INTRODUCTION

Intervertebral disc degeneration is a multifactorial process that mainly affects aging individuals, although in some cases it can affect youths as well. The degenerative process frequently results in low back pain, which is often detrimental to the working capacity, and the existing treatments do not prevent its progression, resulting in high costs for the health system both for clinical and surgical treatment. Nowadays, it is known that disc degeneration is due to cellular, biochemical and structural alterations progressing to a reduction in the number of cells in the intervertebral disc and of the extracellular matrix components.1-3 The extracellular matrix of the intervertebral disc is a complex structure, made up of water, proteoglycans, elastic fibers and collagen, mainly types I and II present in the annulus fibrosus (AF). When the high molecular weight proteoglycans (PG) (aggrecan, versican and perlecan) found predominantly in the nucleus pulposus (NP) are negatively charged and bound to lateral chains with the glycosaminoglycans (GAG), they play a vital role ensuring disc compression resistance during movements. Low molecular weight proteoglycans (decorin, biglycan, fibromodulin and lumican) interact with other extracellular matrix molecules such as collagens and growth factors, signaling processes in disc degeneration.4-6 Heparan sulfate (HS) is a glycosaminoglycan (GAG) that is an important player in extracellular matrix organization. Its chains are cleaved by heparanase (HPSE), an endo-β-glucuronidase, releasing oligosaccharide that potentiate the action of growth factors, cytokines and components involved in angiogenesis and osteogenesis. Studies indicate that heparanase is also involved in other processes, such as cell invasion and inflammation. Two isoforms of this enzyme were described in the literature: heparanase-1 (HPSE1), which has enzymatic activity, and heparanase-2 (HPSE2), whose function is still unknown.7,9 The inflammatory process in disc degeneration is triggered by...
biochemical mediators such as (TNF-α, prostanglandin E), which stimulate the immunological response, and consequently release proinflammatory cytokines, such as the interleukins (IL-1β, IL-6, IL-8), and growth factors, causing pain.10 Metalloproteinases (MMPs) are enzymes that are also involved in extracellular matrix degradation, related to structural alterations of the intervertebral disc.11

In this study, an animal model was used to evaluate the distribution of the main functional and structural extracellular matrix components that are involved in the intervertebral disc degeneration process, aiming to improve the monitoring of the molecules linked to the progression of the disease, achieving a better understanding of the disc degeneration physiopathology. The results will be able to throw light on molecular mechanisms related to the disc degeneration process, evidencing molecules that can be used in the future for diagnosis, prognosis or targeted therapy.

MATERIAL AND METHODS

Animal Model

Three male Wistar (Rattus norvegicus albinus) rats aged 12 weeks (complete skeletal maturity), and weighing between 300 and 350 grams were used for the animal model of intervertebral disc degeneration. All the rats were submitted to degeneration induction and only the first animal was euthanized soon after the surgical procedure, classified as control (or Day 0). The second animal was euthanized 15 days after the induction of the intervertebral disc degeneration process and the third animal at 30 days. The animals remained in the care of the vivarium of the faculty between the degeneration induction and the euthanasia. For the intervertebral disc degeneration induction the animal underwent tail antisepsis with an iodized alcohol solution then were anesthetized with an association of ketamine (88 mg/kg) and 2% xylazine (12 mg/kg) administered intraperitoneally. Deep anesthetic plane was confirmed by the absence of corneal reflex and of reaction to the profound pain caused by interdigital compression. The levels between the sixth and seventh and between the eighth and ninth coccygeal vertebrae were identified by palpation. The degeneration induction was performed by percutaneous puncture using a 20G needle. The needle was introduced up to the nucleus pulposus, rotated 360° and kept in the same position for 30 seconds, in accordance with the study by Zhang et al.12 The intermediary level, located between the seventh and eighth coccygeal vertebrae, was adopted as control, since it was not submitted to puncture. Following confirmation of the deep anesthetic plane, 5mL of arterial blood were drawn from the abdominal aorta, via transabdominal approach, causing euthanasia due to hypovolemic shock. After euthanasia, the tail segments with the discs for study were extracted using a no. 15 scalpel blade. The removed intervertebral discs were stored in properly identified flasks with formaldeyde solution (10%) and sent to the Molecular Biology laboratory for immunohistochemical analyses. This study was approved in the Committee of Ethics in Animal Experimentation, process 003/2011. It should be noted that the disc degeneration model used on rats reproduces morphological and biochemical alterations that are similar to those observed in the degenerative processes of human intervertebral discs.13

