The effects of zoledronic acid and hyperbaric oxygen on posterior lumbar fusion in a rabbit model

We studied the effects of hyperbaric oxygen (HBO) and zoledronic acid (ZA) on posterior lumbar fusion using a validated animal model. A total of 40 New Zealand white rabbits underwent posterior lumbar fusion at L5-6 with autogenous iliac bone grafting. They were divided randomly into four groups as follows: group 1, control; group 2, HBO (2.4 atm for two hours daily); group 3, local ZA (20 μg of ZA mixed with bone graft); and group 4, combined HBO and local ZA. All the animals were killed six weeks after surgery and the fusion segments were subjected to radiological analysis, manual palpation, biomechanical testing and histological examination.

Five rabbits died within two weeks of operation. Thus, 35 rabbits (eight in group 1 and nine in groups 2, 3 and 4) completed the study. The rates of fusion in groups 3 and 4 (p = 0.015) were higher than in group 1 (p < 0.001) in terms of radiological analysis and in group 4 was higher than in group 1 with regard to manual palpation (p = 0.015). We found a statistically significant difference in the biomechanical analysis between groups 1 and 4 (p = 0.024). Histological examination also showed a statistically significant difference between groups 1 and 4 (p = 0.036).

Our results suggest that local ZA combined with HBO may improve the success rate in posterior lumbar spinal fusion.

A successful spinal fusion involves a combination of biological and mechanical factors. The former include removal of arthritic cartilage, decortication and grafting. The latter relate to the instrumentation involved, which makes fusion easier, but cannot achieve it without biological influence. The incidence of pseudarthrosis is reported to be as high as 56% and a reduced rate of fusion is associated with age, inadequate rehabilitation, smoking, disorders which include osteoporosis, neurofibromatosis and spondyloepiphyseal dysplasia, and surgical factors such as the operative technique and complications.

Bisphosphonates are anti-catabolic agents which help to reduce the risk of fracture. The effects of second- and third-generation bisphosphonates on the repair of fractures have been reported in animal studies and, in high doses, they increase the volume of callus, the mineral content and the power against loading in distraction osteogenesis. Zoledronic acid (ZA) has been shown to have a positive effect on lumbar fusion in a rabbit model and it was suggested that this may be enhanced by anabolic treatment.

It has been reported that hyperbaric oxygen (HBO), applied at 2.5 atm for two hours a day, increases bone formation by causing callilage hypertrophy. In another study, it improved bone formation in the distraction zone of a rabbit model of tibial lengthening to the extent that 22% more force was needed to unscrew the titanium implants. It was also shown in a model of lumbar fusion that HBO improved the rate of fusion by increasing bone formation.

Therefore, our aim in this study was to evaluate the effects of third-generation ZA and HBO using a lumbar fusion model in rabbits.
Materials and Methods
This study had ethical approval. After clinical and laboratory examination, New Zealand white rabbits underwent prophylactic treatment for prevention of diarrhoea with coccidin for a week in which 0.5 ml of coccidin was mixed in their drinking water. They were left in their cages to adapt to the environment for ten days, after which 40 male animals with a mean weight of 2506 g (2060 g to 3050 g) and a mean age of 4.8 months (4.0 to 6.0) were selected. They were divided randomly into four groups of ten. Group 1, a control group, underwent bilateral posterior lumbar fusion of L5-6 with autogenous corticocancellous iliac grafting. Group 2 had similar bilateral posterior fusion, with administration of HBO and autogenous corticocancellous iliac grafting. Group 3 had bilateral posterior fusion, administration of local ZA and autogenous corticocancellous grafting and group 4 had bilateral posterior fusion, corticocancellous grafting and administration of both local ZA and HBO.

The animals were housed individually in standard wire cages which were cleaned daily. The experiment was carried out in an airy room and the rabbits were subjected to a daily 12-hour-light-12-hour-dark cycle. Room temperature was 19° (SD 1) and humidity 55% (SD 10) and it was ensured that the animals were comfortable and stress-free. They were given 160 g of pellet bait and between 300 g and 350 g of tap water daily. They were killed at the end of the sixth week post-operatively.

