Marquette University

e-Publications@Marquette

Biomedical Engineering Faculty Research and Publications/College of Engineering

***This paper is NOT THE PUBLISHED VERSION;* but the author's final, peer-reviewed manuscript.**
The published version may be accessed by following the link in the citation below.

The Spine Journal, Vol. 17, No. 12 (December, 2017): 1897-1906. DOI. This article is © Elsevier and permission has been granted for this version to appear in e-Publications@Marquette. Elsevier does not grant permission for this article to be further copied/distributed or hosted elsewhere without the express permission from Elsevier.

Contents

Abstract 2
Keywords 3
Introduction 3
Materials and methods 4
- Radiographic scores 5
- Manual palpation 5
- RNA extraction, cDNA synthesis, and quantitative reverse transcription-polymerase chain reaction 6
- Histology 6
- Data and statistical analysis 7

Results 7
- Histology of the fusion mass 9
- Quantitative RT-PCR 11

Discussion 14
References 16

The temporal expression of adipokines during spinal fusion

Sohrab Virk
Department of Orthopaedics, Ohio State University Wexner Medical Center, Columbus, OH
Alicia L. Bertone
Comparative Orthopedic Research Laboratory, Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH
Hayam Hamaz Hussein
Comparative Orthopedic Research Laboratory, Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH
Jeffrey M. Toth
Department of Orthopaedic Surgery, The Medical College of Wisconsin, Milwaukee, WI
Biomaterials Lab, William Wehr Physics, Marquette University, Milwaukee, WI
Mari Kaido
Comparative Orthopedic Research Laboratory, Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH
Safdar Kahn
Department of Orthopaedics, Ohio State University Wexner Medical Center, Columbus, OH

Abstract

Background Context

Adipokines are secreted by white adipose tissue and have been associated with fracture healing. Our goal was to report the temporal expression of adipokines during spinal fusion in an established rabbit model.

Purpose

Our goal was to report the temporal expression of adipokines during spinal fusion in an established rabbit model.

Study Design

The study design included a laboratory animal model.

Methods

New Zealand white rabbits were assigned to either sham surgery (n=2), unilateral posterior spinal fusion (n=14), or bilateral posterior spinal fusion (n=14). Rabbits were euthanized 1–6 and 10 weeks out from

surgery. Fusion was evaluated by radiographs, manual palpation, and histology. Reverse transcription-polymerase chain reaction on the bone fusion mass catalogued the gene expression of leptin, adiponectin, resistin, and vascular endothelial growth factor (VEGF) at each time point. Results were normalized to the internal control gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) ($2^{\Delta Ct}$), and control bone sites ($2^{\Delta\Delta Ct}$). Quantitative data were analyzed by two-factor analysis of variance ($p<.05$).

Results

Manual palpation scores, radiograph scores, and histologic findings showed progression of boney fusion over time ($p<.0003$). The frequency of fusion by palpation after 4 weeks was 68.75%. Leptin expression in decortication and bone graft sites peaked at 5 weeks after the fusion procedure ($p=.0143$), adiponectin expression was greatest 1 week after surgery ($p<.001$), VEGF expression peaked at 4 weeks just after initial increases in leptin expression ($p<.001$), and resistin decreased precipitously 1 week after the fusion procedure ($p<.001$).

Conclusions

Leptin expression is likely associated with the maturation phase of bone fusion. Adiponectin and resistin may play a role early on during the fusion process. Our results suggest that leptin expression may be upstream of VEGF expression during spinal fusion, and both appear to play an important role in bone spinal fusion.

Keywords

Adipokines; Animal model; Bone fusion; Fracture healing; Gene expression; Leptin; Spinal fusion

Introduction

Obesity creates a low-grade inflammatory state that affects the pathophysiology of numerous diseases.[1,2,3] Obesity has been implicated in the delay of bone healing after a fracture, osteoarthritis, and rheumatoid arthritis.[4,5,6,7,8,9,10,11] There is evidence that obesity negatively affects bone physiology.[12,13,14] Understanding the biochemical pathways that govern the relationship between obesity and bone metabolism is vital for understanding the efficacy of orthopedic interventions for bone healing.

Adipokines are a group of cytokines produced by white adipose tissues that play a role in the low-grade inflammatory state associated with obesity.[15,16] Adipokines include leptin, resistin, visfatin, adiponectin, and others.[17] The role of adipokines on the risk of fracture and fracture healing is influenced by the type of adipokine as well the amount of adipokine present both locally and systematically.[18,19,20,21] Leptin has been implicated in fracture healing in a mouse model.[11] Leptin receptors have been found on osteoblasts, implicating its role in bone mineralization, and there is evidence that leptin is vital during callous formation for fracture healing.[11,22,23] Leptin upregulates vascular endothelial growth factor (VEGF) in cancer cells, and there is research showing the role of VEGF in early vascularization, which precedes bone formation.[24,25,26,27] These processes and growth factors are necessary for spinal fusion.[28]

The spinal fusion process involves a balance of chemical signals promoting bone formation that is needed to span the distances between vertebrae. Based on the role of adipokines in bone formation, adipokines may be essential for the formation of a fusion mass. Khan et al. have identified leptin's role in

organized callous fracture healing using a leptin knockout mouse model.[11] The authors concluded that leptin was critical to fracture callous formation. Our group hypothesized that leptin and other adipokines play a pivotal role in a rabbit spine fusion model. We selected a well-established rabbit spine fusion model and modified the design to provide controls for general anesthesia, as well as the local effects of surgical approach on soft-tissue injury.[29,30] Our specific objectives were to identify the gene expression profile of adipokines during the spinal fusion process and to determine the temporal and relative relationship of adipokine expression. We hypothesized that the gene expression of leptin, adiponectin, VEGF, and resistin would be greater than, and specific to, the generation of bone callus necessary for spinal fusion.