Immunohistochemical Analysis

Representative intervertebral disc degeneration areas were chosen based on the staining of the tissue sections with hematoxylin-eosin (HE). Formalin-fixed, paraffin-embedded 3µm thick sections were deparaffinized and rehydrated. Antigen recovery was performed by heating the slides to 100°C for 30 minutes in a 10 mmol/L citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide aqueous solution for 35 minutes. The sections were then incubated overnight at 4°C with the primary antibodies: anti-HPSE1 (HPA1 L-19), anti-HPSE2 (HPA2 C-17), anti-aggrecan (4F4), anti-decorin (N-15), anti-biglycan (L-15), TGFβ1 (sc-146), anti-MMP-9 (H-129), anti-interleukin-6 (SC-130326) and anti-interleukin-10 (H-160) (Santa Cruz Biotechnology, CA, USA). Finally, the slides were incubated with a peroxidase-labeled streptavidin complex (LSAB®, DakoCytomation, Glostrup, Denmark) for 30 minutes. The sections were developed using 3,3’-diaminobenzidine (DAB), for 1 minute and were counterstained with hematoxylin. Some samples were incubated with 1M phosphate buffer in the absence of the primary antibody, as negative controls. The presence of brown coloration was considered evidence of positive expression of the respective molecules in the cell.

Digital quantification

The slides were analyzed using of a TS100 Nikon Eclipse® light microscope to identify the areas that best represented the immunostaining of the molecules analyzed (hot spots). In each case, the immunostaining quantification was performed using a computer-assisted digital analysis method. The photomicrographs of 640x480 pixels were obtained from non-coincident consecutive fields with magnification of 400X using a 4300 Nikon Coolpix® digital camera adjusted to the same parameters. The resulting images were analyzed using the ImageLab® image processing and analysis system (Softium Informática®, São Paulo, Brazil), adjusted to the micrometric scale (µm).

Positivity index (PI)

In each case, at least 1,000 cells were counted by ImageLab®, and the observer classified them as positive or negative cells. For this reason, the percentage of marked cells was determined according to the following equation:

\[ \text{PI} = \frac{\text{number of marked cells}}{\text{total cells counted}} \times 100 \ [\%] \]

Intensity of expression (ItE)

ImageLab® was used to quantify the intensity of the brown color that resulted from the immunostaining. For each case, the same photomicrographs that were used to determine the PI were considered. Twelve cytoplasmic regions of different randomly marked cells were accessed with the same-sized square (a tool of the ImageLab® system). The average optical density (OD) of these areas was automatically calculated and represents the mean of the red, green and blue (RGB) color compositions per area of cytoplasm analyzed; the OD was expressed in optical units per square micrometer (or/µm²). The same procedure was applied to obtain the background optical density (BOD) of an area without tissue or vascular space for each photomicrograph. For this purpose, a single area was sufficient, as the background...
is homogeneous in each image. The color absolute white that corresponds to the maximum optical density (320.7 ou/µm²) is composed of a complete mixture of red, green and blue, while the color black represents the absence of these colors. Therefore, the optical density values calculated by the program involved a decreasing scale, in which the highest values corresponded to the colors that were clearly visible. The equation shown below was used to calculate the immunostaining intensity expression (IIE) in each case. Its values involve an increasing scale that is subtracted from the BOD proportional to the optical density of absolute white.

\[
\text{IIE} = \frac{320.7 - 320.7 \times \Sigma \text{DOF}}{\Sigma \text{DO}} \quad [\text{ou/µm}^2]
\]

**Index of expression (IE)**

The index of digital expression \( (I_d) \) was obtained by multiplying the percentage of labeled cells (PLC) by the digital immunostaining intensity \( (I_d)\) for each case, according to the following equation:

\[
IE = \frac{PI \times I_d}{100} \quad [\text{ou/µm}^2]
\]

**RESULTS**

Sections of the intervertebral disc of each animal were stained with hematoxylin-eosin and analyzed by a pathologist, for evaluation of the histological parameters. It was noted that the alterations were mainly concentrated in the cartilaginous (central) region, except for the presence of neovessels in the fibrous (peripheral) region of the intervertebral disc. The disc degeneration induced at distal level, between the eighth and ninth coccygeal vertebrae, (Table 1) demonstrated moderate levels of apoptosis throughout the degenerative process, besides reduction of the regeneration process in the samples collected 30 days after degeneration induction. Over the course of the degenerative process, the following phenomena were observed in the extracellular matrix: slight presence of calcification, decreased mixoid degeneration, gradual growth of eosinophilic degeneration and formation of neovessels only at 30 days after disc degeneration induction. The degenerated proximal and distal intervertebral discs were collected, but no significant differences were observed between the discs.

The immunostaining analysis for the high molecular weight proteoglycan aggrecan revealed a reduction of 22% in the expression of this proteoglycan, 15 days after intervertebral degeneration induction, in comparison with the control group. However, the results show the occurrence of a 37% increase in the aggrecan expression 30 days after disc degeneration, when compared with the control group. (Figure 1) The expression levels were confirmed by the digital quantification of the images. (Figure 2)

The low molecular weight proteoglycans, decorin and biglycan, presented notable reduction of expression at 15 days, respectively, 41% and 59%. (Figures 1 and 4) However, 30 days after the induction of the disc degeneration process an increase of 61% was observed in the decorin expression with one of 19% in the biglycan expression, both compared with the control group. The growth factor TGF-β presented considerable reduction (69%), when compared with the intervertebral discs of the animal euthanized at 15 days, after induction of degeneration and control group samples. It is interesting to note that the TGF-β presented significant reduction (24%) of expression at 30 days after disc degeneration in relation to the control group.