Operative technique. The lumbar areas were shaved ten minutes before surgery. A single dose (75 mg/kg) of cefazolin sodium (Cefozin; Science Medicine Industry, Istanbul, Turkey) was injected intravenously 30 minutes pre-operatively and prophylaxis was continued for 72 hours. General anaesthesia was induced using a 5% solution of Ketamine HCl (35 mg/kg) (Ketalor licensed from Parke Davis; Eczacibasi Medicine Industry, Istanbul, Turkey) and maintained with xylazine HCL (Rompun; Bayer Corporation, Leverkusen, Germany) and Ketamine HCl (0.3 mg/kg intramuscularly). Carprofen (4 mg/kg) (Rimadyl; Pfizer Inc., Zaventem, Belgium) was given for three days post-operatively.

The level of L5-6 was only identified radiologically in the first five rabbits using a Kirschner wire. The method was not used afterwards. The animals were placed prone and, through a midline vertical incision, two straight incisions were made at the edges of the lumbar fascia by palpating the L5-6 spinous processes and facet joints. The facet joints and transverse processes were decorticated and the posterior iliac crests identified through the same incision. Iliac corticocancellous grafts were placed over the decorticated areas. For the rabbits in groups 3 and 4, 20 ?g of ZA (Zolenat; Mustafa Nevzat Medicine Industry, Istanbul, Turkey) was applied locally to the site of the fusion. The fascia was closed with 4/0 polyglycolic acid (Atramat, Mexico City, Mexico) and the skin closed with 4/0 polypropilen (Atramat). All the operations were performed by the same investigator (NY).

Administration of HBO. From the first post-operative day groups 2 and 4 received HBO for three weeks in a single-lock hyperbaric chamber (Hipertech Neoks Arbe Chamber). Each rabbit had 2.4 atm pressure at a treatment depth of 50 feet for 110 minutes daily including diving and surfacing.

Radiological analysis. Before the animals were killed posteroanterior and lateral lumbar radiographs were taken at a standard 20 cm from the table. They were evaluated by two observers (YÖ, NŞ) in a blind fashion by two methods. The first was that described by Bransford et al23 which has proved to be effective (Table I) and the second method used the classification system of Lenke et al28 (Table II).

Manual palpation. After the animals had been killed, the integrity of the fusion areas was tested manually for visible movement, avoiding undue force by two investigators (NY, AO). Each movement segment was graded as fused (no apparent movement) or not fused (movement present).

Biomechanical evaluation. Before the biomechanical evaluation, the L5-6 areas were separated from the neighbouring levels with a scalpel. The ligamentous structures were removed and each specimen was fixed using a Raku-Tool (Modelform Chemical Products Industry, Istanbul, Turkey) in square metal frames aligned in the cranial and caudal directions. The length of the test area in the peripheral
frame was adjusted to 2.6 cm for all specimens. In order to avoid stress rise, no screws or wires were used. An external ring was attached to the metal frame in a cranial direction after which all the test areas were fixed in the specially designed apparatus in a caudal direction. A flexion/extension force was applied to all the test areas in a materials testing machine (Zwick Roell 1475, Leominster, United Kingdom). A load cell was fixed through the metal frame in the cranial direction. A maximum non-damaging mobilisation range was defined as displacement of 3.7 mm in two minutes. Six fatigue tests were applied to each specimen and the values in the last test were recorded. The neutral zone (NZ, mm/N) and stiffness values (N/mm) were calculated from load-displacement curves. Stiffness was defined as the slope of the line fitting the load-displacement curve from -3.7 mm to -2.47 mm on flexion. The neutral zone was measured by fitting a straight line to the load-displacement curve from -1.24 mm to +1.24 mm, and was defined as the distance along this line required to produce a load of 1 N. All the lines used for measuring stiffness and neutral zone were calculated using the least-squares method. There was a reverse relationship between neutral zone and stability in which a lower neutral zone gave more stability.3,29

