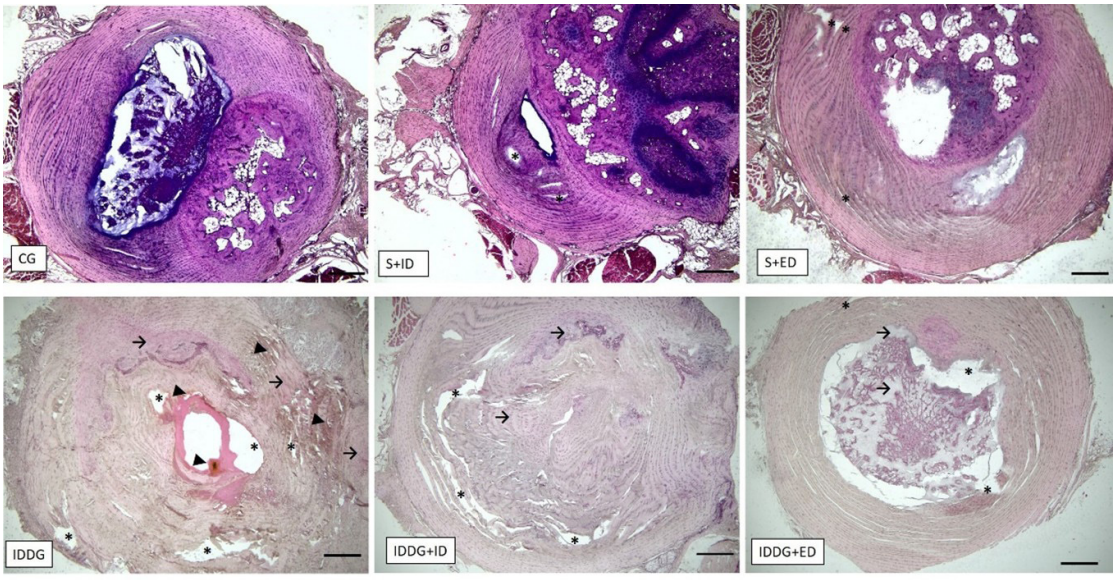
mild disc tearing, and a serpentine appearance. IDDG disc tissues from the tail region exhibited less staining, AF and NP could not be distinguished, disc shapes were unclear, collagens were impaired or irregular, interstitial edema and vacuoles were observed in the disc, and necrotic tissue debris was present in the NP area. The results of previous studies were consistent with our findings for IDD induced in the rat tail (Mert et al., 2025). In the IDDG+ED and IDDG+ID treatment groups, a decrease in vacuoles and edema caused by IDDG, necrotic tissue in the NP area was no longer visible, AF and NP were distinguishable, and the discs exhibited an appearance close to that of those in CG (Figure 5).

Collagen fibers in intervertebral discs stained with VVG exhibited a regular appearance in the CG and S+ED groups. S+ID group discs exhibited vacuoles in AF collagen fibers, mild disc tearing, and a serpentine appearance. In the IDDG discs, AF and NP could not be distinguished; necrotic tissue debris was present in the NP area, collagen fibers were irregular, and interstitial edema and vacuoles were observed. In the IDDG+ED treatment group, the NP and AP borders were clear, collagen fibers were regular, and decreases were observed in vacuoles and edema. Necrotic tissue in the NP area had entirely disappeared, and the disc appearance was close to that in the CG. In the IDDG+ID treatment group, the AP and NP margins were clear, with minimal necrotic tissue and occasional vacuoles observed (Figure 6). In all histological evaluations, we found that healing was better in the homogeneous distribution of NP and organized AF lamellae in the IDDG+ED than in disc degeneration at IDDG+ID.

Grading of intervertebral disc results

H&E histological analysis showed no significant differences between the CG group and the S+ED ($p = 1.000$) or S+ID ($p = 0.685$) treatment groups. However, significant differences were observed between CG and IDDG ($p = 0.001$), as well as between IDDG and the IDDG+ED ($p = 0.001$) or IDDG+ID ($p = 0.001$) treatment groups (Figure 5a).