Materials and methods

All experiments were conducted following approval by our Institutional Animal Care and Use Committee. Thirty 1-year old New Zealand white rabbits were included. The area around the right and left transverse processes at L5 and L6 were used as points of evaluation. The rabbits were divided into three groups. The first group was two control rabbits that were anesthetized and had a sham skin incision with muscular dissection. These rabbits were the control for the surgical procedures. The second group consisted of 14 rabbits that received the same surgical approach to both L5 and L6 sites, but one side received an interpositional fat graft to prevent spinal fusion, and the contralateral site received transverse process decortication and iliac crest autograft application anticipated to form a bony fusion (unilateral fusion [UL] rabbits). Group 3 consisted of 14 rabbits, which received bilateral decortication and iliac crest autograft (bilateral fusion [BL] rabbits). Four rabbits (2 from UL group and 2 from BL group) were euthanized at 1, 2, 3, 4, 5, 6, and 10 weeks after their procedure (Fig. 1). The bone from the non-decorticated transverse processes within the UL group was used as a control for the bone fusion surgical procedure, including for autografting and loss of mobility of the spine. The control rabbits were euthanized at 2 weeks after their sham surgery. Our groups were designed to control for the influence of general anesthesia, surgical approach, and the effect of UL and motion to the expression pattern of adipokines during spinal fusion. All surgical procedures were performed together with surgeons with doctor of medicine and doctor of veterinary medicine degrees.

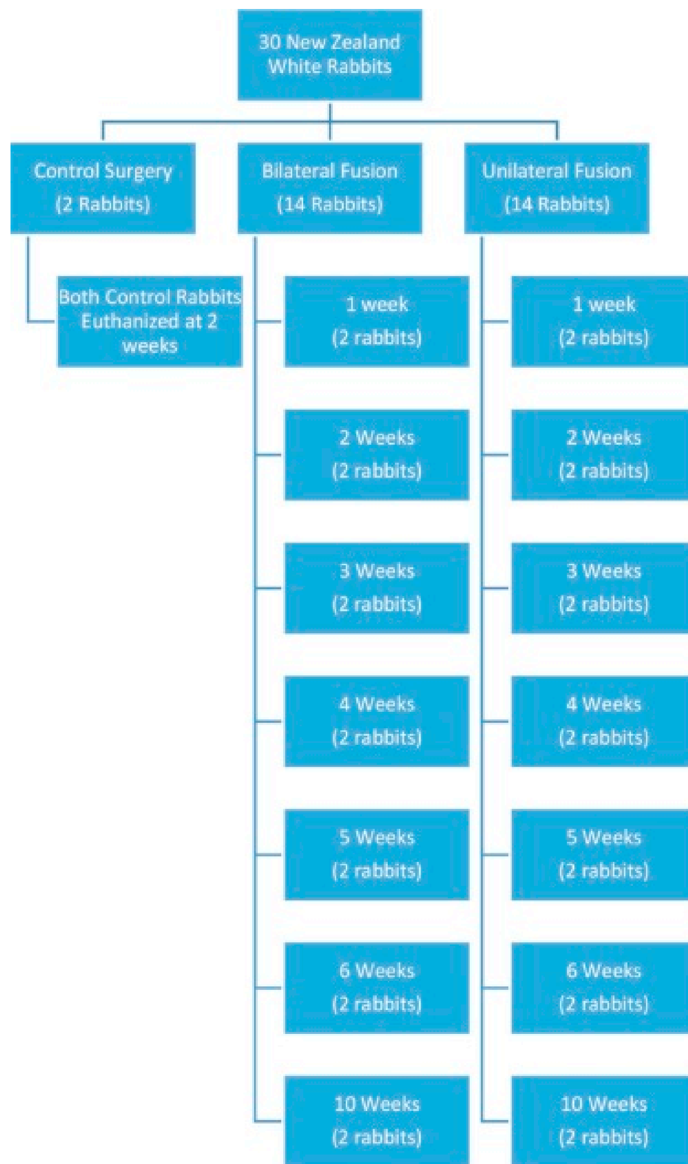

Fig. 1. Illustration of the experimental design.

Radiographic scores

Immediately after euthanasia, dorsal-ventral and lateral digital radiographs of the lumbar spine were obtained from four rabbits (two from the BL group and two from the UL group) at the 2-, 4-, 6-, and 10-week time points to assess the fusion mass. As in previous studies, the radiographs were graded as either 1 (unfused), 2 (partially fused), or 3 (completely fused).[31] The radiographic scoring was performed by one researcher to reduce variability.

Manual palpation

The fusion mass at the level of interest was assessed using manual palpation. Motion with flexion-extension at the level of interest was compared with motion with flexion-extension at adjacent levels.[29] The manual palpation examination was scored using a quantitative system from 0 (no fusion) to 4 (complete fusion), similar to that previously published[32,33,34] (1=minimal, 2=mild, 3=moderate reduction

of motion, and 4=marked or no movement at the level of interest compared with the adjacent levels). A complete fusion was defined as 4 out of 4 on the previously mentioned scale at least 4 weeks out from surgery.[35] Surgeons with a doctor of medicine degree and surgeons with doctor of veterinary medicine degree performed the palpation examination together, and the documented score was agreed upon by all researchers.

RNA extraction, cDNA synthesis, and quantitative reverse transcription-polymerase chain reaction

After manual palpation, a representative portion of the medial fusion mass in both Groups 2 and 3 (UL and BL) was harvested for RNA extraction and gene analysis. The samples were extracted using a sterilized rongeur. The fusion specimens were snap-frozen in liquid nitrogen and placed at −80°C. Additionally, normal transverse process bone specimens were harvested from two levels cephalad to the level of interest and were maintained as separate specimens. The total RNA was extracted using the guanidinium thiocyanate-phenol-chloroform extraction method (TRIzol and chloroform, Invitrogen, Carlsbad, CA, USA) and frozen bone specimens were pulverized in liquid nitrogen. The total RNA was then isolated using standard procedures.[36] Primers were designed and verified using the National Center for Biotechnology Information's Primer-BLAST (Table). Each primer pair was optimized on a heat gradient to ensure amplification efficiency of around 95%–100%. All reactions were run in triplicate for each target gene. The data for the four target genes were presented as mean±standard error of the mean relative mRNA expression normalized to the internal control gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (2^ΔCt). This is a standard technique to analyze the relative expression of a gene as well as to assist as an internal quality control for the polymerase chain reaction (PCR) analysis.[37,38] The four target gene expressions were standardized to independent biological replicate sites (n=4 samples) from the control surgery group using the 2^(ΔΔCt) method to elucidate mean fold changes in gene expression. Relative expression levels were obtained by normalization to *GAPDH* by the comparative 2^**Δ**Ct method.[39] This was performed for both bone from the fusion mass and bone from the non-fused transverse processes in the UL group. These results were plotted on the same graph to illustrate differences in expression attributable to the fusion process. Of note, both 18s ribosomal RNA (18s rRNA) and beta-actin mRNA were tested as potential housekeeping genes during our experiment. Neither 18s rRNA nor beta-actin mRNA provided accurate levels of gene expression when several different primer sequences were tested.

