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Oxidative stress participates in age-related changes in rat lumbar intervertebral discs

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ABSTRACT

Aging is a major factor associated with lumber intervertebral disc degeneration, and oxidative stress is known to play an essential role in the pathogenesis of many age-related diseases. In this study, we investigated oxidative stress in intervertebral discs of Wistar rats in three different age groups: youth, adult, and geriatric. Age-related intervertebral disc changes were examined by histological analysis. In addition, oxidative stress was evaluated by assessing nitric oxide (NO), superoxide dismutase (SOD), malondialdehyde (MDA), and advanced oxidation protein products (AOPPs). Intervertebral disc, but not serum, NO concentrations significantly differed between the three groups. Serum and intervertebral disc SOD activity gradually decreased with age. Furthermore, both serum and intervertebral disc MDA and AOPP levels gradually increased with age. Our studies suggest that oxidative stress is associated with age-related intervertebral disc changes.

1. Introduction

Aging causes the human intervertebral disc to undergo many degenerative biochemical and morphologic changes. In particular, lumbar degeneration, which is the major cause of discogenic lower back pain (DLBP), sharply increases with age. Disc degeneration can begin as early as the second decade of life, while other spinal structures suffer changes much later (Boos et al., 2002; Buckwalter, 1995).

Exposure to reactive oxygen species (ROS) is continuous and unavoidable in aerobic environments (Finkel & Holbrook, 2000). Organisms in an aerobic environment cannot live without oxygen but are simultaneously exposed to toxic ROS, such as superoxide radicals, hydroxyl radicals, and hydrogen peroxide, which damage their cells. Excessive ROS production or impaired antioxidant defenses result in oxidative stress (Brigelius-Flohe, 2009). Disrupting ROS production and antioxidant defense homeostasis determines the degree of oxidative stress, which can result from both intrinsic and extrinsic mechanisms. Cells use intrinsic antioxidants to protect themselves from free radical damage, and naturally occurring extrinsic antioxidants have also been shown to offset and alleviate such damage (Kim et al., 2007). Levels of oxidative stress increase with age due to macromolecular damage and/or an imbalance between reactive oxygen/nitrogen species production and antioxidant defense. The "free radical theory of aging" suggests that organisms age as the result of accumulated free radical damage over time (Harman, 1956, 1972).

Aging is a major factor associated with intervertebral disc degeneration, and strong evidence suggests that ROS production and oxidative stress are related to aging (Finkel & Holbrook, 2000). In this study, we determined whether oxidative stress plays a role in age-related intervertebral disc degeneration in rats.

2. Methods

2.1. Animals

Male Wistar rats were divided into three groups of 20 based on age (three, nine, and 22 months). Animals were obtained from the Animal Center of Southern Medical University in China and were individually housed and fed with standard rat pellets. Arterial blood and intervertebral discs were collected from the rats' nucleus pulposus tissues; the intervertebral discs were then homogenized for oxidative stress analyses. Tissues from the rats' L4/5 intervertebral discs were used for histomorphometry. All

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procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University in China.

2.2. Histology

Five samples from each group were randomly selected for histological examination. The samples were decalcified *en bloc* with a rapid decalcifying solution, and a scalpel was used to cut the center of the vertebrae after decalcification. The L5/S1 or L6/S1 discs and their two adjacent half-vertebrae were sectioned midsagittally, parallel to the initial cut made by the scalpel, and then dehydrated, embedded in paraffin, and sectioned. In addition, two 6-μm sections were mounted and stained with Safranin-O/Fast Green.

2.3. Histological grading

Histological grading was performed as described previously (Han et al., 2008). Samples were graded on a scale based on five categories of degenerative change with scores ranging from five points (one point per category) for a normal disc to 15 points (three points per category). The scaling criteria included: cellularity of the anulus fibrosus, morphology of the annulus fibrosus border between the anulus fibrosus and nucleus pulposus, cellularity of the nucleus pulposus, and morphology of the nucleus pulposus.

2.4. NO measurements

NO concentration was measured using reagents obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). NO concentration was determined by the enzymatic conversion of nitrate to nitrite using nitrate reductase. Nitrite was then detected using the Griess Reaction as measured by colorimetry. In the Griess reaction, acidified NO_2 produces a nitrosating agent that reacts with sulfanilic acid to produce a diazonium ion. This ion couples with N-(1-naphthyl) ethylenediamine to form a chromophoric azo derivative that absorbs light at 540 nm.