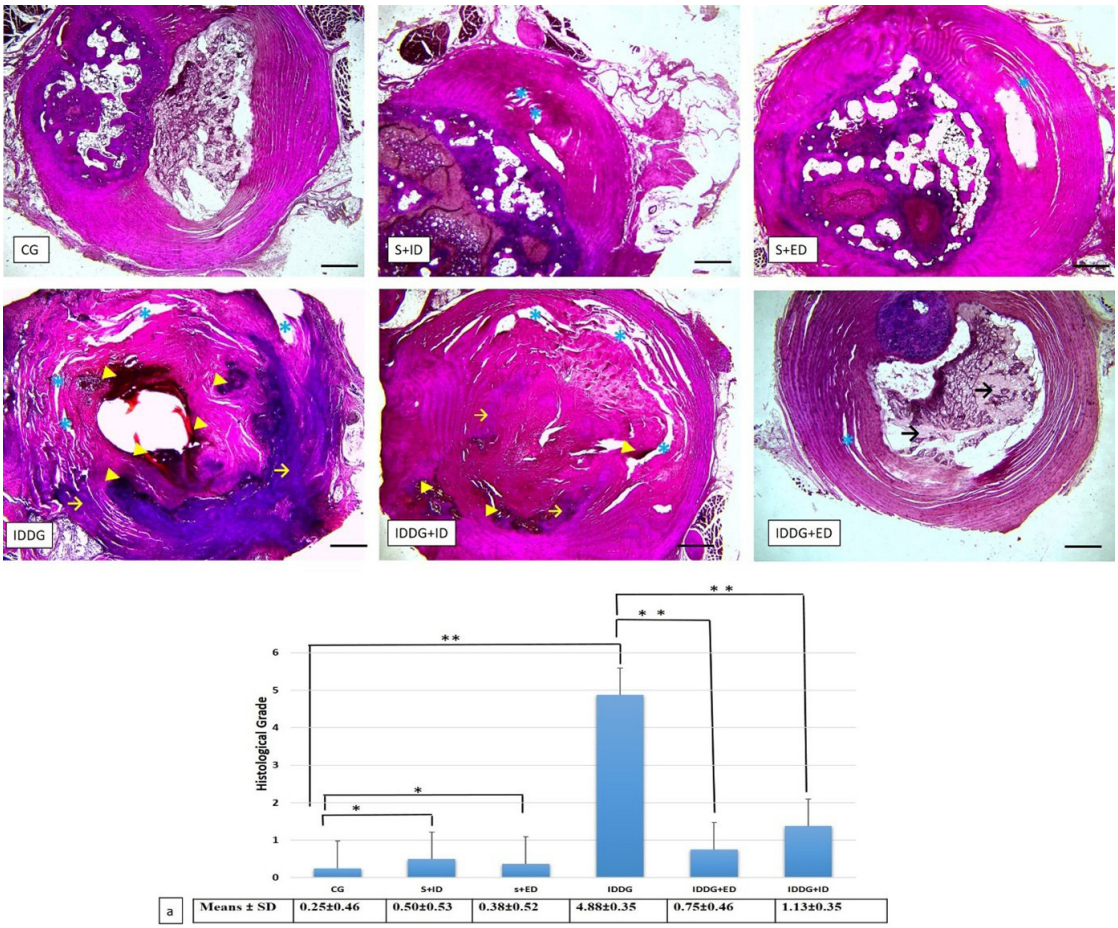
No statistically significant difference was observed between CG and either the S+ED ($p=0.685$) or S+ID ($p=0.351$) treatment groups in terms of VVG histological classification. However, a significant difference was found between the CG and IDDG ($p = 0.001$), as well as between the IDDG and the IDDG+ED ($p = 0.001$) or IDDG+ID ($p = 0.001$) treatment groups (Figure 6a).