Table. Primer sequences for New Zealand white rabbits

Forward primer (5′–3′)	**Reverse primer (5′–3′)**	
Leptin	GCGGAAAGTCCAGGATGACA	CAGGGATGAAGTCCAAACCGA
Adiponectin	AGAGATGGAACCCCTGGTGA	GCTGAATGCTGAGCGGTAGA
Resistin	CACTGTGTCCGGTGGATGAT	CAGGTTCAGGCTCCTCGTTC
VEGF-A	GGCTGCTGCAATGATGAAAG	GTGCTGTAGGAAGCTCATCTC
GAPDH	AGACACGATGGTGAAGGTCG	TGCCGTGGGTGGAATCATAC

VEGF-A, vascular endothelial growth factor A; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Histology

The treated levels were dissected from the rabbit spine, placed in 10% buffered formalin, and subsequently decalcified. Serial sections were stained with Mallory aniline blue (MAB) tissue stain as

well as routine hematoxylin and eosin. Sections were evaluated for evidence of fusion, tissue within the intertransverse process space, and bony remodeling.

Data and statistical analysis

All quantitative data (reverse transcription-polymerase chain reaction [RT-PCR] results, radiographic scores, and palpation scores) were analyzed by a repeated two-way analysis of variance to compare among groups and across time to study the effects of surgical model standardization (ie, UL vs. BL) and time from surgery. The significance level of 0.05 was used in all statistical comparisons. All statistical analyses were performed with SPSS software (SPSS Inc., Chicago, IL, USA). Relative quantitation of leptin, adiponectin, resistin, and VEGF gene expression using real-time quantitative PCR was calculated by the subtraction of the threshold cycle number of the target gene from the internal control reference gene, *GAPDH*, expressed as the power of 2 ($2^{\Delta Ct}$). Additionally, the $2^{\Delta Ct}$ of the target gene was expressed as a ratio to control rabbit $2^{\Delta Ct}$ ($\Delta\Delta Ct$).[39]

Results

All rabbits completed the study and all data were collected as per protocol. The average body weight of rabbits was 3.53 (±0.38) kg.

Manual palpation scores for rabbits in the UL and BL groups significantly increased with time (duration since surgery) ($p<.0001$, Fig. 2). Although not statistically significant ($p=.11$), there was a trend toward lower palpation scores (more motion) in UL spines. Both radiographic and manual palpation scores indicated spine fusion at the Week 5–6 time period. Fusion frequency at 4–10 weeks after surgery was 68.75% (11 of the 16 rabbits). This result did not significantly differ by fusion type as 62.5% (5/8) were fused in the UL group and 75% were fused in the BL group.

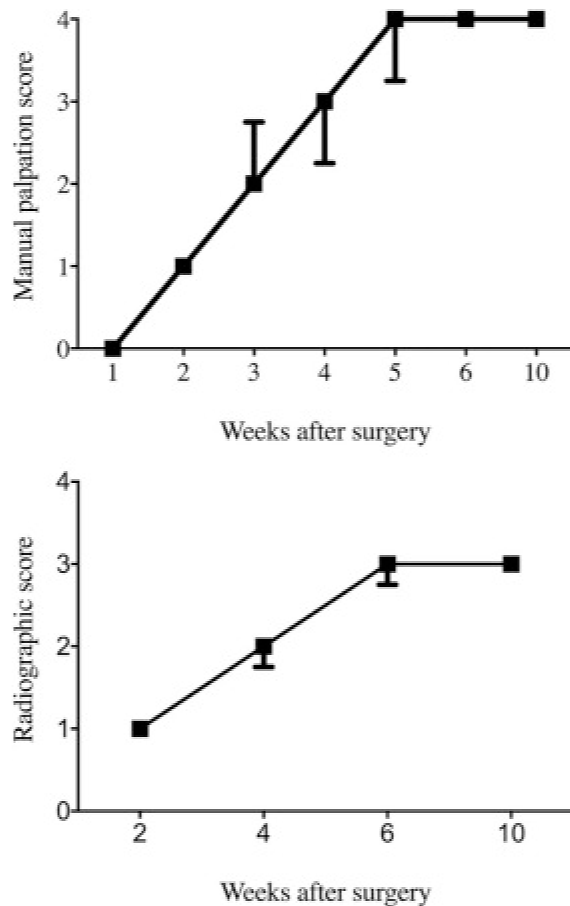

Fig. 2. Graph of mean with quartiles marked of rabbit manual palpation scores (Top) across the weeks evaluated. Rabbits with unilateral and bilateral decortications and bone grafting were combined for graphic purposes because there was no statistically significant difference in the score for these two groups (0=no fusion and no reduction in motion, 1=minimal, 2=mild, 3=moderate reduction of motion and limited movement, and 4=marked reduction of motion or no movement at the level of interest compared with adjacent levels). Of note, at Weeks 1, 2, 6, and 10, all manual palpation scores were the same. Graph of mean and quartiles of radiographic scores (Bottom) across the weeks evaluated. Scores for only decortication and bone graft sites were included. All other groups were scored 1 (1=no fusion, 2=partial fusion, and 3=completely fused). At Week 2, all radiographic scores were 1, and at Week 10, all radiographic scores were 3. Data show a linear increase in score for manual palpation and radiographic assessments of spine fusion that plateaus sometime between 5 and 6 weeks.

Radiographic scores significantly increased with time, reaching a plateau at 6 weeks (Fig. 2). Radiographic imaging over the weeks of spine fusion (Fig. 3A–H) showed no signs of radiographic fusion at 2 weeks, but bone graft could be identified at sites of decortication and bone grafting. (Fig. 3A and B). Radiographic fusion was seen in nearly all decortication and bone graft sites (83.33%) by Week 6 and in 0% of the interpositional fat graft sites (Fig. 3F) in all UL rabbits out to 10 weeks. Radiographic scores increased over time, representing radiographic evidence of progressive fusion ($p<.0003$) in decorticated and bone graft sites only. At Week 10, all decorticated and bone-grafted sites had a radiographic score indicating fusion. (Fig. 3G and H).