<table>
<thead>
<tr>
<th>Points</th>
<th>Histological grading according to classification of Emery et al⁹⁰</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Bone tissue only</td>
</tr>
<tr>
<td>6</td>
<td>Bone tissue, more than fibrocartilage tissue</td>
</tr>
<tr>
<td>5</td>
<td>Fibrocartilage tissue, more than bony tissue</td>
</tr>
<tr>
<td>4</td>
<td>Fibrocartilage tissue only</td>
</tr>
<tr>
<td>3</td>
<td>Fibrocartilage tissue more than fibrosis tissue</td>
</tr>
<tr>
<td>2</td>
<td>Fibrosis tissue more than fibrocartilage tissue</td>
</tr>
<tr>
<td>1</td>
<td>Fibrosis tissue only</td>
</tr>
<tr>
<td>0</td>
<td>Empty islets</td>
</tr>
</tbody>
</table>

Histopathological examination. The tissues were soaked in 10% formaldehyde for 24 hours and then decalcified in 10% formic acid indoors for ten days. The decalcification solution was changed every three days. Specimens were dehydrated in ethanol series, cleaned with xylene and embedded in paraffin. Longitudinal sections of 5 μm were made with a microtome and the tissues stained with haematoxylin and eosin and Toluidine Blue. All were evaluated by an experienced histopathologist (AE) using an Olympus CX21 (Tokyo, Japan) light microscope and photomicrographs were taken. Histopathological evaluation was made according to the criteria described by Emery et al.³⁰ The data were evaluated using fusion scores of between 0 and 7 and classifying them into three sections (Table III).

Statistical analysis. The Kruskal-Wallis test was used as a non-parametric test in the analysis of data on stiffness, the neutral zone, and the histopathological findings. If there was a significant difference between the groups, Dunn’s multiple comparisons test was used for intergroup comparison to determine which group caused that difference. The data from the Lenke criteria, the radiological evaluation and manual palpation were designed to be evaluated as a 2 x 2 design. The analysis of categorical data taken from the Lenke criteria, the radiological evaluation and manual palpation was done using Fisher’s exact test. The median, variance (minimum to maximum) and interquartile range values were used as descriptive values. The level of statistical significance was set as p < 0.05 and statistical analysis was done with SPSS version 16.0 (SPSS Inc., Chicago, Illinois) and GraphPad InStat 3 (Graphpad Software, San Diego, California).

Results
During the study, five rabbits, two from the control group and one from each of the other groups died because of diarrhoea and were excluded, leaving 35 rabbits available for evaluation.