a

Means ± SD	0.25±0.46	0.25±0.46	0.38±0.52	7.88±0.35	3.00±0.00	3.75±0.46

Figure 5. *Images of intervertebral discs stained with hematoxylin & eosin from all the study groups. After degeneration (IDDG), disc tissues exhibited less staining, the annulus fibrosus (AF) and nucleus pulposus (NP) could not be distinguished, AF region collagen layers became disorganized, interstitial edema and vacuoles occurred in the disc, and necrotic tissue was observed in the NP. In the IDDG+ED and IDDG+ID treatment groups, a decrease in vacuoles and edema, disappearance of necrosis in the NP area of the disc, and differentiation between AF and NP were observed. (►) necrotic tissue, (*) vacuoles, (→) interstitial edema. Scale bar=500 µm, 2X. (a) Histological grading for the various study groups. Data are shown as mean ± standard deviation (SD). **p<0.05 statistically significant and *p>0.05 not statistically significant. Statistical analysis was performed using two-way ANOVA followed by a post-hoc Tukey test.*



a	Means ± SD	0.25±0.46	0.50±0.53	0.38±0.52	4.88±0.35	0.75±0.46	1.13±0.35

Figure 6. *Images of intervertebral discs stained with Verhoeff Van Giemsa stain from all the study groups. After degeneration (IDDG), the nucleus pulposus (NP) and annulus fibrosus (AF) could not be distinguished, AF region collagen layers were disorganized, vacuoles and interstitial edema occurred in the disc, and necrotic tissue was observed. In the IDDG+ED treatment groups, a decrease in vacuoles and edema, disappearance of necrosis in the NP area of the disc, and clear AF and NP borders were observed. In the IDDG+ID treatment groups, the AP and NP borders were clear, together with a small amount of necrotic tissues and occasional vacuoles. (►) necrotic tissue, (*) vacuoles, (→) interstitial edema. Scale bar=500 µm, 2X. (a) The histological grades of the different groups. The values are presented as mean ± SD. **p<0.05 statistically significant and *p>0.05 not statistically significant. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test.*

DISCUSSION

IDD has become a health and economic problem for many countries. In this study, the therapeutic effects of HA applied using different methods (ED and ID) after IDD were evaluated through inflammation and histopathological examinations. In a study, animal deaths following infection with IDD were reported (Fernández-Susavila et al., 2018). Larger numbers of animals were therefore included in the experimental groups. However, our study's findings show that IDD induced in the rat tail and HA administration did not result in weight changes or death in the animals used. We found no previous reports on this subject in the literature.

Inflammation plays a critical role in the onset and progression of IDD by driving the ongoing loss of extracellular matrix, promoting apoptosis, inducing oxidative stress, and triggering endoplasmic reticulum stress (Kang et al., 2023; Zhao et al., 2010). In our study, proinflammatory cytokines TNF-α and IL-6 were evaluated in rat blood serum levels. It was found that IDDG rats had increased serum levels of IL-6 and TNF-α. An increase in inflammation values is expected after degeneration (Risbud & Shapiro, 2014). Additionally, high TNF-α is an essential factor in detecting disc degeneration at an early stage (Kang et al., 2015). HA is known for its pharmacological effects, including anti-oxidative, anti-apoptotic, anti-inflammatory, and analgesic properties (Cooper et al., 2017; Marinho et al., 2021; Zhang et al., 2022). In this study, it was determined that serum levels of IL-6 and TNF-α decreased when the HA application groups (IDDG+ID and IDDG+ED) were compared with the IDDG group. A study examined the short-term effects of HA application with the ID method after IDD was created in rabbits. The study's findings revealed a decrease in IL-6 and TNF-alpha levels, although the reduction in average values was not statistically significant (Inoue et al., 2021). No comprehensive study was found regarding the ED method. In our research, it was determined that TNF-alpha and IL-6 values decreased as a result of examining the long-term effects of HA applied using the ID and ED methods. These results confirm that HA applied to ED and ID has an anti-inflammatory effect on IDD.

