# Inhibition of vertebral endplate perfusion results in decreased intervertebral disc intranuclear diffusive transport

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## Abstract

Impaired nutrition of the intervertebral disc has been hypothesized to be one of the causes of disc degeneration. However, no causal relationship between decreased endplate perfusion and limited nutrient transport has been demonstrated to support this pathogenic mechanism. To determine the importance of endplate perfusion on solute diffusion into the nucleus pulposus and to show causality of endplate perfusion on intranuclear diffusion in large animal lumbar intervertebral discs, diffusive transport into ovine lumbar intervertebral discs was evaluated after inhibiting adjacent vertebral endplate perfusion. Partial perfusion blocks were created in vertebrae close and parallel to both endplates of lumbar discs of anaesthetized sheep. To assess diffusivity of small molecules through the endplate, $N_2O$ was introduced into the inhalation gas mixture and concentrations of intranuclear $N_2O$ were measured for 35 min thereafter. Post mortem, procion red was infused through the spinal vasculature and perfusion through the endplate was assessed by quantifying the density of dye-perfused endplate vascular buds in histology sections. Perfusion of the endplates overlying the nucleus pulposus was inhibited by almost 50% in the partially blocked discs relative to the control discs. There was also a nine-fold decreased transport rate of intranuclear $N_2O$ in partially blocked discs compared with control discs. The density of perfused endplate vascular buds correlated significantly to the amount of transported intranuclear $N_2O$ ($r^2 = 0.52$, $P = 0.008$). The vertebral endplate was demonstrated to be the main route of intravascular solute transport into the nucleus pulposus of intervertebral discs, and inhibition of endplate perfusion can cause inhibited solute transport into the disc intranuclear tissue.
**Key words** diffusion; endplate; intervertebral disc; perfusion; sheep.

## Introduction

Low back pain is one of the major health problems in western society. About 85% of people at the age of 50 recall an episode of low back pain (Bigos et al. 1991). The costs are estimated to be $20 billion yearly in the USA alone (Cats-Baril & Frymoyer, 1991). In the Netherlands the direct and indirect costs in 1991 were estimated to be 1.7% of the gross national product (van Tulder et al. 1995). Low back pain is a multifactorial problem, which includes intervertebral disc degeneration (Adams, 2004). Besides other factors such as loading (Adams, 2004), heredity (Battie et al. 2004) and aging, it is believed that limited nutrition of the disc can be an aetiological factor in intervertebral disc degeneration.

The intervertebral disc is the largest avascular structure in the human body. Because of its avascularity, the cells inside the disc depend on diffusion for the transport of nutrients and waste products. Small capillary buds with a diameter of about 20–50 μm run through the pores of the bony endplate (Oki et al. 1994). Nutrients diffuse from these vascular buds and the small blood vessels in the outer part of the outer annulus to all cells inside the disc. Less permeable endplates could thus result in nutrient deficiency and very likely in cell death and intervertebral disc degeneration.

Almost 40 years ago, Nachemson et al. (1970) found a significant association between permeability of the endplate and intervertebral disc degeneration. In addition, Benneker et al. (2005) found a significant correlation between the amount of pores in the endplate and disc degeneration in a human cadaver study. Discs of grade 4 degeneration on the Thompson scale (Thompson et al. 1990) had about 50% fewer pores of 20–50 μm (corresponding in size to the capillary bud openings (Oki et al. 1994)) than discs of grade 1.

Magnetic resonance imaging has been used extensively to study diffusion into the disc. With the help of a contrast fluid, diffusion into the disc can be imaged and quantified.