The heparanase isoform expression analysis evidenced reduction of 29% in the immunostaining of the anti-heparanase-1 antibody, in the samples obtained on the 15th day after induction of the disc degeneration process, maintaining slight reduction (6%) on the 30th day, in comparison with the control group. (Figure 3) Comparing immunostaining of the heparanase-2 isoform (HPSE2) with the control group, we also observed slight reduction in the expression (4%) on the 15th day and an increase of approximately 15% on the 30th day, after intervertebral degeneration induction. The expression levels were confirmed by the digital quantification of the images. (Figure 4)

Metalloproteinase-9 (MMP-9) presented a reduction of 36% in expression on the 15th day, in comparison to the non-degenerated discs. Once again, an increase of 31% in the MMP-9 expression could be observed on the 30th day, in relation to the control group.

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**Table 1.** Pathological evaluation after induction of intervertebral disc degeneration in rats.

<table>
<thead>
<tr>
<th>Days after degeneration induction</th>
<th>Apoptosis</th>
<th>Regeneration</th>
<th>Fracture/Fissure</th>
<th>Calcification</th>
<th>Mixoid Degeneration</th>
<th>Eosinophilic degeneration</th>
<th>Inflammatory Infiltrate</th>
<th>Neovessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days</td>
<td>(+)</td>
<td>(+++)</td>
<td>0</td>
<td>0</td>
<td>(++)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15 days</td>
<td>(+++)</td>
<td>(+)</td>
<td>0</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 days</td>
<td>(+)</td>
<td>(++)</td>
<td>0</td>
<td>(++)</td>
<td>(+)</td>
<td>(++)</td>
<td>0</td>
<td>(++)</td>
</tr>
</tbody>
</table>

Grading of the alterations: 0. Absent; (+). Slight; (++) Moderate; (+++). Intense.
As regards the molecules involved in the inflammatory process, the decrease of interleukin-6 (IL-6) expression was clearly evident in the intervertebral disc samples that underwent the degeneration process. Regardless of the time elapsed from induction of the degenerative process, 15 days or 30 days, the observed decrease was, respectively, (15% and 3%), when compared with the control samples. (Figure 5) The interleukin-10 (IL-10) expression presented notable reduction at 15 days and slight decrease at 30 days after the induction of the degenerative process, respectively, (51% and 1%) in relation to the control group, as shown in Figure 3. The digital quantification confirms the results obtained in the immunohistochemical reactions. (Figure 6) In general, the analyses of the immunostaining with specific antibodies in the intervertebral disc samples from rats evidenced reduction in the expression of the extracellular matrix components on the 15th day after degeneration induction and, on the 30th day, return to levels close to those observed in the non-degenerated disc samples obtained.
DISCUSSION

The results observed in this pilot study of induction of intervertebral disc degeneration in rats show a variation in the expression of the extracellular matrix components in different periods. On the 15th day there was reduction of the expression of extracellular matrix components (proteoglycans, TGF-β), as well as of enzymes that take part in the matrix remodeling process (heparanase and MMP-9), while on the 30th day, the evaluation of the expression of such components was similar to the values obtained in the control group (without disc degeneration). Thirty days after disc degeneration, we noticed a decrease in the regeneration, calcification and neovessel formation process, characterizing a tissue that had already undergone remodeling when compared at 15 days after the degeneration induction where we noticed intense tissue regeneration.

Data from the literature evidence a decrease in the activity of enzymes involved in degradation of the proteoglycan aggrecan, the aggrecanases, associated with the progression of enzymes involved in degradation of the proteoglycan where we noticed intense tissue regeneration.

when compared at 15 days after the degeneration induction characterizing a tissue that had already undergone remodeling processes.

Thirty days after disc degeneration, we noticed a decrease in the expression of such components was similar to the values obtained in the control group (without disc degeneration).

The fact that the HPSE1 isoform dose not present significant alteration of expression at 15 days after the degeneration process can explain the decrease of the HPSE1 isoform expression, which possibly suffers negative modulation by HPSE2.

The HPSE2 isoform does not have enzymatic activity and its function is still unknown. It was described that HPSE2 can modulate the enzymatic activity of HPSE1.21

Considering that the 30th day, in the experimental model of disc degeneration in adult rats, is equivalent to the late degeneration process, we can affirm that the results found confirm the findings obtained in the literature.

CONCLUSION

The results show that in the acute phase of the disease (15th day) there were significant alterations of the extracellular matrix components, while in a later phase (30th day), the tissue returned to a molecule expression profile similar to the normal tissue, probably due to the occurrence of an intense extracellular matrix remodeling process.

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