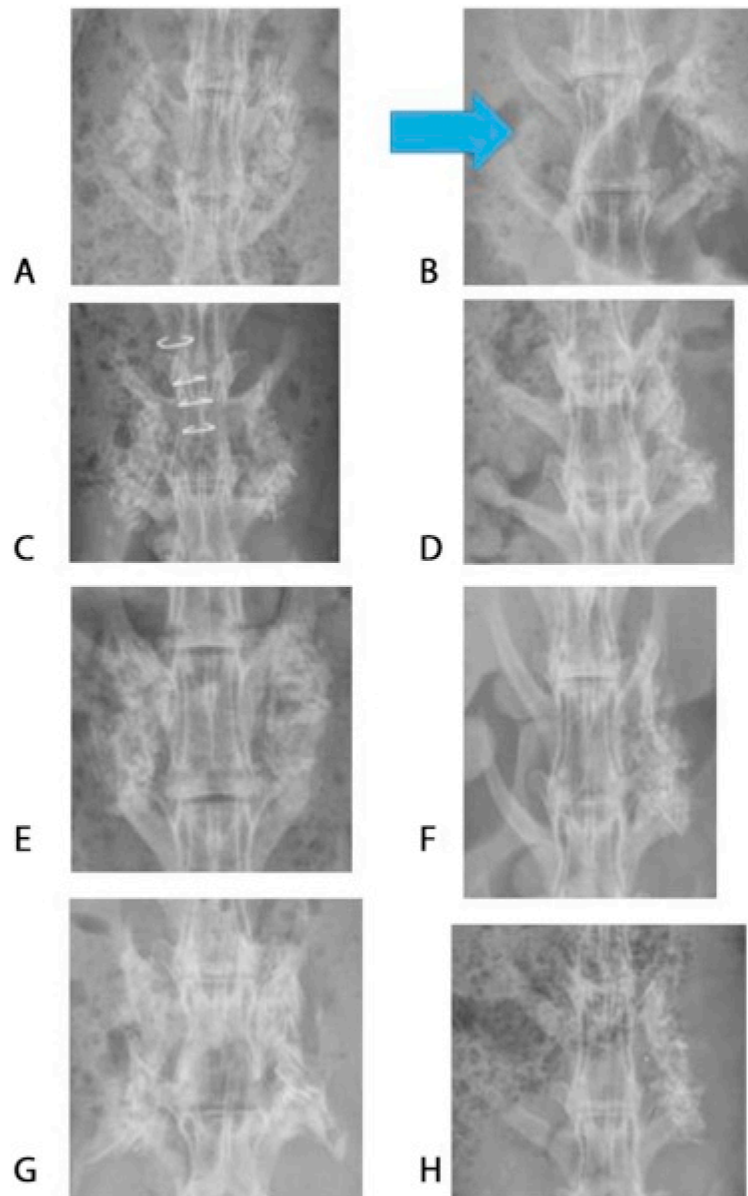

Fig. 3. Dorsal-ventral radiographs of rabbit lumbar spines with bilateral decortication and bone graft (left column) and unilateral decortication and bone graft on one side with interpositional fat graft on the contralateral side (right column). At 2 weeks, bone graft can be seen bilaterally (A) and unilaterally (B) without bony bridging. The interpositional fat graft obviated fusion between transverse processes (arrow in B). There was no significant lucency within the bone graft and evidence of further consolidation of the fusion mass between transverse processes in radiographs at 4 weeks in C and D. (E and F) Radiographs from rabbits at 6 and 10 postoperative weeks, respectively. There was a significant bony growth with developing cortical and trabecular bones between transverse processes by Week 10 (G and H).

Histology of the fusion mass

Bone autograft resorption and endochondral ossification was seen at 2 weeks (Fig. 4A). Cartilage had formed directly adjacent to autograft fragments within the intertransverse process space. The amount of endochondral ossification and new bone formation was minimal (Fig. 4B). At 4 weeks, greater osteoid (Fig. 4C) was present within the intertransverse process space, directly adjacent to the autograft. Bone

had grown within the intertransverse process space and was in the process of developing lamellar bone by Week 4 (Fig. 4C and D).