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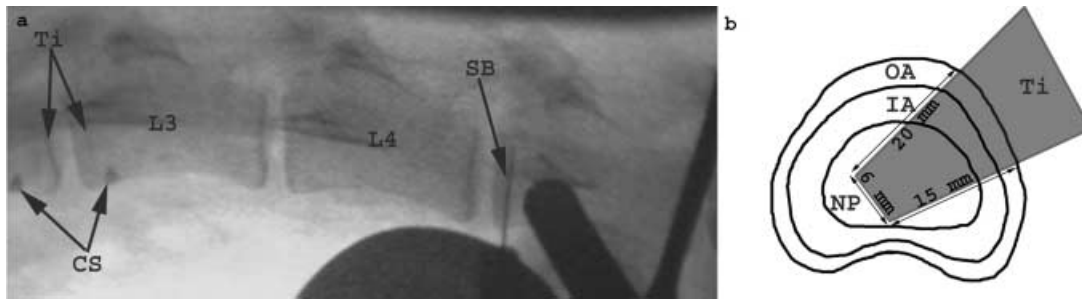*Dr K. Ito, AO Research Institute, Davos, Switzerland.*
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**Fig. 1** (a) Fluoroscope image during the surgery. The defects at the L2–L3 level have already been made. The saw blade is cutting the defect in the L5 vertebrae. (b) A schematic of the titanium foil overlaying the nucleus pulposus. Ti = titanium foil, CS = cortical screw, SB = saw blade, OA = outer annulus, IA = inner annulus, NP = nucleus pulposus.

Most of these studies focused on the effect of maturation (Ibrahim et al. 1995) and degeneration (Nguyen-minh et al. 1997, 1998) on diffusion. But one study focused on the diffusion pathway and showed that diffusion of the contrast fluid into the nucleus pulposus derives mostly from the subchondral bone through the endplate (Rajasekaran et al. 2004).

Ogata & Whiteside (1981) showed that blockage of this endplate route had great effects on diffusion in a dog model. They disrupted the blood supply to the endplate by osteomization of the adjacent vertebrae close to the bone–disc interface and putting a metal plate into this slot. They found that this endplate blockage had a greater effect on the diffusion than disrupting the blood vessels over the annulus fibrosus, indicating that the endplate route is the most important.

So far, it is clear that nutrients reach the nucleus pulposus of the disc through diffusion, and that the endplate route is the most important route. However, the mechanistic relationship between endplate perfusion and diffusion remains unclear. Therefore, the goal of this study is to demonstrate the relationship between a decrease in functional capillary buds in the endplate and diffusion inhibition into the nucleus pulposus of intervertebral discs.

## Materials and methods

### Animals

The animal experiments were approved by the Animal Experimentation Commission of the Veterinary Office of the Canton of Grison, Switzerland, and followed the guidelines of the Swiss Federal Veterinary Office for the use and care of laboratory animals. Four Swiss Alpine sheep between 5 and 7 years old and approximately 50 kg body weight were sedated and anaesthetized with an inhalation mixture of oxygen and 2–3% isoflurane (Baxter, Volketswil, Switzerland). At the end of the experiment they were euthanized with an overdose of pentobarbital (100 mg kg$^{-1}$, i.v. Pentobarbitalum natricum, Streuli & Co. AG, Uznach, Switzerland).

### Surgical preparation

After the L1–L6 vertebrae were identified with a fluoroscope, the lateral aspect of the L2–L5 discs were exposed through a retro-peritoneal approach. A slot, approximately 2 cm deep and 1 cm wide and 0.6 mm thick was sawn in the vertebrae close and parallel to both endplates of the L2–L3 and L4–L5 intervertebral discs under fluoroscopic guidance (Fig. 1a). The slot was started on the antero-lateral aspect of the vertebral body approximately 1 mm from the edge of the endplate. The slot was oriented toward the opposite postero-lateral quadrant of the endplate to cover the central portion of the nucleus pulposus (Fig. 1b). A CP-titanium-foil (Allegheny Rodney Metals, Sprockhövel, Germany), approximately 25 mm long and 0.050 mm thick, (Fig. 1b) was inserted into the slot and the part extending from the slot was folded toward the bone and secured to the vertebrae with a cortical screw (1.5 × 6 mm, Synthes Inc., Oberdorf, Switzerland).