Radiological. The fusions on the right and left sides were evaluated separately according to the classification of Bransford et al.²³ The data were categorised as degree 1 to 2 and degree 3 to make the 2 x 2 design. Regarding the right side, there were more fusions in group 4 than in groups 1 and 2 (Fisher’s exact test p = 0.009, odds ratio (OR) 3, 95% confidence interval (CI) 1.191 to 7.558; Fisher’s exact test p = 0.05, OR 16, 95% CI 1.315 to 194.623). There was no significant difference in the rate of fusion in the other groups. On the left side there were more fusions in group 4 than in groups 1 and 2 (Fisher’s exact test p = 0.015, OR 3, 95% CI 1.785 to 24.5; Fisher’s exact test p = 0.015, OR 28, 95% CI 2.067 to 379.247). There was no statistically significant difference in the rate of fusion in the other groups. On the left side there were more fusions in group 4 than in groups 1 and 2 (Fisher’s exact test p = 0.015, OR 3, 95% CI 1.785 to 24.5; Fisher’s exact test p = 0.015, OR 28, 95% CI 2.067 to 379.247). There was no statistically significant difference in the rate of fusion in the other groups. On the left side there were more fusions in group 4 than in groups 1 and 2 (Fisher’s exact test p = 0.015, OR 3, 95% CI 1.785 to 24.5; Fisher’s exact test p = 0.015, OR 28, 95% CI 2.067 to 379.247). There was no statistically significant difference in the rate of fusion in the other groups. On the left side there were more fusions in group 4 than in groups 1 and 2 (Fisher’s exact test p = 0.015, OR 3, 95% CI 1.785 to 24.5; Fisher’s exact test p = 0.015, OR 28, 95% CI 2.067 to 379.247). There was no statistically significant difference in the rate of fusion in the other groups. On the left side there were more fusions in group 4 than in groups 1 and 2 (Fisher’s exact test p = 0.015, OR 3, 95% CI 1.785 to 24.5; Fisher’s exact test p = 0.015, OR 28, 95% CI 2.067 to 379.247). There was no statistically significant difference in the rate of fusion in the other groups. On the left side there were more fusions in group 4 than in groups 1 and 2 (Fisher’s exact test p = 0.015, OR 3, 95% CI 1.785 to 24.5; Fisher’s exact test p = 0.015, OR 28, 95% CI 2.067 to 379.247).
(Fisher’s exact test $p = 0.015$, OR 24; 95% CI 1.741 to 330.804). No statistically significant difference was detected in the other groups.

**Biomechanical testing.** Although no significant difference was detected in the groups in terms of stiffness, the values increased from group 1 to a maximum in group 4. According to the evaluation of neutral zone, values in group 4 were higher and therefore more stable than in group 1 (Dunn’s multiple comparison test $p = 0.024$, 95% CI 0.17 to 0.45). No statistically significant difference was found in the neutral zone values in the other groups (Table V).

**Histopathological.** According to the criteria of Emery et al., higher histopathological values were found in group 4 than in group 1 (Dunn’s multiple comparison test $p = 0.036$, 95% CI 6.04 to 6.85). No statistically significant difference was detected in the other groups (Table V, Figs 5 to 8).

**Discussion**

These results indicate that the addition of HBO to single-dose local ZA gives enhanced stability and improves the rate of fusion. When used separately, ZA and HBO did not show any biomechanical differences. However, the rate of fusion with ZA was greater than that with HBO.
ZA may cause this by inhibition of protein prenylation, stimulation of osteoclast apoptosis, suppression of mature osteoclast function, reduction of cytokine production and its anti-tumour and anti-angiogenic properties.\textsuperscript{31-37} ZA inhibits the maturation and gathering of osteoclasts on the bone surface\textsuperscript{31} and suppresses the functional effectiveness of mature osteoclasts. These effects are 850 times greater than those with pamidronate.\textsuperscript{31} In their \textit{in vivo} study, Pataki et al\textsuperscript{38} compared treatment of growing rats with 2.8 \(\mu\)g/kg ZA for ten days with 370 \(\mu\)g/kg of pamidronate and found that ZA significantly raised calcium and hydroxyproline levels and radiological density in trabecular bone, without affecting cortical bone. In other studies it has been shown that nitrogenous (N-containing) bisphosphonates inhibits catabolism in bones and increases the formation of callus in fractures and distraction osteogenesis.\textsuperscript{21,22,39,40} It was also reported that in ovariectomised rats, a weekly subcutaneous ZA injection of 1.5 \(\mu\)g/kg protected the density and strength of lumbar vertebrae.\textsuperscript{41} In another study on monkeys, it was shown that long-term treatment with ZA was tolerated well and that, based on dose, it suppressed bone loss without damaging mineralisation.\textsuperscript{42,43}

