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ORIGINAL ARTICLE

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Comparison of BMP-2 and combined IGF-I/TGF-ß1 application in a sheep cervical spine fusion model

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Abstract Growth factors have proven to promote spine fusion. However, no comparative evaluation of growth factors in spinal fusion has yet been performed. The purpose of this study was to compare the efficacy and safety of combined IGF-I and TGF-ß1 application with BMP-2 application and autologous cancellous bone graft at an early time point in a sheep cervical spine fusion model. Thirty-two sheep underwent C3/4 discectomy and fusion. They were divided into four groups, according to their treatment: group 1, titanium cage (*n*=8); group 2, titanium cage filled with autologous cancellous iliac crest bone grafts (*n*=8); group 3, titanium cage coated with a poly-(D,L-lactide) (PDLLA) carrier including BMP-2 (5% w/w) (*n*=8); group 4, titanium cage coated with a PDLLA carrier including IGF-I (5% w/w) and TGF-ß1 (1% w/w) (*n*=8). Blood samples, body weight and temperature were analysed. Radiographic scans were performed pre- and postoperatively and after 1, 2, 4, 8 and 12 weeks. At the same time points, disc space height and intervertebral angle were measured. After 12 weeks, the animals were killed and fusion sites were evaluated using functional radiographic views in flexion and extension. Quantitative computed tomographic scans were performed to assess bone mineral density, bone mineral content and bony callus volume. Biomechanical testing was carried out and the values for range of motion, and neutral and elastic zone were determined. Histomorphological and histomorphometrical analysis were performed and polychrome sequential labelling was used to determine the time frame of new bone formation. The results showed that, in comparison to the group treated with the cage alone (group 1), the cage plus BMP-2 group (group 3) and the cage plus IGF-I and TGF-ß1 group (group 4) demonstrated a significantly higher fusion rate in radiographic findings, a higher biomechanical stability, a more advanced interbody fusion in histomorphometrical analysis, and an accelerated interbody fusion on fluorochrome sequence labelling. In comparison to the bone graft group (group 2), the BMP-2 (group 3) and IGF-I/TGF-ß1 group (group 4) showed significantly less residual motion on functional radiographic evaluation, higher bone mineral density of the callus and higher biomechanical stability in extension, rotation and bending. The BMP-2 group showed significantly less residual motion on functional radiographic evaluation and higher intervertebral bone matrix formation on fluorochrome sequence labelling at 9 weeks in comparison to the IGF-I/TGF-ß1 group. In contrast, the IGF-I/TGF-ß1 group showed a significantly higher bone mineral density of the callus than the BMP-2

group. In comparison to the autologous cancellous bone graft group, both growth factors (BMP-2 and combined IGF-I and TGF-ß1) significantly improved the biomechanical results of interbody fusion. No systemic side effects were observed for either growth factor. On the basis of these preliminary results, it would appear that combined IGF-I/TGF-ß1 application yields equivalent results to BMP-2 application at an early time point in anterior sheep cervical spine fusion.

Keywords Cervical spine · Sheep · Animal model · Interbody fusion · BMP-2 · IGF-I · TGF-ß1 · Growth factor

Introduction

More than 30 years ago, Urist [42] determined the osteoinductive capacity of demineralized bone matrix. Advances in protein isolation and molecular cloning technology subsequently yielded several soluble, low-molecular-weight growth factors, such as transforming growth factors (TGFs), bone morphogenetic proteins (BMPs), platelet-derived growth factors (PDGFs), insuline-like growth factors (IGFs), fibroplast growth factors (FGFs) and epidermal growth factors (EGFs) [41]. Meanwhile, many of these growth factors have been produced as recombinant molecules in virtually unlimited quantities using genetically modified cell lines. However, only some of them have demonstrated a significant osteoinductive capacity, and only two have been precisely evaluated in experimental spine fusion. Presently, only BMP-2 [4, 5, 6, 7, 8, 11, 16, 26, 27, 33, 34, 45, 46] and OP-1 (BMP-7) [10, 23] have proven to accelerate spinal fusion and to overcome the disadvantages of an autologous bone graft. Therefore, the optimum growth factor or growth factor combination to promote spinal fusion is still a matter of discussion.

Recently, in vitro and in vivo studies have demonstrated an osteoinductive effect of isolated IGF-I and TGF-ß1 or combined IGF-I/TGF-ß1 application [24, 25, 29, 41]. IGF-I stimulates the replication of osteoblasts and the synthesis of bone matrix [13]. TGF-ß1 regulates different cell types that are directly involved in bone remodeling and bone matrix formation, such as mesenchymal cells, chondrocytes, osteoblasts, and osteoclasts [31, 32]. In vivo studies have shown that decreased levels of IGF-I and TGF-ß1 are associated with bone loss and osteoporosis [1, 7, 44], whereas the local application of isolated IGF-I or TGF-ß1 can positively influence fracture healing [15, 30, 38]. Additionally, in vitro and in vivo studies have demonstrated a significant osteoinductive effect of combined IGF-I and TGF-ß1 application [20, 36]. Schmidmaier [36] demonstrated that the combined application of IGF-I and TGF-ß1 had a significantly higher stimulating effect on bone matrix formation in rat tibia fractures than a single application of IGF-I or TGF-ß1. Kandziora [20] was able to show an increased intervertebral bone matrix formation comparing combined IGF-I and TGF-ß1 application with an autologous tricortical iliac crest bone graft in a sheep cervical spine fusion model.

Presently, no comparative evaluations of different growth factors in experimental spine fusion are available. The purpose of this study was to compare the efficacy and safety of combined IGF-I and TGF-ß1 application with BMP-2 application and autologous cancellous bone grafts at an early time point in an in vivo sheep cervical spine interbody fusion model.

Materials and methods

Study design

Thirty-two adult female merino sheep (2 years old) underwent C3/4 discectomy and fusion. No sheep was lost to follow-up. The sheep were randomly assigned to the following groups:

- Group 1: titanium cage (n=8)
- Group 2: titanium cage filled with autologous cancellous iliac crest bone grafts (n=8)
- Group 3: titanium cage coated with a biodegradable poly-(D,L-lactide) (PDLLA) carrier including BMP-2 (5% w/w) (n=8)
- Group 4: titanium cage coated with a biodegradable PDLLA carrier including IGF-I (5% w/w) and TGF-ß1 (1% w/w) (n=8)

After 12 weeks all the sheep were sacrificed and radiographic, biomechanical and histological evaluations were performed. All animal experimental work was approved by local authorities.