### Diffusion

Custom-made electrodes (Barron et al. 1997) were inserted into the centre of the nucleus pulposus of the L1–L2, L2–L3, L4–L5 and L5–L6 discs with fluoroscope guidance. On some occasions the L5–L6 disc could not be approached because the length of the needle did not allow clearance of the iliac crest, in which case diffusion measurements were taken in the L3–L4 disc. A reference electrode was inserted into the paraspinal musculature. Anaesthesia gas was then switched from $O_2$ with 2–3% isoflurane to a mixture of 30%/70% $O_2$/$N_2O$ with isoflurane, and amperometrical measurement of intranuclear concentrations of $N_2O$ (Urban et al. 2001) were started ($t_0$). The measurements were done in triplicate and carried out every 5 min for 35 min.

The working electrodes used for the diffusion measurements were constructed with two silver wires (diameter = 0.125 mm with a 0.0125-mm coating) inserted into stainless steel tubes (outer diameter = 1.1 mm) separated by an insulating polymer. A silver/silver chloride electrode saturated with potassium chloride was used as combined

reference and counter electrode. A multichannel potentiostat (Perkin Elmer Instruments VMP, Ametek, Illingen, Germany) connected to a laptop computer with complementary software was used for measurements. A potential from 0 to –1.5 V (at a rate of –40 mV/s) was applied and the current was measured at a frequency of 50 Hz. At –0.6 V and –1.2 V a plateau in the current-potential curve was observed. The first plateau was caused by the presence of $O_2$, and the second by the presence of $N_2O$ (Urban et al. 2001). At these plateaux the current was averaged over five measured points. The calibration curves for each electrode were constructed afterward by measuring the currents in phosphate-buffered saline saturated with known gas concentrations ($N_2/O_2$ or $N_2O/O_2$ mixtures).

### Perfusion

Five minutes after 25 000 IU of heparin (Fresenius Medical Care, Stans, Switzerland) were injected intravenously, the animal was euthanized. Post-mortem, femoral arteries were ligated and the abdominal aorta was cannulated as close to the diaphragm as possible in the abdominal cavity. The renal arteries as well as the thoracic aorta were ligated. Then, 50 000 IU of heparin in 1 L of normal saline was injected through the cannulated abdominal aorta at 100 mmHg to evacuate vasculature in the lumbar vertebrae. The vertebral vasculature was then infused with 800–1000 mL of 5% (w/w) procion red (BASF, H8BN) in water at 100 mmHg.

The lumbar spines were dissected into bone-disc-bone segments, snap frozen in liquid $N_2$, freeze substituted in acetone to retain the water-soluble procion red in the specimens (Steinbrecht & Müller, 1987), and embedded in methylmethacrylate. Freeze substitution was done in four steps of a minimum of 2 days each at –80 °C, –20 °C, 4 °C and room temperature, before embedding in methylmethacrylate. Sections of 200 μm were cut with a rotating blade diamond saw (Leitz 1600, Leica AG, Glattbrugg, Switzerland). From each bone-disc-bone segment the three most mid-sagittal sections overlying the nucleus pulposus were ground and polished on a micro grinding system (Typ AW-10, Exakt Apparatebau, Norderstedt, Germany) to approximately 100 μm thickness. Images, centred over the nucleus pulposus, were recorded on a fluorescent microscope (Zeiss Axioplan 2, Carl Zeiss AG, Feldbach, Switzerland) with three-band excitation (Zeiss filter set #25: exciter filter TBP 400/495/570, beam splitter FT 410/505/ 584, barrier filter TBP 460/530/610). Quantification of the density of patent endplate capillary buds was done semiautomatically with an image analysis program (AXIOVISION, Carl Zeiss AG) and custom macro (EXCEL, Microsoft, Microsoft, Redmond, WA, USA). Because of autofluorescence of the bone, the boundary between the bony and avascular cartilaginous endplates could be traced by hand. The area within 60 μm of the traced line over the bony endplate,

extending between the inner annulus/nucleus pulposus borders, was set as the region of interest (ROI). Within this ROI the pixels were then thresholded for red values such that capillary buds were distinguished from variable amount of dye leakage from the vessel (threshold based initially on histogram and adjusted manually on each image). Based on the findings of Benneker et al. (2005) islands of threshold pixels larger than 15 $μm^2$ were counted automatically.