When HA application methods were compared, anti-inflammatory effects were observed in both application methods (IDDG+ID and IDDG+ED). However, when the average values in the IDDG+ID application are taken into account, it was found to be more effective. In the analyses performed in the sham groups, it was determi-

ned that IL-6 values were statistically increased in the S+ID group compared to the control group, and TNF-α values were also increased. However, their average values were not statistically significant. As a result of these findings, it was found that there was an increase in inflammation values in the S+ID injection group. Considering that HA does not have an inflammation-promoting effect, it suggests that the ID injection method may cause an increase in blood serum levels of inflammation. Studies have reported an inflammatory reaction at the injection site, pain, and swelling (Michou et al., 2004) and acute aseptic arthritis mimicking septic arthritis (Kaçmaz et al., 2017) following ID application. Although HA applied with the ID method after IDD has a greater anti-inflammatory effect, we think that it is more appropriate to choose the ED method because it causes tissue damage.

In histological evaluations, we observed disc repair with homogeneous NP distribution and organized AF lamellae in the IDDG groups injected with HA using ED and ID methods compared to the IDDG. These results show that HA applied to the discs treated with IDD by ED and ID injection methods morphologically restructures AF and NP. Previous studies have shown that HA enters the cell as a result of disruptions in the cell membrane, can alter the properties of cell membranes, serve as a cytoskeleton, and can change and control the shape of the cell (Liang et al., 2016; Marinho et al., 2021). A study shows that HA could safeguard mitochondrial function and enhance IDD by promoting mitophagy in NP cells (Zhang et al., 2022).

Although it is stated that HA treats disc degeneration, no study has been found showing which application method is more effective. In our study, the histological effects of ID and ED injection methods were compared. First, the ID injection method was evaluated in the S+ID and IDDG+ID groups. The intervertebral discs of the S+ID group had vacuoles in AF collagen fibers, mild disc rupture, and a serpentine appearance. In the IDDG+ID groups, the AP and NP borders were clear, with minimal necrotic tissue and occasional vacuoles observed. No studies have been found that indicate HA will cause histopathological changes in the intervertebral disc. In fact, a study states that the problems occurring in the tissue after ID injection are not related to HA (Lussier et al., 1996).

However, studies have shown that pathological consequences may occur in the tissue after ID injection. Studies indicate that changes such as pain and swelling occur in the ID injection area (Michou et al., 2004), and acute aseptic arthritis septic arthritis (Kaçmaz et al., 2017). Our results and literature reviews suggest that the reason for the histopathological changes in the intervertebral discs in the ID injection groups is the application method. Another application performed on the intervertebral disc tissue is ED. No study has been found showing the histological changes in the intervertebral disc tissues of ED after IDD. However, Isık et al. (2015) observed positive effects such as the prevention of fibrosis and a decrease in the density of fibrotic tissue as a result of ED HA injection. The histological results in our study show that HA enters the cells and creates a therapeutic effect as a result of its application with the ED and ID methods but the ED method is more effective. This may be due to changes in tissues being caused following ID application.

In conclusion, IDD and HA caused no weight changes, or mortality. Both epidural and intradiscal HA administration reduced inflammatory and degenerative changes in IDD, with the epidural route demonstrating superior histological recovery by better preserving disc morphological integrity and promoting tissue repair. HA application caused an anti-inflammatory effect by preventing contact with receptors of inflammatory cytokine signaling cascades such as TNFα and IL1β as a result of its reaction with membrane receptors such as CD44 and RHAMM. Especially in recent years, there has been widespread use of HA-containing drugs. This study attempts to provide information on the effects of HA and application methods. Considering the study results, it is thought that the effectiveness of the methods should be taken into account in human applications. In a short time, the appropriate dose, appropriate method and effective results should be determined.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Data Availability Statement

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

Ethical approval

All animal procedures were approved by the institutional ethics committee at Niğde Ömer Halisdemir University (protocol number 2019/19 dated 08.05.2019).

Financial disclosure

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Drug statement

Truvisc sodium hyaluronate, 32 mg/2.0 ml, VSY Biotechnology, Germany

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