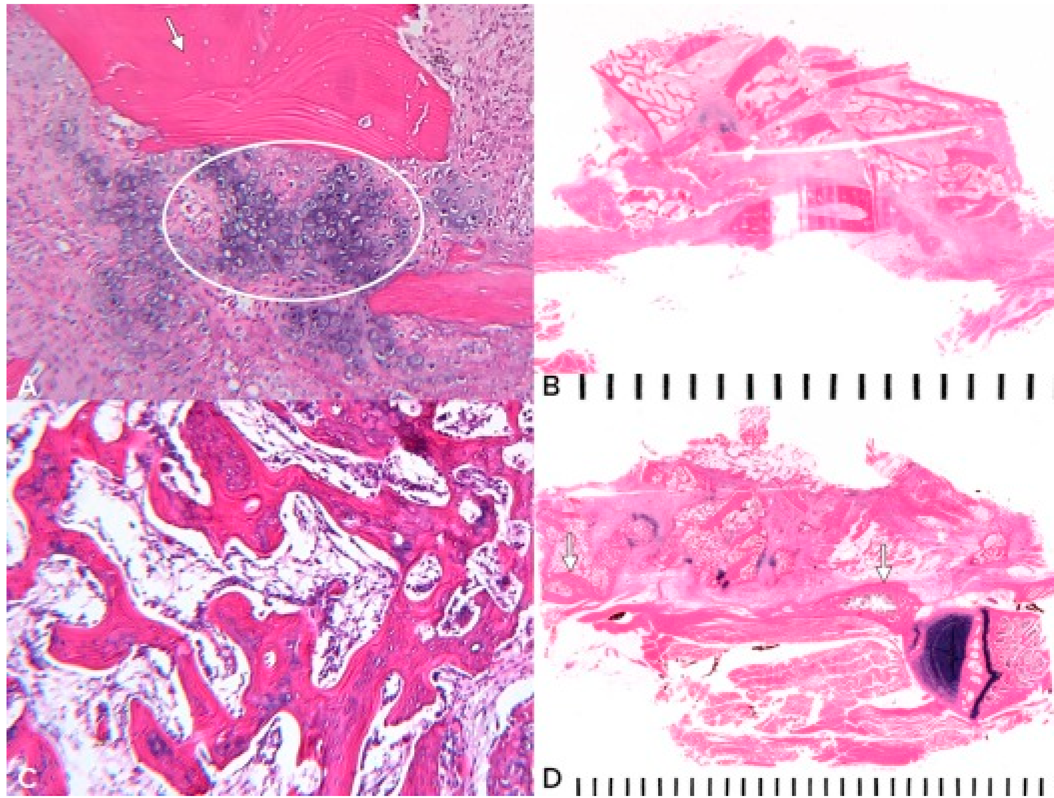

Fig. 4. (A) Micrograph from the intertransverse process space from a UL-treated rabbit at 2 postoperative weeks showing a focus of endochondral ossification (white circle) directly adjacent to an unincorporated bone graft fragment with empty lacunae (white arrow) (H&E, original magnification=79×). (B) Macroscopic sagittal histology image of the intertransverse process space from a UL-treated rabbit at 2 postoperative weeks showing unincorporated bone graft fragments within the intertransverse process space at 2 postoperative weeks (H&E, either a millimeter scale in the field or 4×). (C) Micrograph from the intertransverse process space from a UL-treated rabbit at 4 postoperative weeks showing a trabecular pattern of de novo bone dorsal to the decorticated transverse process (H&E, original magnification=79×). (D) Macroscopic sagittal histology image of the intertransverse process space from a BL-treated rabbit at 4 postoperative weeks showing the development of a bony fusion mass with the incorporation of bone autograft fragments dorsal to and between the decorticated transverse processes (white arrows) at 4 postoperative weeks (H&E, millimeter scale in the field). UL, unilateral fusion; H&E, hematoxylin and eosin; BL, bilateral fusion.

The fusion mass continued to mature at 6 and 10 weeks (Fig. 5A–D) as demonstrated by consolidating bone with the incorporation of the autograft fragments. There was evidence of osteoblasts facilitating bone growth (Fig. 5A). By 10 weeks, bone growth and maturation resulted in a de novo fusion mass (Fig. 5C and D). A cement line was found between the autograft and newly formed de novo bone. Histology confirmed substantial bone growth and maturation of the fusion mass that coincided with loss of spine mobility as assessed by a manual palpation score. There was a greater amount of de novo bone formation within the 4- and 6-week groups as compared with the 10-week group. This result may reflect the normal coupling of bone formation and bone remodeling that results in a smaller final bone callous volume.[40,41]

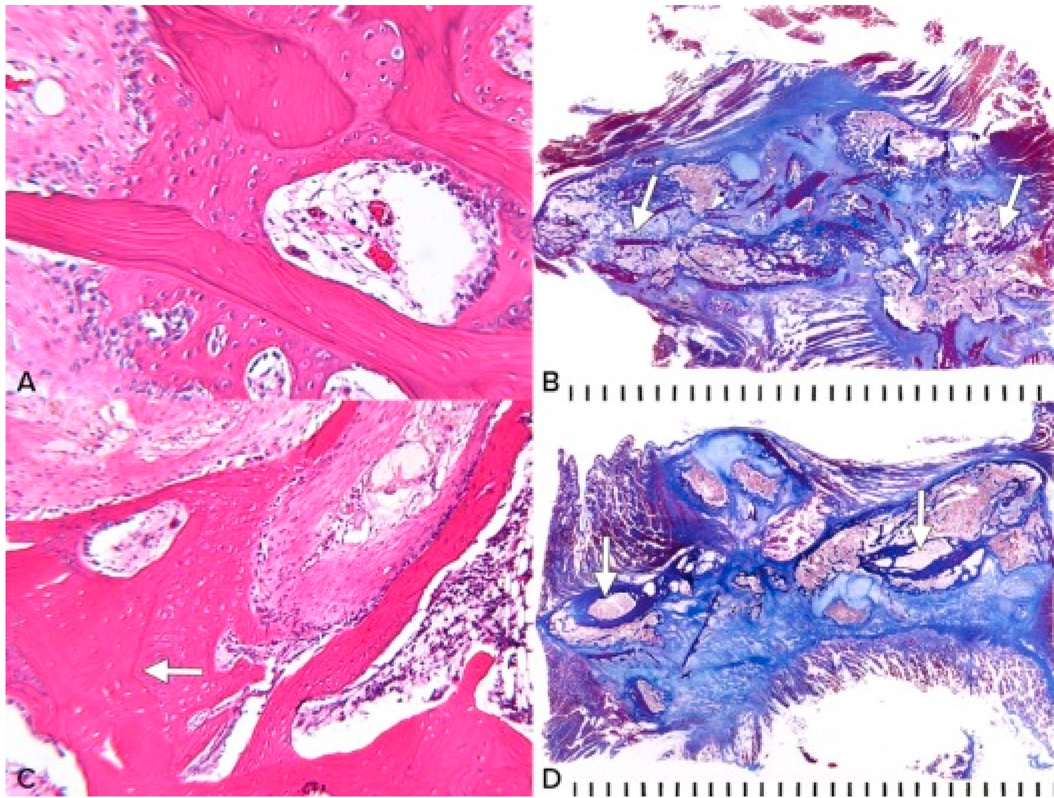

Fig. 5. (A) Micrograph from a BL-treated rabbit at 6 postoperative weeks showing hypertrophied osteoblasts and several foci of intramembranous ossification. Bone autograft fragments (with empty osteocyte lacunae) are incorporated into the developing fusion mass within the intertransverse process space at 6 postoperative weeks (H&E, original magnification=125×). Macroscopic sagittal histology image (B) of the intertransverse process space from a UL-treated rabbit at 6 postoperative weeks showing substantial bony growth and the development of a bony fusion mass with the incorporation of bone autograft fragments dorsal to and between the decorticated transverse processes (white arrows) at 6 postoperative weeks (MAB, millimeter scale in the field). Micrograph (C) from a BL-treated rabbit at 10 postoperative weeks showing hypertrophied osteoblasts and foci of intramembranous ossification. Bone autograft fragments (with empty osteocyte lacunae) are incorporated into the developing fusion mass within the intertransverse process space at 10 postoperative weeks. A cement line is observed between autograft and newly formed de novo bone (white arrow) (H&E, original magnification=79×). (D) Macroscopic sagittal histology image of the treated intertransverse process space from a BL-treated rabbit at 10 postoperative weeks showing the development of a bony fusion mass with the incorporation of bone autograft fragments dorsal to and between the decorticated transverse processes (white arrows) (MAB, millimeter scale in the field). H&E, hematoxylin and eosin; BL, bilateral fusion.