### Statistics

For each disc, the $N_2O$ concentrations per time point were normalized to the $t_0$ level. This was done because not all measurements could be taken simultaneously, resulting in delay from change in anaesthesia gas mixture to initial measurement for some probes. The intervals between measurements were the same for all probes. Per time point, the triplicate values were averaged and then pooled into control and blocked discs.

The number of perfused vascular buds per mm of endplate was averaged per section (proximal and distal adjacent endplate) and pooled into control or blocked discs. Differences in diffusion between the control and blocked discs were tested with analysis of variance (ANOVA) for repeated measures. Differences in perfusion between control and blocked discs were tested for significance using the independent Student $t$-test (SPSS v 13, SPSS AG, Zurich, Switzerland). A $P$-value lower than 0.05 was considered significant.

## Results

### Surgical procedure

No major complications occurred during the operations. In one animal, a caudal lumbar segmental artery was damaged during the approach. In this sheep, the L4–L5 disc was excluded from the analysis ($n = 7$ partially blocked discs and $n = 8$ control discs for diffusion data analysis). In two sheep the procion red infusion did not reach the most cranial vertebra of the lumbar spine, and in these levels no perfusion analysis could be performed ($n = 21$ partially blocked sections and $n = 18$ control sections for perfusion data analysis).

### Diffusion

A steady increase in $N_2O$ concentration was measured in the control discs over time, whereas in the blocked discs this increase was less pronounced. The increase of $N_2O$ concentration in 35 min was on average 360% compared to the $t_0$ level for control discs. In the experimental discs this increase was only 41%. The difference in increase between the control and blocked discs was significant ($P = 0.005$,
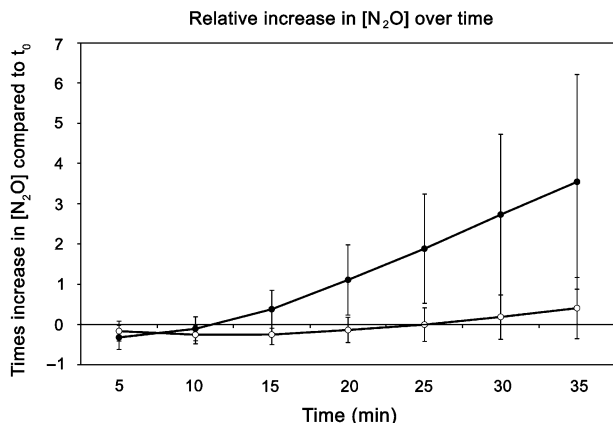
**Fig. 2** The increase in nuclear nitrous oxide concentration over time after the inhalation gas has been changed from 98% oxygen to 30% $O_2$ and 70% $N_2O$ ($t_0$). In the blocked discs this increase is significantly lower than in the control discs. The values have been normalized to $t_0$. The increases are significantly different between the control and blocked discs ($P = 0.005$, $n = 8$ controls, and $n = 7$ partially blocked).



**Fig. 4** Correlation between perfusion and diffusion measurements. The more vascular buds were perfused, the more $N_2O$ diffused into the disc at 35 min, after changing the inhalation gas mixture. Data points represent the average perfusion and diffusion of the 12 discs analysed both for diffusion and perfusion, $r^2 = 0.5229$, $P = 0.008$.

Fig. 2), but there were no significant differences between the disc levels.