It was reported that spinal fusion was delayed and bone maturation inhibited the daily administration of alendronate in rabbit fusion models.\textsuperscript{14} Although increased rates of fusion were seen with both high and low doses of alendronate, the rates of fusion were lower in the high-dose group, but this finding was not statistically significant.\textsuperscript{43}
Delayed fusion was reported in a rabbit lumbar fusion model in which pamidronate was used continuously in a high dose. Because different dose-dependent results were reported in these studies in which bisphosphonates were used continuously, the use of single-dose ZA was suggested. In our study, we used a single dose of 20 mg of ZA as suggested by Bransford et al., in order to avoid the inhibitory effects caused by dose-dependence and prolonged treatment. In the rabbit model of Bransford et al., using single-dose ZA, a greater rate of fusion (60%) was detected compared with a control group (25%) in which ZA was not used. Furthermore, they reported that single-dose local ZA slowed the remodelling of the fusion mass temporarily and that there might not be a sufficient rate of fusion without anabolic stimulus. Our study showed better radiological results in the group in which autogenous iliac bone grafting and ZA had been used than in the group in which only autogenous iliac bone grafting alone was used. This supports the findings of others.

For example, in a rat model of femoral defects which studied the combined effect of ZA and osteoprotegerin-1 (an osteoinductive growing factor), these agents together produced better bone mineral density and strength in callus than in other groups. In our study, we combined the anabolic effects of HBO with ZA. In men, the former has two basic effects. The first is mechanical which is useful in the reduction of the size of air bubbles caused by diving accidents or air emboli. The second is the effect of the increased partial pressure of oxygen on all tissues, resulting in increased neovascularisation, antimicrobial activity, vasoconstriction and the reperfusion rate. HBO has been known for a long time to be effective in wound healing. Some studies underlined that its most important effect was the increase of nutrition and oxygenation in repaired tissues, with oxygen under pressure acting as a healing mechanism in chronic hypovascular tissues. Sawai et al. demonstrated the effectiveness of HBO and autogenous iliac bone grafting in defects in the rabbit mandible and a study of the effect of HBO in a rabbit model of lumbar fusion showed that HBO increased fusion in the fourth and eighth weeks. However, fusion zones were found in seven of the 12 control rabbits compared with ten of the 12 HBO rabbits, this favouring the latter. In our study, although there was no biomechanical or radiological difference between the control and HBO groups, the results were relatively better in the latter. Also, the rates of fusion were better on biomechanical, radiological and histopathological analysis in the ZA-HBO group compared with the control group. Although no statistically significant difference was found between the ZA and ZA-HBO groups, there were more meaningful values in the biomechanical and radiological analyses in the ZA-HBO group when it was compared with the control group. The radiological fusion results were better in the ZA-HBO group and it was considered that this combination might be a reasonable treatment option in patients.

At the end of the sixth week, new bone formation was demonstrable histologically in every group. Fibrous tissue was not detected although rarely, there was fibrocortilage. There was also a greater rate of fusion histologically in the ZA-HBO group than in the other groups. No histological examination was performed in the only other published report of the effects of single-dose ZA in a rabbit lumbar fusion model. In a similar model, Lehman et al. administered 0.005 mg/kg of alendronate sodium daily for eight weeks and found a lower rate of fusion on histological analysis in the alendronate group compared with a control group. Their explanation for this was that bisphosphonates decreased the secondary effects of osteoblasts and the formation of new bone and that high dosage of alendronate.
sodium reduced the rate of fusion. In their rabbit model, Bezer et al. applied instrumentation with Kirschner wires to one group and with polylactide rods in another. On histological examination, they detected bony fusion in all the rabbits at the end of the 12th week and reported no statistically significant difference in the groups. There were similar rates of fusion in all the groups on histological analysis; but rate of bony fusion was higher in the ZA-HBO group. We therefore believe that the anabolic effect of HBO contributes to the generation of fusion.

In conclusion, when the radiological, biomechanical and histopathological findings are considered, we have shown for the first time that the addition of HBO to single-dose ZA improves the rate of fusion and provides stability in an animal model of lumbar fusion. Accordingly, we believe that this approach may be applicable to clinical practice.

The authors would like to thank M. A. Beyoğlu and M. Yetiş for their kind help in evaluating the biomechanical tests.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References