Quantitative RT-PCR

The results of RT-PCR adipokine gene expression are presented as ($2^{\Delta Ct}$) in Fig. 6A–D. There were no significant differences in gene expression between bone from two levels cephalad to the level of interest in the BL and UL groups, bone from the level of interest in control rabbits, and bone from the contralateral non-fused side at the level of interest in the UL group ($p>.05$), indicating representation of a non-fusing bone. We selected the interpositional fat graft procedure as the control comparison to the decortication and bone graft fusion mass for illustrative purposes for PCR data, but results did not differ when decortication and bone graft data were compared with sham control or adjacent spine levels. Expression of adiponectin, resistin, VEGF, and leptin within the decortication and bone graft fusion mass

showed significant ($p<.05$) time-dependent changes corresponding to different histologic findings, radiographic results, and manual palpation scores at different time points (Fig. 6A–D). There were no significant changes in leptin, VEGF, resistin, or adiponectin within the control interpositional fat graft mass, indicating that these variations in gene expression of these adipokines was bone fusion specific. Leptin increased during the 3- to 6-week time frame, spiking at 5 weeks. This finding corresponded to increased bone formation, incorporation of bone graft, and development of woven bone between transverse processes (Fig. 4, Fig. 5). The timing of this increase in leptin corresponded to the incorporation of bone graft with the remodeling of the fusion mass.

Adiponectin expression was greatest 1 week after surgery only in the sites receiving decortication and bone graft (Fig. 6B). This corresponded to the initial reactive phase to bone graft and the decortication procedure. Adiponectin expression decreased by 2 weeks and no longer differed from the control interpositional fat graft site for the rest of the study.

Resistin expression decreased during the middle, reparative phase of the spinal fusion. There was a mixture of woven and lamellar bones developing between transverse processes during these time periods (Fig. 4, Fig. 5). At 6–10 weeks, resistin returned to Week 1 levels as the fusion mass matured.

Vascular endothelial growth factor expression had two peaks of expression at Weeks 4 and 10, which were significantly greater than the control sites ($p<.05$). These time points corresponded to histopathology findings that had bone development as the dominant tissue activity (Fig. 5A and B).

Normalization of adipokine expression to the control rabbit's bone at Week 2 ($\Delta\Delta Ct$) demonstrated a similar pattern to the changes in gene expression across time for leptin, adiponectin, vascular endothelial growth factor A, and resistin (Fig. 7). Specifically, 1.0 was equivalent to baseline expression from control rabbits. As in the ΔCt data (Fig. 6), leptin downregulated over the first 2 weeks and increased during the bone formation phase between Weeks 3–5. The increase and the decrease in VEGF expression occurred just after the increase in leptin and after the decrease in leptin, respectively.

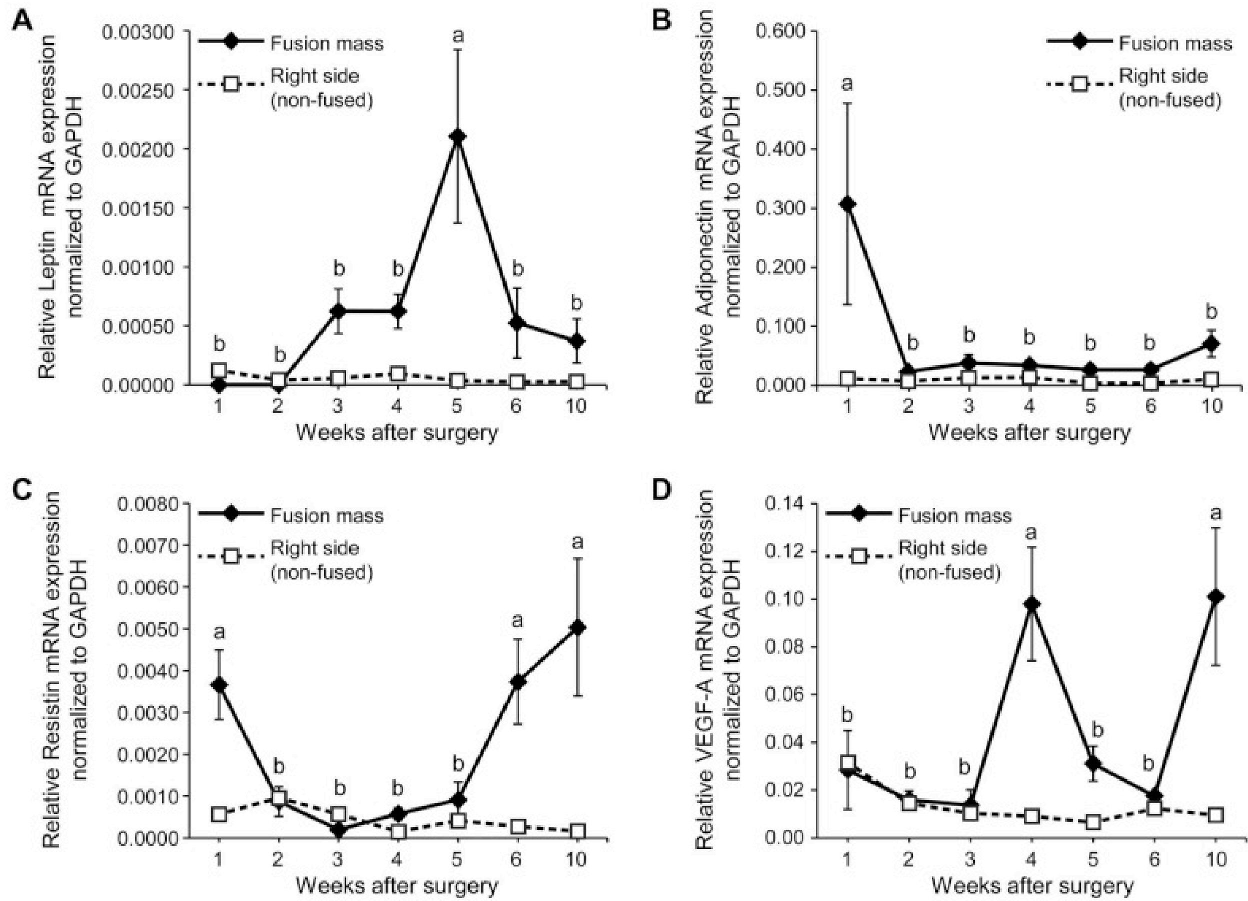

Fig. 6. Illustrations of mean±standard error of the mean gene expression differences in the threshold cycle (2^ΔCt) number between the target gene and the internal control reference gene, *GAPDH*. Means without a common superscript letter differ (p<.05), as analyzed by one-way analysis of variance of leptin (A), adiponectin (B), resistin (C), and VEGF (D). Leptin starts to increase at Week 3, significantly spiking at Week 5. The increase in leptin occurs just before a significant spike in VEGF expression at Week 4. Both resistin and adiponectin spike in expression during the initial stage of healing at Week 1. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; VEGF, vascular endothelial growth factor.