2.5. Antioxidant measurements

SOD activity was measured using reagents obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Xanthinexanthine oxidase (XOD) was used to generate superoxide radicals, which react with 2-iodophenyl-3-(4-nitrophenyl)-5-phenyltetrazolium (INT) chloride to form a red formazan dye. SOD activity was then determined by the degree of inhibition of this reaction at 505 nm. Results were expressed as SOD U/ml for serum and SOD U/mg protein for intervertebral disc homogenates.

2.6. Lipid peroxidation measurements

MDA is a secondary product of lipid peroxidation that is generated by exposure to ROS and free radicals. Thiobarbituric acid reactive substances were used to measure the reaction between MDA and thiobarbituric, and lipid peroxidation was determined as described previously (Ohkawa, Ohishi, & Yagi, 1979). Briefly, 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml 20% acetic acid (pH 3.5), 1.5 ml of 0.8% thiobarbituric acid, 0.2 ml serum or homogenate, and water were combined for a total volume 4.0 ml. Samples were then heated in a 95 °C water bath for 1 h and then cooled under running tap water. Next, 1.0 ml chilled water and 5.0 ml of butanol and pyridine (15:1, v/v) were added, and samples were vigorously vortexed for 15 s and then centrifuged at 3500 rpm for 10 min. The intensity of the supernatant was measured at 532 nm with 1,1,3,3-tetra-ethoxy-propane as reference.

2.7. Protein damage measurements

AOPP was measured as described previously (Witko-Sarsat et al., 2003). Briefly, 200 μl of plasma was diluted 1:5 with PBS; chloramines-T (0–100 μmol/l) was used for calibration measurements, and PBS alone was used as a blank. Samples were added to a 96-well microtitre plate, and 10 μl 1.16 M potassium iodide and 20 μl acetic acid were added. The absorbance of the reaction mixture was immediately read using a microplate reader at 340 nm.

2.8. Statistical analysis

Statistical analyses were performed using SPSS, version 13.0 (Echo Soft Corp., USA). Data are expressed as mean ± standard deviation (SD). One-way analyses of variance and post hoc multiple comparisons were performed to analyze differences between groups. A p-value < 0.05 were considered statistically significant.

3. Results

3.1. Histological intervertebral disc changes with age

The histologic appearances of the rats' intervertebral discs are shown in Fig. 1. Histological grading was significantly different between the three age groups (Table 1). Discs from the youth group (three months old) scored lower than those of the adult group (nine months old). In addition, discs from both these groups scored lower than those from the geriatric group (22 months old).

3.2. NO concentrations

NO concentrations in the serum and intervertebral discs gradually increased with age (Table 2). Serum NO concentrations between the three groups were not significantly different ($F = 0.261$, $p = 0.771$). In contrast, intervertebral disc NO concentrations were significantly different ($F = 9.374$, $p < 0.001$). Intervertebral disc NO concentrations in the adult and geriatric groups were significantly higher than those in the youth group ($p < 0.001$ and $p = 0.010$, respectively). However, no significant differences between the adult and geriatric groups were observed ($p = 0.114$).

3.3. SOD activity

SOD activity in the serum and intervertebral discs gradually decreased with age (Table 3). Serum SOD activity in the youth group was significantly higher than that in the adult and geriatric groups ($p = 0.009$ and $p < 0.001$, respectively); no significant differences between the adult and geriatric groups were observed ($p = 0.114$). Intervertebral disc SOD activity in the geriatric group were significantly lower than that in youth and adult groups ($p = 0.001$ and $p = 0.039$, respectively), but no significant differences between the youth and adult groups were observed ($p = 0.150$).

3.4. MDA levels

MDA levels in the serum and intervertebral discs gradually increased with age (Table 4). Serum MDA levels in the youth group was significantly lower than those in the adult and geriatric groups ($p = 0.050$ and $p < 0.001$, respectively), and serum MDA levels in the adult group were also significantly lower than those in the geriatric group ($p < 0.001$). Intervertebral disc MDA levels in the geriatric group was significantly higher than those in the youth or adult groups ($p = 0.012$ and $p = 0.045$, respectively). However, no significant differences between the youth and adult groups were observed ($p = 0.578$).