Coating of the cages

PDLLA (Boehringer Ingelheim, Germany) was chosen as the drug carrier system. The properties of the PDLLA coating and the coating technique have been described previously [12, 35]. In group 3, recombinant human BMP-2 (rhBMP-2, R&D Systems, Abingdon, UK) (5% w/w) was incorporated in the PDLLA coating. In group 4, recombinant human IGF-I (R&D Systems) (5% w/w) and recombinant human TGF-ß1 (R&D Systems) (1% w/w) were incorporated in the PDLLA coating. The average PDLLA coating mass of the cages was 3.02±0.12 mg. Therefore, approximately 150 µg (5% w/w) BMP-2 and 150 µg (5% w/w) IGF-I plus 30 µg (1% w/w) TGF-ß1 were incorporated in the coating of each cage in the respective groups.

Surgical technique and postoperative care

The animals underwent the surgical procedure under general endotracheal anaesthesia. The anterior part of the neck and the left iliac crest (group 2) was prepped in a sterile fashion and a left anterolateral approach to the cervical spine was carried out through a longitudinal skin incision. The longus colli muscle was incised in the midline, and the intervertebral disc C3/4 was exposed. After distraction of the motion segment with a Caspar distractor, anterior discectomy C3/4 was performed. The endplates were uniformly shaved with a 2-mm high-speed diamond drill down to bleeding

bone. For interbody stabilization, meshed titanium cages (Motech GmbH, Schwenningen, Germany, height 8 mm, diameter 14 mm) were used. In group 2, the cages were filled with autologous cancellous bone grafts taken from the left iliac crest. Prior to insertion, the volume of the bone grafts was determined using the water displacement technique (Archimedes principle). The average volume of the bone grafts was 1.42±0.1 cm^3. In the growth factor groups and the cage alone group (groups 1, 3 and 4) the cages were not filled with bone graft. Finally, all cages were inserted uniformly into the intervertebral space. The wound was irrigated with saline, and the longus colli muscle, the subcutaneous tissue and the skin were reapproximated with sutures and a soft bandage was applied to the neck.

After surgery, the animals were maintained under observation until fully recovered from general anaesthesia. They received two doses of 0.5 g metamizol-natrium (Novaminsulfon, Lichtenstein) per day for 5 days intramuscularly. Clinical examination was performed daily for the first 10 days, then weekly. The sheep were allowed ad libitum activity for the remainder of the experiment. Fluorochrome sequential labels were administered at 3, 6 and 9 weeks postoperatively, consisting of oxytetracycline (25 mg/kg IV) at 3 weeks, calcein green (15 mg/kg IV) at 6 weeks, and xylenol orange (90 mg/kg IV) at 9 weeks. Twelve weeks after surgery, the animals were killed after induction of anaesthesia by an intravenous injection of potassium chloride. The complete cervical spine including parts of the occiput and T1 was then excised and cleaned from the surrounding tissue.

Blood and serum analysis

Blood and serum samples were taken from the saphenous vein of the hind leg of the sheep pre- and postoperatively, and after 1, 2, 4, 8 and 12 weeks. The blood samples were analysed for routine laboratory parameters (blood count, electrolytes, alkaline phosphatase, thyroid values and glucose).

Body weight and body temperature

Preoperatively and after 1, 2, 4, 8 and 12 weeks, rectal body temperature and body weight were determined.

Radiographic analysis

Radiographic evaluations have been described in detail earlier [18]. Lateral and posteroanterior digital radiographic scans (X-ray unit: Mobilett Plus, Siemens AG, Germany; X-ray films: Fuji CR 24×30, Fuji, Germany) were performed pre- and postoperatively and after 1, 2, 4, 8 and 12 weeks. At the same time points, anterior, middle and posterior intervertebral disc space heights (DSH) and intervertebral angle (IVA) of the motion segment C3/4 were measured on lateral radiographic scans. Average intervertebral DSH was calculated from anterior, middle and posterior DSH measurements (anterior+middle+posterior DSH/3). After 12 weeks, bony fusion was categorised on lateral radiographs using the following parameters [20]: (A) no bony fusion, (B) maximum intervertebral gap in the cranio-caudal direction of more than 5 mm, (C) maximum intervertebral gap in the cranio-caudal direction of less than 5 mm, (D) complete bony fusion. The maximum intervertebral gap in the cranio-caudal direction was measured directly on lateral radiographs using a ruler. All radiographic measurements were evaluated by three independent observers.

Functional radiographic analysis

Functional radiographic evaluation of the sheep cervical spine has been described in detail earlier [18]. After sacrifice, fusion sites were evaluated using lateral digital functional radiographic scans in flexion and extension (X-ray unit: Mobilett Plus, Siemens AG, Germany; X-ray films: Fuji CR 24×30, Fuji, Germany). For this purpose, T1 was rigidly fixed with a Steinmann pin, while a 60-N load was applied through C1 using a dynamometer (Newtonmeter, Inha GmbH, Berlin, Germany). Flexion/extension differences in intervertebral angle (IVA) and lordosis angle (LA) were calculated. All functional radiographic measurements were evaluated by three independent observers.

Quantitative computed tomographic analysis

Quantitative computed tomographic scans (QCT) were performed using a Siemens Somatom plus 4 scanner (Siemens Inc., Erlangen, Germany). Axial cuts with 1-mm slice thickness were made parallel to the intervertebral disc space. Bone mineral density (BMD) and bone volume measurements of the callus have been described in detail earlier [18]. BMD measurements were calibrated with a six-point bone mineral density phantom, and were performed using the specific software of the scanner (Sienet Magic View VA 30A, Siemens, Inc.). Bony callus volume (BCV) was measured using an image analysing system (Zeiss KS 400, Zeiss GmbH, Germany). Bone mineral content (BMC) was calculated from BMD and BCV measurements (BMC=BCV×BMD). After 12 weeks, bony fusion was categorised on sagittal and coronal two-dimensional (2D) CT reconstructions using the A–D parameters described earlier [20]. The maximum intervertebral gap in the craniocaudal direction was measured directly on midsagittal 2D CT reconstructions using the scanner software described above. All radiographic CT measurements were evaluated by three independent observers.