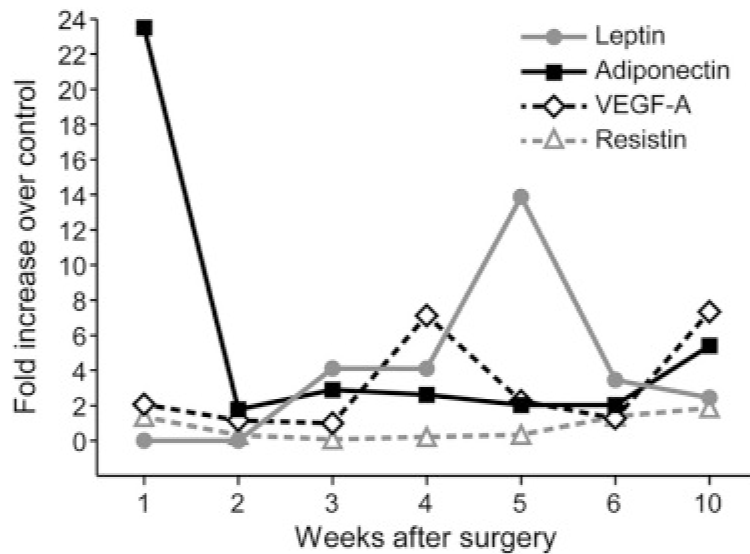

Fig. 7. Mean fold change in leptin, adiponectin, resistin, and VEGF-A gene expression standardized to the control sham surgery group using the 2(−ΔΔCt) method. Note the uptick in leptin expression that is upstream from an increase in vascular endothelial growth factor. There is a substantial increase in leptin expression by Week 5. Also, the same trends in Fig. 6 are reflected in the patterns of mean fold expression shown in this figure. This includes the increase in adiponectin and resistin in the initial week of healing. VEGF-A, vascular endothelial growth factor A.

Discussion

Our study showed significant patterns of adipokine gene expression unique to each adipokine, which varied during the spinal fusion process. Leptin expression spiked within the fusion mass during the bony remodeling phase at 3–6 weeks. Adiponectin expression contrastingly is relatively greater initially during the reactive phase and decreases dramatically shortly thereafter. When comparing expression profiles, there was evidence that the expression of leptin is upstream to that of VEGF, given that the increases in leptin occurred just before the increases in VEGF expression. These findings are consistent with previous research on the role of leptin within bony healing.[11,42,43,44,45] Our data support this importance of leptin for bony healing phases of spinal fusion. The results of our experiment were consistent with our initial hypothesis in that leptin and VEGF expression increased during the remodeling phase of callus formation for the spinal fusion process. Results related to the differing expression of adiponectin and resistin, however, were unexpected given that these adipokines increased initially during the inflammatory phase of healing and only rose again during the final late or remodeling phase for the fusion mass.

Our results indicate that adipokine expression varies significantly during the spinal fusion process. As mentioned previously, there is an established link between adipokines and the low-grade inflammatory state of obesity.[15,16] Therefore, the change in normal homeostasis of adipokine expression at the local and systemic level for obese patients might contribute to the poor clinical outcomes for the obese population.[46,47,48] Further research is needed to determine how adipokine expression at the local or systemic level changes for obese patients undergoing spinal fusion surgery and how these changes might affect clinical outcomes.

The coordinated expression profile of leptin and VEGF suggested that these factors are connected. The high expression of VEGF within the bony remodeling phase of healing as well as the final remodeling

phase of healing (at 10 weeks) has been demonstrated in previous studies.[49,50] Leptin has been shown to increase the expression of the VEGF and the VEGF receptor on mouse mammary cancer cells.[27] Although we did find in vitro reports on the impact of leptin on the VEGF or the VEGF receptor within a fusion mass, our data suggested that this same upstream position of leptin might exist in vivo for bony healing. We found that VEGF expression was more transient than leptin, as it decreased after the initial spike in leptin. Leptin, therefore, may trigger angiogenesis in spinal fusion, as during fracture healing.[51]

Adiponectin expression during the initial reactive response to decortication and bone grafting decreased after Week 1, with a significantly greater decrease than in the interpositional fat graft sites, and our data suggested this drop might be linked to initial bone healing. Previous research has shown contrasting results in terms of the effect of adiponectin on bone remodeling.[52,53,54,55,56] There was a complex role of adiponectin in bone healing, and the impact of adiponectin may depend on the timing and the amount of the adiponectin activity.[52,55] Our results suggested that local expression of adiponectin might precede initial bone reaction during the inflammatory phase of spinal fusion and might be downregulated during the growth of a spinal fusion mass.

Our study has several limitations. We were forced to use *GAPDH* as a housekeeping gene as a control because of our inability to find the correct primer sequence for New Zealand white rabbits for either 18s rRNA or beta-actin mRNA. The authors acknowledge that there may be a variation in the expression of *GAPDH* that could have influenced our results as has been mentioned in previous literature.[57,58] We used the manual palpation examination to assess spinal fusion. Although previous research has demonstrated the validity of this examination, we acknowledge the subjective nature of this outcome.[29,35] In addition to this assessment, we provided radiographic and histologic assessments of bone remodeling.

Our study goal was to measure the variations and patterns of adipokine expression with bone fusion of the spine. To assist with this goal, we normalized our data by several methods, and our results showed similar findings with each of the control comparisons. One comparison for analysis was bone from sham control rabbits at 2 weeks to UL and BL groups, serving to control for general anesthesia and skin incision with muscle dissection. Another comparison for analysis was the lumbar sites cephalad and caudal to L5 and L6 to control for time effects in healthy bone on these same rabbits. The last comparison for analysis was the interpositional fat graft site at the L5–L6 lumbar space, which provided comparative information for the effect of soft-tissue injury, tissue grafting, and, potentially, the retention of limited motion at a surgical site on the adipokine gene expression profile. Given that changes in leptin, adiponectin, resistin, and VEGF were only seen in the decortication and bone fusion masses, it followed that these fluctuations in gene expression were likely associated with the bone fusion process. Future research will be required to examine adipokine expression during the human spinal fusion process and whether strategic influence through adipokine pathways could be effective in accelerating this process. There are still many unanswered questions about the precise interplay of adipokines and bone healing in general and in spinal fusion in particular. We hope our results encourage research into this topic.