## Perfusion

The histological sections clearly exhibited the procion red-infused capillary buds in the bony endplates (white arrows in Fig. 3).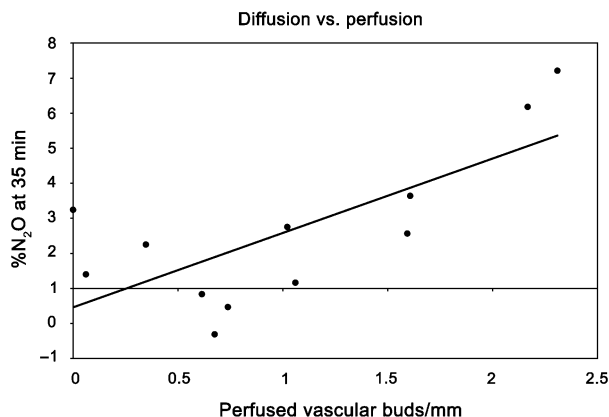 The difference between the blocked and normal discs was very clear. The perfusion block left many vascular buds unperfused (red arrows in Fig. 3). Over the nuclear region, in the control discs, 1.52 ± 0.99 vascular buds per mm were perfused, whereas in the blocked discs, this was only 0.78 ± 0.58 ($P = 0.006$). There was a weak ($R^2 = 0.52$) but significant ($P = 0.008$) correlation between the amount of perfused vascular buds and the [$N_2O$] diffused into the disc at 35 min (Fig. 4).
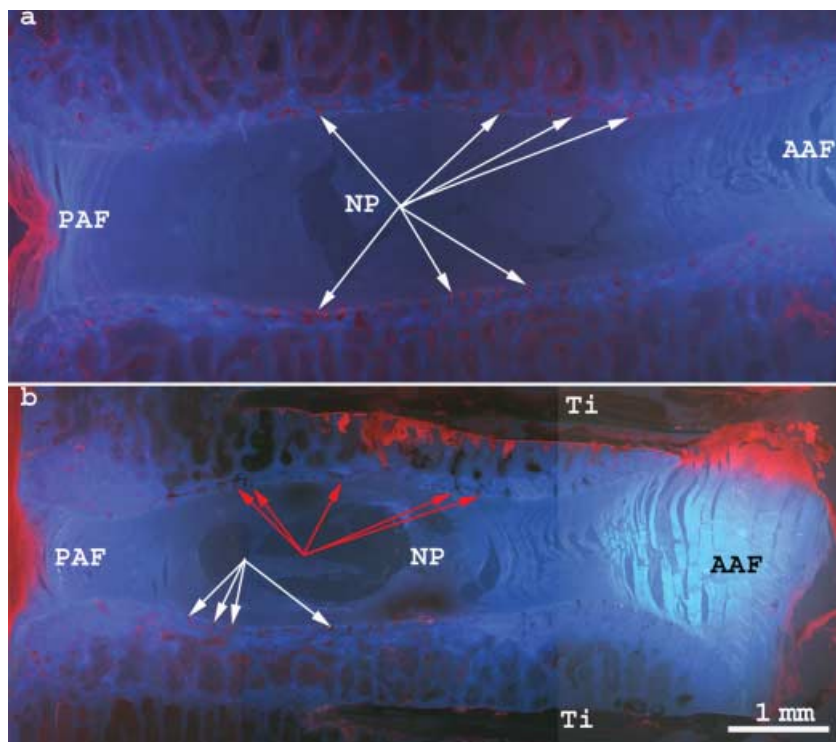


**Fig. 3** Procion red-filled endplate vascular buds (white arrows) and non-filled buds (red arrows). AAF anterior annulus fibrosus, PAF = posterior annulus fibrosus, NP = nucleus pulposus, Ti = titanium foil. (a) Control, (b) experimental.

## Discussion

In this study the foil only covers about 30% of the disc surface, overlying the nucleus pulposus. Despite this small area of coverage, but because of its central location, endplate perfusion adjacent to the nucleus was decreased by almost 50% on average. The central part of the endplate is more permeable than the edge (Nachemson et al. 1970; Oki et al. 1996). Oki et al. (1996) suggest that this difference in permeability lies in the different structure of the vascular buds in the nucleus region of the endplate, where they are complex coils, compared with the region over the inner annulus, where they are single loops.