Biomechanical analysis

After euthanasia, biomechanical testing was performed by a nondestructive flexibility method using a nonconstrained testing apparatus described in detail earlier [17, 18]. Pure bending moments of 6 Nm were applied to the motion segments C3/4 using a system of cables and pulleys to induce flexion, extension, left and right lateral bending and left and right axial rotation. Tension was applied to the cables with a uniaxial testing machine (1456, Zwick GmbH, Ulm, Germany). Three-dimensional displacement of each motion segment was measured using an optical measurement system (Qualysis Inc., Sävebalden, Sweden). Triangular markers with three diodes (Qualysis Inc.) were attached to the bodies of C3 and C4. Marker positions were detected with two cameras and recorded with a computerized motion analysis system (PC-Reflex, Qualysis Inc). Angular displacement of the upper vertebra (C3) in relation to the lower vertebra (C4) was calculated from marker positions using custom-made computer software. The measurement error associated with this method was ±0.1° [21]. Range of motion (ROM), and neutral (NZ) and elastic (EZ) zones were determined.

Histomorphological, histomorphometrical and fluorochrome analysis

All C3/4 motion segments were harvested at 12 weeks for bone histology. The motion segments had been fixed for 7 days in 10% normal buffered formaldehyde followed by dehydration in ascending concentrations of ethanol, and embedded undecalcified in methylmethacrylate (Technovit 9100, Heraeus Kulzer GmbH, Germany).

For histomorphological and histomorphometrical analysis, longitudinal sections in the sagittal plane were cut at 6 µm with a Leica SM 2500 S microtome and a 40° stainless steel knife. Afterwards, the residual parts of the cages were removed and the following stains were used: (1) Safranin-O/Lightgreen, (2) Safranin-O/v. Kossa, (3) Astrablue and (4) Masson-Goldner.

Masson-Goldner stainings were used for histomorphological analysis, which included evaluation of bony fusion using the A–D parameters described earlier [20]. The maximum intervertebral gap in the cranio-caudal direction was measured directly on mid-sagittal sections using an image analysing system (Zeiss KS 400, Zeiss GmbH, Germany). Histomorphometrical parameters were measured on the residual stainings using a Leica DM-RB microscope, and the image analysing system (Zeiss KS 400). Parameters were measured at a magnification of ×1.6.

The sagittal diameter distance (S) of C3 and the average preoperative DSH were determined to define the size of the region of interest (ROI) for histomorphometrical evaluation [20]. The entire intervertebral fusion area was included in this ROI. The following structural indices were calculated in the ROI: bone volume/total volume (BV/TV), cartilage volume/total volume (CV/TV), mineralised cartilage volume/cartilage volume (mCV/CV).

For fluorochrome analysis, longitudinal sections in the parasagittal plane were cut at 400 µm with a precise macro grinding machine (Fa. Exact, Norderstedt, Germany). These slices were then ground to a thickness of 80 µm using a precise micro grinding machine (Fa. Exact). Fluorochrome markers were analysed under appropriate lighting conditions using a Leica DM-RB microscope and an image analysing system (Zeiss KS 400). Parameters were measured at a magnification of ×1.6.

Fluorochrome analysis of intervertebral fusion areas has been described in detail earlier [45]. The first appearance of the marker served to time formation of new bone matrix. The presence or absence of each marker around or within the cage was used to determine the relative time frame of new bone formation.

Statistical analysis

Comparison of data was performed using one way ANOVA for independent samples followed by TUKEY post-hoc analysis for multiple comparison procedures with Bonferroni correction for multiple measurements. Intraobserver variability for radiographic measurements, functional radiographic evaluation and CT measurements was determined using kappa statistics. The A–D score [20] was used to categorise semiquantitative bony fusion on plain radiographs, CT scans and histological stainings; however, no statistical evaluation of this score was performed. Statistically significant differences were defined at a 95% confidence level. The values are given as mean±SD. SPSS (release 7.0, SPSS Inc., Chicago, Illinois) software supported statistical evaluation.

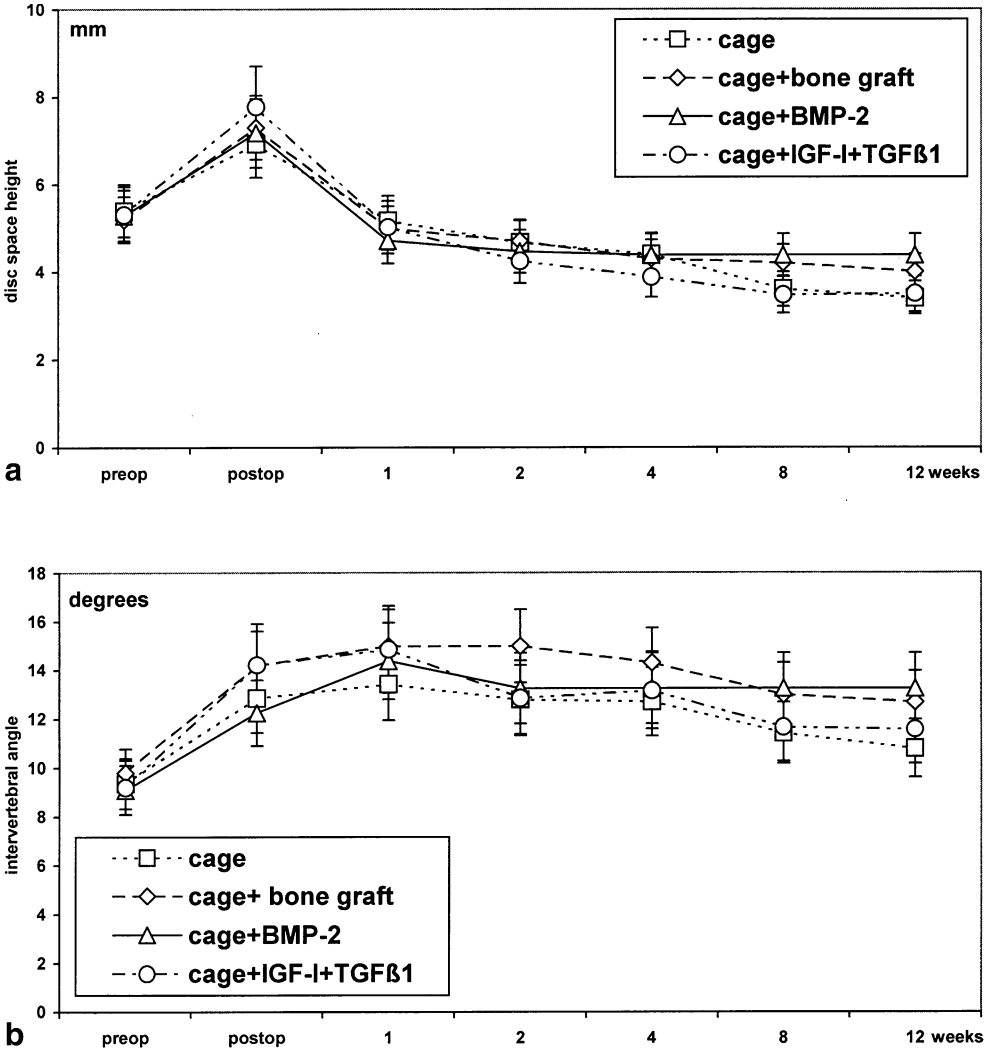

Fig. 1a, b Radiographic analysis. **a** Average disc space height of the different groups throughout the observation period. **b** Average intervertebral angle of the different groups throughout the observation period

