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Nucleus pulposus inhibits the axonal outgrowth of cultured dorsal root ganglion cells

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Abstract Although it is well established that nucleus pulposus cells may induce structural and functional changes in adjacent nerve roots when placed epidurally, it is not known whether this is due to direct neurotoxic effects or whether the nerve roots are affected indirectly by reduction of nutrition and inflammatory/immunologic mechanisms. In the present study we assessed the effects of various tissues on cultured dorsal root ganglions from newborn rats. Nucleus pulposus was found to have a toxic effect on the axons by blocking axonal outgrowth, but no similar effects on the nerve cell bodies (extra-ganglionic nerve cell density, nerve cell arborisation) were found as compared to the series with only culture medium. Sterile water for 1 or 24 h (positive controls) induced significant effects by all four criteria, whereas medium without nerve growth factor, fat and frozen

nucleus pulposus had no statistically significant effects. The study thus showed that there are direct axonotoxic effects induced by the nucleus pulposus, and since frozen nucleus pulposus did not have any effects, it may be assumed that the mechanisms are related to substances produced by the nucleus pulposus cells. The presented model allows for future studies on the neurotoxic properties of nucleus pulposus cell-derived candidate substances.

Key words Sciatica · Disc herniation · Cell culture · Nerve cells · Nerve root

Introduction

The biologic activity of nucleus pulposus has recently been documented. Instead of merely being an inert substance that induces mechanical deformation of nerve roots in case of disc herniation, it has been shown to induce various pathophysiologic mechanisms such as structural, functional and vascular alterations in adjacent nerve roots after epidural application [3, 4, 10–12, 18–25]. Although certain cytokines, such as TNF- α , IL-1 β and IFN- γ , produced by the nucleus pulposus cells, seem to initiate these pathophysiologic changes, it is not known whether the ef-

fects are directly neurotoxic, or whether the nerves are affected indirectly by interference with the nutrition or by initiating inflammatory reactions [12, 18, 19]. In the present study, we assessed whether or not there might be direct neurotoxic effects of nucleus pulposus on cultured dorsal root ganglion cells.

Materials and methods

A total of 15 Sprague-Dawley rats, 1–2 days old, were killed, and the spinal canal was opened under sterile conditions with the aid of a surgical microscope. After the spinal cord had been gently re-

Fig. 1 A–C Photomicrographs showing the number of nerve cells outside the ganglion. There is no staining used and the live cells are observed using phase-contrast technique. The magnification is the same in all three figures and the bar in **C** is 500 μm . **A** Dorsal root ganglion from a newborn rat placed in the culture dish. There are no nerve cells observed outside the ganglion. **B** A similar ganglion with few cells growing in the collagen in the culture dish outside of the ganglion. Using this focus and magnification it is not possible to observe the axons or dendrites in the collagen layer. **C** There is a high number of ganglion cells growing outside the ganglion tissue. Similar to **B**, it is not possible to see any axons or dendrites in this photo

moved, the dorsal root ganglia were harvested. Approximately 40 ganglia were obtained from each animal. The ganglia were placed in a collagen layer in collagen-coated culture trays (Calf skin collagen III, Sigma Chemical Co., St Louis, Mo., USA), one

ganglion in each tray. The ganglia were cultured in 1 ml DMEM medium (Life Technologies AB, Täby, Sweden, pH 7.2–7.4), including 10% Fetal Calf Serum (Göteborgs Termometer Fabrik, Gothenburg, Sweden), 0.5% Bovine Serum Albumin (Life Technologies AB), and 10 ng/ml nerve growth factor (NGF 2.5 S, Life Technologies).

The ganglia were incubated for 24 h in a cell culture incubator at 37.0°C and 95% relative humidity (Biocenter 2001, Berman & Beving, Stockholm, Sweden). After this 24 h, the ganglia were assessed microscopically according to four criteria. Of the approximately 600 ganglia that were harvested, 388 had adhered to the collagen-coated tray, resulting in nerve cell growth. The other ganglia were floating freely in the culture medium, and these ganglia were thus omitted from the study. The average outgrowth of all the axons from one ganglion into the collagen layer was estimated by the observer and measured both in absolute terms (μm) and also as a ratio to the ganglion diameter. The number of nerve cells outside the ganglion (cell density) was assessed according to an arbitrary three-point scale, where 0 = no cells observed outside the ganglion (Fig. 1 A), 1 = few cells observed outside the ganglion (Fig. 1 B), and 2 = a high number of cells present outside the ganglion (Fig. 1 C). The number of intercellular connections by dendrites between these free nerve cells (arborisation) was assessed according to an arbitrary three-point scale similar to the scale used for assessing the number of cells. Three unbiased and independent observers performed the assessment.

After the assessments had been performed, the ganglia were exposed to one of the seven following treatments.