$N_2O$ was used as a tracer for small solutes instead of measuring glucose directly. Because $N_2O$ can be measured amperometrically and does not occur in the body naturally, the efficacy of the perfusion block could be measured. This would not be possible with glucose as all discs would have been saturated before the block would have been created. It is assumed that the inhibition of diffusion for $N_2O$ (mw 44) would be similar for other small nutrients such as glucose (mw 180) and oxygen (mw 32) as all three molecules are similar in size and have no charge. The technique used in this study for post mortem infusion of procion red may seem cumbersome, but it enabled us to infuse a large amount of the dye which would not be possible *in vivo*, hereby increasing the signal-to-noise ratio in the images.

For diffusion, the distance from the nearest blood supply to the cells is of great importance. For this reason a sheep model was chosen. The geometry of the sheep lumbar spine is comparable to that of humans and the discs are sufficiently large (Wilke et al. 1997a,b). Furthermore, the sheep has been generally accepted as a model in spinal research.

Diffusion was only measured for a very short time (35 min), therefore the measurements do not necessarily comply with steady state conditions. As can be seen in Fig. 3, the procion red stains the outer annulus quite intensively. This was also seen in a study by Brodin (1955) and is probably because of the small arteries in the outer part of the annulus fibrosus. One could argue that diffusion through the annulus could play a much bigger role under steady state conditions than in such a short-term study. However, literature on normal discs does not support this. Rajasekaran et al. (2004) have shown that 24 h after injection of contrast fluid the diffusion followed the pattern of the endplates, and no clear diffusion though the annulus was observed. An analytical study by Ferguson et al. (2004) also shows that after 24 h small nutrients do not diffuse though the annulus fibrosus into the nucleus pulposus. Furthermore, an *in vitro* study in which sheep intervertebral discs were cultured with endplates under static or diurnal loading showed that even after 4 days of culture with TMR-dextran (a fluorescent dye), the dye intensity dropped off steeply from the outer annulus into the inner annulus (Gantenbein et al. 2006). So, even with a blockage of the endplate the gradient from the inner annulus to the centre of the disc would be very small, which is not favourable for diffusion.

In the case of endplate blockage, fewer nutrients can diffuse from the capillary buds to the cells inside the disc (Roberts et al. 1996). Vice versa, waste products cannot be disposed of, resulting in a build up of lactic acid, thus lowering the pH in the centre of the disc. *In vitro* studies by Horner & Urban (2001) and Bibby et al. (2005) showed that cell viability and metabolism depend on the cell density, glucose, oxygen concentrations and pH. At high cell densities, the viable distance (distance from diffusion source and cell viability level of 95%) was much lower than under low cell densities. Under low oxygen conditions the cells survive up to 12 days but do not produce much proteoglycan; for that, oxygen is required (Horner & Urban, 2001). Under low glucose conditions or at a pH of 6 the cells do not survive for longer periods (Bibby et al. 2005). This supports the hypothesis that nutrient deficiency could result in intervertebral disc degeneration; however, *in vivo* studies should be done to test this.

This animal model is being developed to be used in investigating the effect of nutrient deprivation on cellular responses. To be able to exclude the effect of changes in the mechanics of the disc by creating such defects, this was tested. In previously frozen cadaver sheep lumbar motion segments the intranuclear pressure was measured before and after defect creation. Measurements were done with a needle pressure transducer described by Adams et al. (1996). There was no significant effect on intranuclear pressure.

Like the study by Ogata & Whiteside (1981) this study shows that a blockage of the endplates results in an inhibition of diffusion of small solutes into the nucleus pulposus. This indicates that this is the most important route for solute transport to the nucleus of the disc. The mechanism through which this happens is demonstrated by the correlation between the functional capillary buds and the diffusion. By creating such a partial perfusion block of the endplate, cellular responses to nutrient insufficiency can be investigated *in vivo* in longer term studies.

## Acknowledgements

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