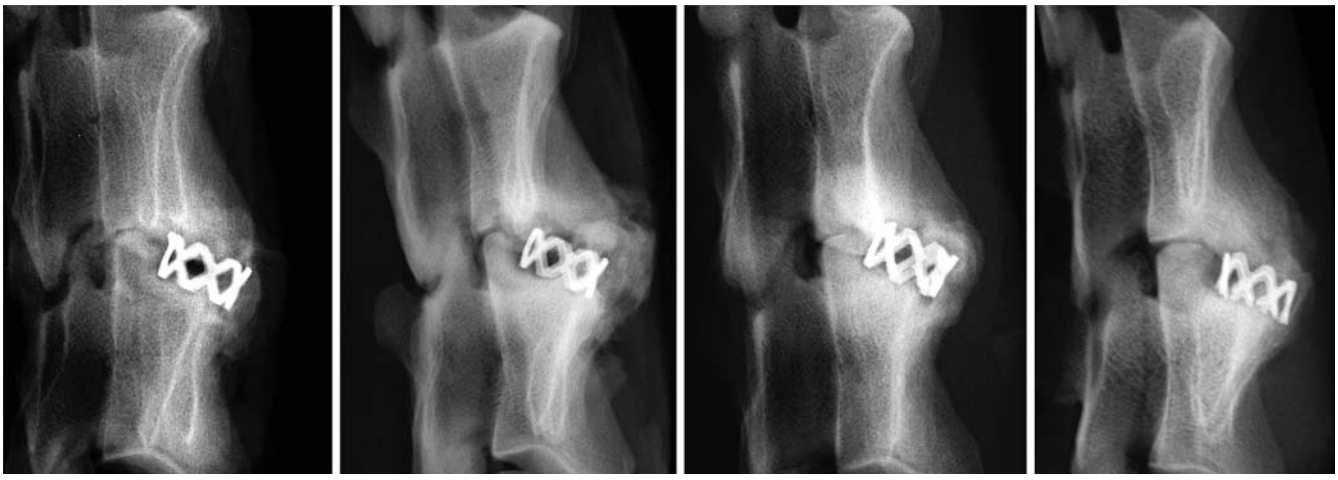

Fig. 2 Radiographic analysis. After 12 weeks, bony fusion was evaluated on lateral radiographic scans (animal 8 of each group)

Results

Blood and serum analysis results

Full blood count did not show any significant differences either between the groups or at different time points throughout the course of the experiment. Even in the initial period, 1 week postoperatively, no significant changes were found for average haemoglobin, erythrocyte and leukocyte levels compared to the preoperative baseline levels.

Levels of electrolytes (Na, K, Cl, Ca) did not show significant changes during the experimental period. Furthermore, no differences in thyroid hormones, alkaline phosphatase or glucose levels were found throughout the observation period or between the groups.

Body weight and body temperature

No significant differences were found between all groups in mean body temperature and body weight throughout the experimental period. Postoperatively, a slight and constant increase in body weight for all sheep of all groups was determined.

Radiographic results

Intraobserver agreement for radiographic measurements was good, showing kappa values ranging between 0.74 and 0.93.

Preoperative baseline values of all radiographic parameters did not show any differences between the groups. No significant differences were found for average disc space height and intervertebral angle between all groups throughout the observation period (Fig. 1). However, at 12 weeks there was a trend for a smaller intervertebral angle and disc space height in the cage alone group (group 1) compared to the other groups.

After 12 weeks, bony fusion was evaluated on radiographic scans (Fig. 2). In the bone graft group (group 2), the BMP-2 group (group 3) and the IGF-I/TGF-ß1 group (group 4), a more advanced interbody fusion was found in comparison to the cage alone group (Table 1). No noticeable difference in fusion rate was found between the bone graft group (group 2) and the BMP-2 group (group 3) or the IGF-I/TGF-ß1 group (group 4).

Functional radiographic results

Intraobserver agreement for functional radiographic measurements was excellent, showing kappa values ranging between 0.86 and 0.93.

After 12 weeks there were no significant differences between flexion/extension values of intervertebral and lordosis angle (Table 2) between the cage alone group (group 1) and the cage plus bone graft group (group 2).

Table 1 Radiographic analysis. After 12 weeks, bony fusion of the four groups was determined on radiographic scans using the following parameters: (A) no bony fusion, (B) maximum intervertebral gap in cranio-caudal direction of more than 5 mm, (C) maximum intervertebral gap in cranio-caudal direction of less than 5 mm, (D) complete bony fusion

Bony fusion parameter (score)	Group 1 (n=8) Cage	Group 2 (n=8) Cage+bone graft	Group 3 (n=8) Cage+ BMP-2	Group 4 (n=8) Cage+IGF-I+ TGF-ß1
A	0	0	0	0
B	6	4	2	2
C	2	3	4	5
D	0	1	2	1

Table 2 After 12 weeks, functional radiographic evaluation in flexion/extension of the four groups was determined. Flexion/extension differences of intervertebral angle (IVA) and lordosis angle (LA) were calculated: values are presented as mean ±SD, with range in parentheses

Flexion/ extension difference in degrees	Group 1 (n=8) Cage	Group 2 (n=8) Cage+bone graft	Group 3 (n=8) Cage+ BMP-2	Group 4 (n=8) Cage+IGF-I+ TGF-ß1
IVA	8.6±2.6 (6.0–10.0)	7.7±3.0 (4.5–11.0)	2.7±2.1[a,b,c] (0–7.0)	5.9±1.9[a] (3.5–7.5)
LA	8.6±3.1 (6.0–12.0)	7.9±3.4 (4.5–11.5)	3.9±2.3[a,b,c] (0–9.0)	6.3±1.6[a] (5.0–8.0)

[a] $P<0.05$ in comparison to the cage alone group (group 1)
[b] $P<0.05$ in comparison to the cage plus bone graft group (group 2)
[c] $P<0.05$ in comparison to the cage plus IGF-I/TGF-ß1 group (group 3)

Flexion/extension differences in the IGF-I/TGF-ß1 group (group 4) were significantly lower than in group 1 ($P<0.05$). Functional radiographic assessment revealed significantly lower residual flexion/extension movement in the cages with BMP-2 group (group 3) than in any other group ($P<0.05$).