1. To form a control group, the culture medium was changed to a new culture medium.
2. The culture medium was changed to culture medium without nerve growth factor.
3. Fifty milligrams of fat taken from the shoulder region of the mother rat was diluted in 10 ml of the culture medium by gently stirring the test tube for 1 h. Each ganglion in this series was cultured in 1 ml of this preparation.
4. Fifty milligrams of nucleus pulposus taken from the mother was diluted in 10 ml of the culture medium in a similar way to fat, and the ganglia were cultured in 1 ml of this preparation.
5. Fifty milligrams of nucleus pulposus from the mother was frozen at -20°C for 24 h before dilution in 10 ml of the culture medium. The ganglia were cultured in 1 ml of this preparation.
6. The culture medium was changed to sterile water for 1 h and then changed back to the culture medium.
7. The culture medium was exchanged for sterile water for the remaining part of the study (24 h).

Twenty-four hours after the first assessment, the ganglia were re-assessed in the same manner as before the treatment was initiated. The ratio between the values for the 48 h and the 24 h assessment for each ganglion preparation and from each observer was calculated. A ratio of 1.0 thus indicated that there was no change between the two recordings and a ratio higher than 1.0 that the value for that specific criterion had increased. All the obtained ratios, from all three observers, were averaged for each specific treatment. The data were assessed statistically by ANOVA (Fisher's PLSD at 5%). The experimental protocol for this experiment was approved by the local animal ethics committee.

Results

The results are presented in Figs. 2–5. Sterile water for 24 h, used as positive control, induced a reduction to 0 in all four measurement criteria, whereas sterile water for 1 h induced a statistically significant, although not com-

Fig. 2 The average outgrowth of axons from the cultured dorsal root ganglions was measured in micrometres. The ganglions were assessed the first time 24 h after initiating the culture, just before being exposed to the various test substances. The second assessment was performed 24 h later, i.e. 48 h after initiating the cultures. The presented data are the ratios between the second and the first recording, averaged for each test series, expressed as average $\pm$ SD. In the culture medium, fat and frozen nucleus pulposus series, the length of the axons increased by a factor of 2.2–2.4. Culture medium without nerve growth factor limited the axonal outgrowth, but the difference was not statistically significant. However, nucleus pulposus and sterile water for 1 h and 24 h significantly reduced the outgrowth compared to the culture medium series ($P < 0.05$). Sterile water application induced lysis of the newly formed extra-ganglionic nerve cell in the culture dish, so the second assessment of cells in the series with sterile water for 1 h was performed on cells formed after this treatment, which were, therefore, only 24 h old. The value 1.2 thus implies an outgrowth similar to the cells that were lysed. However, the value 1.3 in the nucleus pulposus series implies that the average outgrowth during the 24 h when nucleus pulposus was present was only 30% of that during the first 24 h

plete, reduction. Nucleus pulposus induced a reduction in axonal outgrowth, but not in cellular density nor arborisation. Frozen nucleus pulposus and fat did not differ from control (culture medium), and depletion of nerve growth factor in the culture medium induced a slight, but not statistically significant, reduction in axonal outgrowth.

Discussion

The data indicate that nucleus pulposus may impair the outgrowth of axons in culture, but will not affect the

Fig. 3 Since the size varied considerably between the ganglions, there was a suspicion that the outgrowth length might be related to the ganglion diameter. To assess this hypothesis we also measured outgrowth in relation to the ganglion diameter and otherwise proceeded as for the absolute measurements in Fig. 2, also expressed as average $\pm$ SD. Although the outgrowth ratio generally seemed to be slightly higher than in Fig. 2, the relationship between the series was the same as for the absolute measurements in Fig. 2

growth of nerve cells or dendrites. Since frozen nucleus pulposus had no similar effects, one may assume that the effect of nucleus pulposus on the axons was related to substances produced by the nucleus pulposus cells.

Nucleus pulposus has recently been shown in experimental studies to induce structural, functional and vascular changes in adjacent nerve roots when placed epidurally [3, 4, 10–12, 18–23]. Although the cells of the nucleus pulposus have been defined as being intimately related to these effects, the exact mechanisms are not fully understood [12, 19, 24]. It has been suggested that the nucleus pulposus cells may injure the nerve roots either by releasing bioactive substances or by possessing antigens that render them “non-host” to the immune system, and that products from the resulting autoimmune reaction may injure the nerve roots [18]. Neither is it known whether any potentially toxic substances may act directly on the axons and Schwann cells, i.e. direct neurotoxicity, or whether there is a secondary neurotoxicity induced by, for instance, impairment of the blood supply. Recent studies have shown that nucleus pulposus may induce an increase in vascular permeability with an increase in intraneural fluid pressure and subsequent reduction in intraneural

Fig. 4 Compared to the culture medium series, the average number of nerve cell bodies that had established in the culture tray outside the ganglions (cellular density) was similar in the fat and the frozen nucleus pulposus series, whereas there was a slight but not statistically significant increase in the culture medium without nerve growth factor and the nucleus pulposus series, (average $\pm$ SD). However, sterile water for 1 h and 24 h induced statistically significant reductions ($P < 0.05$)

blood flow [3, 4, 25]. However, although secondary effects on nerve structure and function by nutritional deprivation seem to be present, it is not known whether there are also direct effects caused by nucleus pulposus cell-derived substances.