Quantitative computed tomographic results

Intraobserver agreement for CT measurements was excellent, showing kappa values ranging between 0.84 and 0.98.

After 12 weeks, bone mineral content (BMC) and bony callus volume (BCV) were significantly lower in the cage alone group (group 1) than in any other group ($P<0.05$) (Fig. 3). There were no significant differences in BMC and BCV between the cage plus bone graft group (group 2), the cage plus BMP-2 group (group 3) and the cage plus TGF-I/IGF-ß1 group (group 4). Bone mineral density of the callus (BMD) in the cage plus IGF-I/TGF-ß1 group (group 4) was significantly higher than in any other group ($P<0.05$). The cages with BMP-2 group (group 3) showed significantly higher values for BMD ($P<0.05$) than groups 1 and 2 (Table 3). There was no significant difference for BMD of the callus between the cage alone group (group 1) and the cages filled with cancellous bone grafts (group 2).

Fusion was evaluated on sagittal 2D CT reconstructions (Table 4). In the bone graft group (group 2), the BMP-2 group (group 3) and the IGF-I/TGF-ß1 group (group 4), a more advanced interbody fusion was found in comparison to the cage alone group (Table 1). No significant difference in fusion score was found between the bone graft group (group 2) and the BMP-2 group (group 3) or the IGF-I/TGF-ß1 group (group 4).

Biomechanical results

Biomechanical results for range of motion (ROM), neutral zone (NZ) and elastic zone (EZ) are depicted in Table 5.

Lowest ROM, NZ and EZ values were consistently found for the growth factor groups (groups 3 and 4). ROM in all directions, NZ and EZ in rotation and NZ in lateral bending were significantly ($P<0.05$) lower in the growth factor groups (groups 3 and 4) than in the cage alone group (group 1) and the cage plus bone graft group (group 2). No significant difference for ROM, NZ and EZ in any direction was found between the cage alone group (group 1) and the cage plus bone graft group (group 2). Additionally, no significant difference was found between the cage plus BMP-2 group (group 3) and the cage plus IGF-I/TGF-ß1 group (group 4).

Histomorphological results

Histomorphological analysis supported the findings of radiographic and biomechanical examinations (Fig. 4).

In the cage alone group (group 1), mainly fibroblasts and occasionally cartilage cells were observed between the endplates. Cages were surrounded by a distinct small line of fibroblasts. Group 2, stabilised with cages plus cancellous bone grafts, showed some bony islands between the endplates with cartilage and fibrous tissue com-

Fig. 3 Computed tomographic (CT) analysis. After 12 weeks, interbody fusion was evaluated using quantitative computed tomography (QCT). Depicted are axial CT scans performed parallel to the intervertebral space (animal 6 of each group)

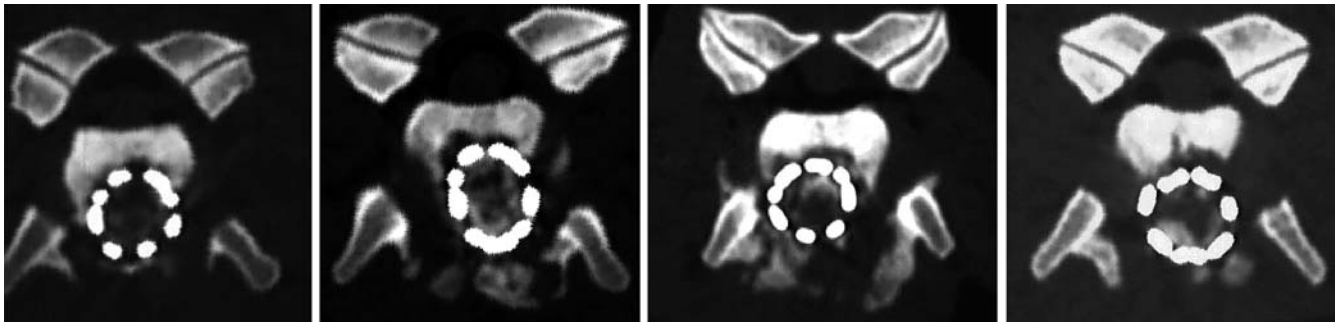

Table 3 After 12 weeks, quantitative computed tomographic analysis (QCT) was performed to measure bone mineral density (BMD), bone mineral content (BMC) and bony callus volume (BCV): values are presented as mean ±SD, with range in parentheses

QCT	Group 1 (*n*=8) Cage	Group 2 (*n*=8) Cage+bone graft[a]	Group 3 (*n*=8) Cage+ BMP-2	Group 4 (*n*=8) Cage+IGF-I+ TGF-ß1
BMD (g/cm^3)	0.58±0.04 (0.65–0.56)	0.55±0.05 (0.52–0.59)	0.62±0.04[b,c] (0.65–0.58)	0.65±0.04[b,c,d] (0.68–0.59)
BMC (g)	1.9±0.7 (1.2–2.8)	3.2±0.3[b] (2.8–3.7)	3.1±1.1[b] (2.0–5.5)	3.4±1.6[b] (2.4–5.5)
BCV (cm^3)	3.3±1.2 (2.0–4.7)	5.4±1.4[b] (3.8–6.3)	5.4±1.9[b] (2.1–7.4)	5.2±1.2[b] (4.1–6.8)

[a] Initial callus volume of the bone graft was 1.42 cm^3
[b] $P<0.05$ in comparison to the cage alone group (group 1)
[c] $P<0.05$ in comparison to the cage plus bone graft group (group 2)
[d] $P<0.05$ in comparison to the cage plus BMP-2 group (group 3)

Table 4 CT evaluation. After 12 weeks, bony fusion of the four groups was determined on sagittal two-dimensional CT reconstruction using the following parameters: (A) no bony fusion, (B) maximum intervertebral gap in cranio-caudal direction of more than 5 mm, (C) maximum intervertebral gap in cranio-caudal direction of less than 5 mm, (D) complete bony fusion

Bony fusion parameter (score)	Group 1 (*n*=8) Cage	Group 2 (*n*=8) Cylinder cage+ bone graft	Group 3 (*n*=8) Cage+ BMP-2	Group 4 (*n*=8) Cage+PDLLA+ IGF-I+TGF-ß1
A	0	0	0	0
B	6	4	2	2
C	2	3	5	5
D	0	1	1	1

ponents. The tissue surrounding the cages appeared similar to that in group 1. Inside the cage of group 2 sheep, osteoclastic activity was noted as an indication of graft resorption. Groups 3 and 4, stabilised with cages coated with BMP-2 and IGF-I/TGF-ß1, respectively, showed extensive callus formation and bony islands between the endplates, with cartilage and small fibrous tissue components. Most of the callus was seen ventrally. These findings were accompanied by capillary ingrowth and resorptive lacunae, without major differences between the two growth factor groups. No ossifications of the spinal ligaments were observed in any group.

Bony fusion was evaluated histomorphologically (Table 6). No major differences in the fusion score were found between the bone graft group (group 2) and the BMP-2 group (group 3) or the IGF-I/TGF-ß1 group (group 4). However, in comparison to the cage alone group (group 1), all these groups showed more advanced interbody bone matrix formation.

Table 5 Biomechanical analysis. After 12 weeks, biomechanical analysis was performed to measure range of motion (ROM), neutral (NZ) and elastic zone (EZ) for the different test modes: values (in degrees) are presented as mean ±SD

Test modes	Group 1 (*n*=8) Cage	Group 2 (*n*=8) Cage+bone graft	Group 3 (*n*=8) Cage+ BMP-2	Group 4 (*n*=8) Cage+IGF-I+ TGF-ß1
Flexion				
ROM	3.9±0.8	3.9±1.3	2.9±1.2	2.9±1.2
NZ	1.3±0.6	1.3±0.9	0.9±1.1	0.7±0.7
EZ	2.6±0.8	2.6±0.8	2.0±0.7	2.3±0.8
Extension				
ROM	3.9±1.3	3.8±1.2	2.7±0.7[a]	2.2±0.7[a,b]
NZ	1.0±0.6	1.4±0.9	0.6±0.5	0.6±0.5
EZ	2.9±0.9	2.4±1.0	2.1±0.3[a]	1.6±0.3[a,b]
Right rotation				
ROM	2.3±1.0	2.0±1.0	1.0±0.3[a,b]	1.4±0.3[a]
NZ	0.5±0.1	0.4±0.2	0.2±0.1[a,b]	0.2±0.1[a,b]
EZ	2.0±0.9	1.6±0.9	0.8±0.3[a,b]	1.2±0.3[a]
Left rotation				
ROM	2.3±0.8	2.1±1.0	1.1±0.4[a,b]	1.5±0.4[a]
NZ	0.4±0.1	0.5±0.3	0.2±0.1[a,b]	0.2±0.1[a,b]
EZ	1.9±0.7	1.6±0.9	0.9±0.4[a,b]	1.3±0.3[a]
Right bending				
ROM	3.8±1.0	4.2±2.2	2.1±0.7[a,b]	2.5±0.8[a,b]
NZ	1.0±0.4	1.2±1.2	0.6±0.2[a,b]	0.6±0.3[a,b]
EZ	2.8±0.7	3.0±1.9	1.5±0.7[a,b]	1.9±0.7[a,b]
Left bending				
ROM	3.9±1.2	4.1±2.1	2.1±0.9[a,b]	2.5±0.8[a,b]
NZ	1.0±0.4	1.3±1.2	0.5±0.2[a,b]	0.5±0.4[a,b]
EZ	2.9±0.8	2.8±1.9	2.0±0.7[a,b]	2.0±0.5[a,b]

[a] $P<0.05$ in comparison to the cage alone group (group 1)
[b] $P<0.05$ in comparison to the cage plus bone graft group (group 2)

Histomorphometrical results

The results of histomorphometrical analysis are presented in Table 7. Histomorphometrical analysis showed no significant differences in sagittal diameter index (baseline) between the groups. Compared to the cage alone group (group 1), histomorphometrical parameters revealed significantly more advanced bone matrix formation (bone volume/total volume ratio) in groups 2, 3 and 4 ($P<0.05$). No differences were found in the bone volume/total volume ratio between the bone graft group (group 2) and the BMP-2 group (group 3) or the IGF-I/TGF-ß1 group (group 4). There were no differences in the other histomorphometrical parameters for any of the groups.

Fluorochrome analysis results

The results of fluorochrome analysis are depicted in Table 8. The BMP-2 and IGF-I/TGF-ß1 coated cages exhibited

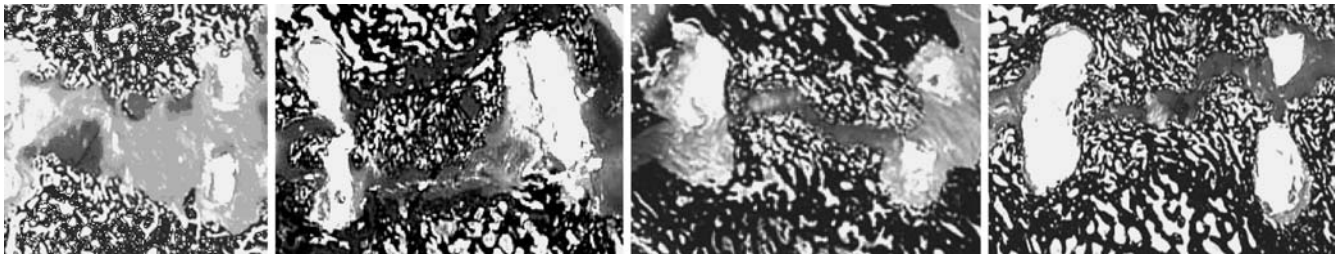

Fig. 4 Histomorphological analysis of the intervertebral fusion area. After 12 weeks, interbody fusion was evaluated histomorphologically and histomorphometrically on midsagittal slides. Depicted is the intervertebral bone matrix formation within the cage (animal 6 of each group; Safranin O/v. Kossa staining, magnification ×2.4)

Table 6 Histomorphological analysis. After 12 weeks, bony fusion of the four groups was evaluated histomorphologically using the following parameters: (A) no bony fusion, (B) maximum intervertebral gap in cranio-caudal direction of more than 5 mm, (C) maximum intervertebral gap in cranio-caudal direction of less than 5 mm, (D) complete bony fusion

Bony fusion parameter (score)	Group 1 (*n*=8) Cage	Group 2 (*n*=8) Cage+bone graft	Group 3 (*n*=8) Cage+ BMP-2	Group 4 (*n*=8) Cage+IGF-I+ TGF-ß1
A	0	0	0	0
B	6	4	3	3
C	2	3	4	4
D	0	1	1	1