Nerve cell cultures have been in use for 10 years, and they are now standard procedures in experimental nerve research [2, 7, 16, 32, 34]. In the present study we developed a culture system based on previous studies and experience. Dorsal root ganglions from newborn rats were cultured for 24 h and assessed regarding axonal and nerve cell growth. After treatment with various substances, the ganglions were reassessed 24 h after the first assessment. It was then seen that culture medium without nerve growth factor, fat and frozen nucleus pulposus had no effects on either axons or nerve cells. Since there were considerable differences in the size of the ganglions, we choose to measure axonal outgrowth both in absolute terms in micrometres (μ m) and in relation to ganglion diameter. However, although the outgrowth ratios were generally higher when looking at outgrowth related to the ganglion diameter, there were no differences in outcome for the various groups between these two assessments

Fig. 5 The average number of dendrites linking the extra-ganglionic nerve cells (arborisation) was uniform for all series except for the sterile water for 1 h and 24 h, which both induced statistically significant reductions ($P < 0.05$) compared to the culture medium series. In analogy to the comment in the legend to Fig. 2, the second assessment in the sterile water for 1 h series was performed on cells that were only 24 h old, due to lysis of the cells after the first assessment, (average $\pm$ SD). The cells established after the treatment were thus both fewer in number (0.6, Fig. 4) and had less developed arborisation (0.6) compared to the cells established before the treatment

(Figs. 2, 3). The two positive controls, sterile water for 1 and 24 h respectively, both induced reductions in all criteria compared to medium. In fact, sterile water seemed to induce lysis of the nerve cells growing outside the ganglion. When the sterile water was maintained for 24 h, there were no cells observed 48 h after initiating the cultures. However, when the sterile water was changed back to culture medium after 1 h, cells once again began to grow outside the ganglion. However, at the final assessment, these cells were 24 h younger than the cells in the other series, which may account for the reduction in axonal outgrowth and cellular density. The axonal outgrowth ratio was measured as 1.2–1.3, i.e. the outgrowth of the cells established after the sterile water treatment was 20–30% more than for the cells before the water application. Conversely, the number of cells and the degree of arborisation of the cells established after the lysis were only 60% of the number of cells and degree of arborisation established before the sterile water application.

Application of nucleus pulposus to the culture medium induced a reduction in axonal outgrowth but did not seem

to affect the nerve cell bodies, since both cellular density and arborisation were comparable to the other groups. The outgrowth ratio was 1.2–1.3, indicating that there was only a 20–30% increase in length during the 24 h when nucleus pulposus was present in the culture dish compared to before the nucleus pulposus was added. One may therefore suspect that the nucleus pulposus had an “axono-static” effect on the axonal outgrowth, thus almost preserving the length before application. According to previous studies, epidural application of nucleus pulposus induces both axonal and myelin changes, which is in agreement with the data of the present study [18, 23]. By freezing the nucleus pulposus for 24 h, the cellular content is efficiently killed. Since the frozen nucleus pulposus had no effects, one may assume that the effects are related to live nucleus pulposus cells and that the effects are mediated by substances produced by these cells. Nucleus pulposus is similar in its constitution to cartilage, and it is known that axonal outgrowth is inhibited by cartilage-related molecules such as chondroitin sulphate, proteoglycan, agglutinin, chondroitin-6-sulphate and keratan sulfate [6, 17, 29, 30]. However, there are also cytokines related to chondrocytes that may induce an inhibition of axonal outgrowth and regeneration of peripheral nerves as well as being neurotoxic in vivo [5, 8, 14, 19, 26, 33]. Since such cytokines, IL-1 β , IL-6 and TNF- α , may be

found in disc herniation tissue and are known to be present in disc cell cultures, it may seem reasonable that such substances might be responsible for the effects on the cultured nerve cells [9, 19, 31]. However, the role of such cytokines has been debated and there is other evidence showing that they may have no effect on nerve cells in culture as well as evidence that they promote nerve regeneration [1, 15, 28]. Also, in general terms, local inflammation has been considered to promote nerve regeneration [13, 27]. Although the effects on axons and myelin of nucleus pulposus cell-derived substances are not clear from the current literature, we now have an in vitro system in which specific candidate substances may be tested under controlled circumstances.

In summary, the data of the present pilot study indicate that nucleus pulposus has a direct toxic effect on axons but not on nerve cell bodies, and that this effect is related to the nucleus pulposus cells. Although it is not clear which substances may be involved, we have now developed a controlled in vitro model for assessing the effects of candidate substances that may be found in the nucleus pulposus cells and that may be responsible for the observed effects. Such studies may lead to a better understanding of the basic pathophysiologic events behind disc herniation and sciatica, and hopefully to better diagnosis and treatment modalities in the future.

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