Table 7 After 12 weeks, histomorphometrical analysis was performed and the following structural indices were calculated in the region of interest (ROI): sagittal diameter distance (SDD, baseline), bone volume/total volume (BV/TV), cartilage volume/total volume (CV/TV), mineralised cartilage volume/cartilage volume (mCV/CV): values are presented as mean ±SD, with range in parentheses

	Group 1 (*n*=8) Cage	Group 2 (*n*=8) Cage+bone graft[a]	Group 3 (*n*=8) Cage+ BMP-2	Group 4 (*n*=8) Cage+IGF-I+ TGF-ß1
SDD (mm)	25.6±1.2 (24.6–27.0)	25.5±1.1 (24.6–27.8)	25.8±1.4 (24.7–27.9)	26.6±1.1 (25.3–28.3)
BV/TV (%)	38.3±4.1 (26.4–52.1)	45.5±6.7[b] (38.5–61.3)	44.2±3.1[b] (34.2–52.8)	43.3±2.8[b] (33.8–51.4)
CV/TV (%)	4.3±2.4 (1.4–11.0)	4.6±2.7 (0.8–9.4)	6.1±2.8 (1.9–17.2)	4.8±2.4 (0.8–14.6)
mCV/CV (%)	3.4±1.8 (0.8–5.0)	2.8±1.3 (0.2–7.6)	4.1±1.2 (1.8–6.1)	3.2±1.2 (1.8–5.7)

[a] Initial callus volume of the bone graft was 1.42 cm^3
[b] $P<0.05$ in comparison to the cage alone group (group 1)

earlier new bone formation, both within and around the cages, compared to the other groups. There were no significant differences in new bone matrix formation between groups 2, 3 and 4 after 6 weeks. In contrast to the other groups, all cages coated with BMP-2 (group 4) showed new bone formation around and within the cages at 9 weeks.

Discussion

The purpose of this study was to compare the efficacy and safety of combined IGF-I and TGF-ß1 application with BMP-2 application and autologous cancellous bone grafts at an early time point (12 weeks) in an in vivo sheep cervical spine interbody fusion model.

Regardless of the interbody fixation technique, the sheep cervical spine will normally reach solid fusion after 24 weeks. Therefore, it was not the purpose of this study to achieve solid bony fusion. The study aimed, instead, to analyse a developing "bony fusion" at an early time point (12 weeks), in order to determine subtle distinctions between the various fixation techniques.

It is well known that the surgical intervention, including the decortication of the endplates, seems, in itself, to stimulate intervertebral fusion to a limited degree [34, 37]. To compare this "spontaneous fusion potential" with a growth factor or cancellous bone graft stimulated fusion, the cage alone group (group 1) was included in this study. The growth factor groups, as well as the bone graft group, showed a fusion potential significantly higher than the "spontaneous fusion potential" of the cage alone group.

BMP-2 demonstrated significant acceleration of interbody fusion in this study. In comparison to the cage alone group (group 1), representing the spontaneous fusion potential, the cage plus BMP-2 group (group 3) demonstrated a higher fusion rate in radiographic findings, a higher biomechanical stability, an advanced interbody fusion in histomorphometrical analysis, and an accelerated interbody fusion on fluorochrome sequence labelling.

These results are in concordance with previous animal studies using BMP-2 in experimental spinal fusion [4, 5, 6, 7, 8, 11, 14, 26, 27, 33, 34, 45]. However, the majority of these animal studies used BMP-2 in an intertransverse process fusion model of the lumbar spine [5, 7, 8, 16, 26, 27, 33]. Compared with these models, the biological environment of anterior interbody fusion evaluated in this study provides greater access to cancellous bone and bone marrow elements with osteogenetic potency. This favourable biological environment may, therefore, reduce the

Table 8 After 12 weeks, fluorochrome analysis was performed. Depicted are the number of fusion sites (of the different groups at different time points) at which the fluorochrome marker was present adjacent or within the cage or bone graft

Indices	Group 1 (*n*=8) Cage		Group 2 (*n*=8) Cage+bone graft		Group 3 (*n*=8) Cage+BMP-2		Group 4 (*n*=8) Cage+IGF-I+TGF-ß1	
	Adjacent	Within	Adjacent	Within	Adjacent	Within	Adjacent	Within
3 weeks	1	1	0	1	2	2	2	2
6 weeks	1	5	4	6	3	4	4	4
9 weeks	1	6	4	6	8	8	6	6

amount of growth factors needed to promote fusion, making the use of growth factors more economically feasible. In either case, it should be remembered that the dose of growth factors may need to be adjusted to the different biological environments and to different carriers. In several studies using an intertransverse fusion model and a collagen carrier, high BMP-2 doses (up to 1500 µg) were necessary to achieve intertransverse fusion [5, 7, 8, 16, 26, 27, 33]. In this study, a small dose of BMP-2 (approximately 150 µg) applied with a PDLLA-coated cage was able to accelerate anterior interbody bone matrix formation significantly. Due to the local administration via a PDLLA coating of cages, high and continuously released BMP-2 concentrations can be obtained at the fusion site [35]. The quantity and the release of the incorporated growth factors is small in relation to the total organism. Therefore, no systemic side effects on blood count, electrolytes, glucose levels, thyroid hormones, body weight or body temperature caused by BMP-2 were observed in this study.

Besides that, BMP-2 is also able to induce de novo bone in ectopic soft tissue sites, even in the absence of bone marrow elements [2, 46]. Some authors think that a growth factor is required to induce ectopic bone to promote interbody fusion sufficiently [4, 26, 27]. However, this characteristic of BMP-2 might also be harmful. In previous studies, new bone formation induced in the ligamentum flavum by BMP-2 resulted in flattening of the spinal cord [28]. Hoshi and co-workers [14] showed that BMP-2 induced ossification of the spinal ligaments, resulting even in spinal cord compression. Other authors have suggested that BMP-2 might play an important role in the ossification of spinal ligaments, especially the posterior longitudinal ligament [14, 22]. In this study, anterior interbody discectomy and fusion was performed, preserving the posterior longitudinal ligament. In concordance with Zdeblick et al. [45] and Hecht et al. [11], also using an anterior interbody fusion model, we were not able to determine any ossification of the posterior longitudinal ligament using BMP-2 applied by a PDLLA-coated interbody cage.

In previous anterior interbody fusion models using BMP-2, threaded cages were applied to stabilise the anterior spinal column [4, 6, 11, 45]. Zdeblick and co-workers [45], for example, used BAK-cages in a three-level fusion model in the cervical spine of the goat. In in vitro experiments using sheep cervical spines, Kandziora and co-workers [19] showed profound biomechanical differences between threaded cages and cylindrical cages like the meshed titanium cage used in this study. Especially in bending, the vertical cylinder design cages showed significantly higher stiffness and lower range of motion than the threaded cages. Nevertheless, no relevant differences in interbody bone matrix formation could be observed comparing the results of this study using BMP-2 plus cylindrical cages with previous studies using BMP-2 plus threaded cages [4, 6, 11, 45]. This suggests that the osteoinductive effect of BMP-2 applied in the intervertebral space is, to a certain extent, independent from the biomechanical properties of the intervertebral implant.

In contrast to BMP-2, IGF-I and TGF-ß1 are not able to induce de novo bone growth in ectopic soft tissue sites [24]. IGF-I stimulates the replication of osteoblasts and the synthesis of bone matrix [13]. TGF-ß1 regulates different cell types that are directly involved in bone remodeling and bone matrix formation, such as mesenchymal cells, chondrocytes, osteoblasts, and osteoclasts [31, 32]. Due to these characteristics, both growth factors have demonstrated their ability to accelerate the spontaneous fusion potential [9].

In this study the combined application of IGF-I and TGF-ß1 was able to promote interbody bone matrix formation. In comparison to the cage alone group (group 1) the cage plus IGF-I and TGF-ß1 group (group 4) demonstrated a significantly higher fusion rate in radiographic findings, a higher biomechanical stability, a more advanced interbody fusion in histomorphometrical analysis, and an accelerated interbody fusion on fluorochrome sequence labelling.

These results are in concordance with previous animal studies using IGF-I and TGF-ß1 in other anatomical locations. In these studies, IGF-I and TGF-ß1 individually and in combination have been shown to improve bone matrix formation and bone remodelling by direct and indirect mechanisms [3, 9, 13, 25, 29, 32, 40].

For systemically applied IGF-I and TGF-ß1, side effects have been described depending on concentration [25, 39, 43]. In particular, effects on electrolytes, glucose levels and thyroid hormones have been specified [39, 43]. However, based on these preliminary results, no systemic side effects on blood count, electrolytes, glucose levels, thyroid hormones, body weight or body temperature caused by IGF-I or TGF-ß1 were observed in this study. This may be due to the local administration of IGF-I and

TGF-ß1 via a PDLLA-coating of cages providing high local but low systemic concentrations of the growth factors [35].

One aim of this study was to compare autologous bone grafts (group 1) with growth factors (groups 3 and 4) in experimental spinal fusion. In comparison to the bone graft group, both growth factor groups (BMP-2 and combined IGF-I/TGF-ß1) showed significantly lower residual motion on functional radiographic evaluation, higher bone mineral density of the callus and higher biomechanical stability in extension, rotation and bending after 12 weeks in vivo. Additionally, although there was no significant difference between the bone graft group and the growth factor groups in the total amount of bone in the intervertebral space (bony callus volume and bone volume/total volume ratio) after 12 weeks, the fact that in group 2 the cages were initially filled with 1.42 cm^3 of cancellous bone must be taken into consideration. Therefore, the amount of new-formed bone in the bone graft group was approximately 4 cm^3 (range 5.4–1.42 cm^3; see Table 3). In contrast, the two growth factor groups showed an amount of new formed bone of 5.4 cm^3 (BMP-2) and 5.2 cm^3 (IGF-I/TGF-ß1), respectively (Table 3). This is the first study to describe an acceleration of intervertebral bone matrix formation with combined IGF-I/TGF-ß1 application in comparison to autologous cancellous bone grafts. However, this effect has already been described for BMP-2 [5, 8, 16, 26, 27, 45]. Boden et al. [5] and Zdeblick et al. [45], for example, have demonstrated that in comparison to autologous cancellous bone grafts BMP-2 was able to accelerate spinal fusion.

Another aim of this study was to compare IGF-I and TGF-ß1 with BMP-2 application in the sheep cervical spine fusion model. At present, the "ideal" concentrations for any growth factors to induce fusion are unknown. Some studies on BMP-2 have demonstrated good results in anterior spinal fusion models with BMP-2 doses ranging between 100 and 250 µg [4, 11, 45]. In other studies, the 5:1 ratio of IGF-I and TGF-ß1 applied by a PDLLA-coated implant has proven to be most effective [20, 36]. Therefore, comparable concentrations of BMP-2 (150 µg) and IGF-I (150 µg)/TGF-ß1 (30 µg) were chosen for this study. Using these concentrations, only slight and inconsistent differences could be evaluated comparing the two growth factor groups. The BMP-2 group showed significantly lower residual motion on functional radiographic evaluation and higher intervertebral bone matrix formation on fluorochrome sequence labelling at 9 weeks in comparison to the IGF-I/TGF-ß1 group. This slightly higher amount of bone matrix in the intervertebral space of the BMP-2 group might be due to the specific characteristic of BMP-2 of inducing de novo bone [2, 46]. However, this new formed bone did not result in a higher biomechanical stability of the BMP-2 fused motion segments. In contrast, the IGF-I/TGF-ß1 group showed a significantly higher bone mineral density of the callus than the BMP-2 group. This effect might be correlated with the ability of IGF-I and TGF-ß1 to accelerate bone remodelling, by influencing osteoblasts and osteoclasts [3, 25, 29, 32]. However, this effect was also not correlated with a higher biomechanical stability of the fused motion segments. Beside that, no relevant differences could be evaluated between the BMP-2 and the IGF-I/TGF-ß1 group in radiographic, biomechanical, or histological results. On the basis of these preliminary results, no systemic side effects of either growth factor could be determined.

Conclusion

In comparison to the cage alone group, the local application of growth factors (BMP-2 or combined IGF-I/TGF-ß1) significantly improved results of interbody bone matrix formation in this sheep cervical spine fusion model. In comparison to the cancellous bone graft group, both growth factors significantly improved the biomechanical results of interbody fusion. An additional advantage of the use of growth factors is that donorsite morbidity of the iliac crest graft can be avoided. On the basis of the preliminary results of this study, the combined IGF-I/TGF-ß1 application yields equivalent results to BMP-2 application at an early time point in this anterior sheep cervical spine fusion model. Therefore, combined IGF-I/TGF-ß1 application has proven to have considerable effectiveness in experimental spinal fusion. Although no systemic side effects were observed for either growth factor during the 12-week follow-up period, long-term effects of these growth factors are still unknown. Further, in particular long-term, comparative in vivo studies of different growth factors might elucidate which growth factor or combination of growth factors – also taking into account those not yet tested – could create the basis for spine fusion at an optimised velocity and with minimised risk.

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