In summary, our study reported the temporal changes in expression of adipokines during spinal fusion. Leptin is likely associated with the maturation phase of bone fusion at 3–6 weeks and may play a role in promoting this process as in fracture healing. Adiponectin is associated with the bone reactive and inflammatory phase of bony fusion and may play a role early on in the fusion process. Vascular endothelial growth factor expression was temporally associated with leptin expression. This evidence

supported the hypothesis that leptin expression may be upstream of VEGF expression during spinal fusion. Future research would be required to understand the cascade and interdependence of adipokine expression within the spinal fusion process in humans. Furthermore, these results underscore the varied physiological roles that white adipose tissue plays in bone metabolism through adipokines.

References

C. Herder, S. Schneitler, W. Rathmann, *et al.* **Low-grade inflammation, obesity, and insulin resistance in adolescents** J Clin Endocrinol Metab, 92 (2007), pp. 4569-4574

B.B. Duncan, M.I. Schmidt, J.S. Pankow, *et al.* **Low-grade systemic inflammation and the development of type 2 diabetes the atherosclerosis risk in communities study** Diabetes, 52 (2003), pp. 1799-1805

J. Danesh, P. Whincup, M. Walker, *et al.* **Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses** BMJ, 321 (2000), pp. 199-204

R.C. Koonce, J.T. Bravman **Obesity and osteoarthritis: more than just wear and tear** J Am Acad Orthop Surg, 21 (2013), pp. 161-169

M. Grotle, K.B. Hagen, B. Natvig, F.A. Dahl, T.K. Kvien **Obesity and osteoarthritis in knee, hip and/or hand: an epidemiological study in the general population with 10 years follow-up** BMC Musculoskelet Disord, 9 (2008), pp. 1-5

P. Pottie, N. Presle, B. Terlain, P. Netter, D. Mainard, F. Berenbaum **Obesity and osteoarthritis: more complex than predicted!** Ann Rheum Dis, 65 (2006), pp. 1403-1405

E. Yusuf, R. Nelissen, A. Ioan-Facsinay, *et al.* **Association between weight or body mass index and hand osteoarthritis: a systematic review** Ann Rheum Dis, 69 (2010), pp. 761-765

T.M. Griffin, F. Guilak **Why is obesity associated with osteoarthritis? Insights from mouse models of obesity** Biorheology, 45 (2008), pp. 387-398

F. Guilak, B. Fermor, F.J. Keefe, *et al.* **The role of biomechanics and inflammation in cartilage injury and repair** Clin Orthop Relat Res, 423 (2004), pp. 17-26

Tian G., Liang J.N., Wang Z.Y., Zhou D. **Emerging role of leptin in rheumatoid arthritis** Clin Exp Immunol, 177 (2014), pp. 557-570

S.N. Khan, G. DuRaine, S.S. Virk, *et al.* **The temporal role of leptin within fracture healing and the effect of local application of recombinant leptin on fracture healing** J Orthop Trauma, 27 (2013), pp. 656-662

Cao J.J. **Effects of obesity on bone metabolism** J Orthop Surg Res, 6 (2011), pp. 1-7

A. Goulding, R.W. Taylor, I.E. Jones, K.A. McAuley, P.J. Manning, S.M. Williams **Overweight and obese children have low bone mass and area for their weight** Int J Obes Relat Metab Disord, 24 (2000), pp. 627-632

M. Blum, S.S. Harris, A. Must, *et al.* **Leptin, body composition and bone mineral density in premenopausal women** Calcif Tissue Int, 73 (2003), pp. 27-32

F. Lago, C. Dieguez, J. Gómez-Reino, O. Gualillo **The emerging role of adipokines as mediators of inflammation and immune responses** Cytokine Growth Factor Rev, 18 (2007), pp. 313-325

P. Trayhurn, I.S. Wood **Signalling role of adipose tissue: adipokines and inflammation in obesity** Biochem Soc Trans, 33 (2005), pp. 1078-1081

G. Fantuzzi **Adipose tissue, adipokines, and inflammation** J Allergy Clin Immunol, 115 (2005), pp. 911-919

K.E. Barbour, J.M. Zmuda, R. Boudreau, *et al.* **Adipokines and the risk of fracture in older adults** J Bone Miner Res, 26 (2011), pp. 1568-1576

M. Scotece, J. Conde, R. Gómez, *et al.* **Beyond fat mass: exploring the role of adipokines in rheumatic diseases** ScientificWorldJournal, 11 (2011), pp. 1932-1947

Yan H., Zhang H.W., Fu P., *et al.* **Leptin's effect on accelerated fracture healing after traumatic brain injury** Neurol Res, 35 (2013), pp. 537-544

Wang L., Yuan J.-S., Zhang H.-X., Ding H., Tang X.-G., Wei Y.-Z. **Effect of leptin on bone metabolism in rat model of traumatic brain injury and femoral fracture** Chin J Traumatol, 14 (2011), pp. 7-13

J.E. Reseland, U. Syversen, I. Bakke, *et al.* **Leptin is expressed in and secreted from primary cultures of human osteoblasts and promotes bone mineralization** J Bone Miner Res, 16 (2001), pp. 1426-1433

J.O. Gordeladze, C.A. Drevon, U. Syversen, J.E. Reseland **Leptin stimulates human osteoblastic cell proliferation, de novo collagen synthesis, and mineralization: impact on differentiation markers, apoptosis, and osteoclastic signaling** J Cell Biochem, 85 (2002), pp. 825-836

Wang L., Tang X., Zhang H., Yuan J., Ding H., Wei Y. **Elevated leptin expression in rat model of traumatic spinal cord injury and femoral fracture** J Spinal Cord Med, 34 (2011), pp. 501-509

T. Tanigawa, T. Watanabe, K. Otani, *et al.* **Leptin promotes gastric ulcer healing via upregulation of vascular endothelial growth factor** Digestion, 81 (2010), pp. 86-95

J.F. Whitfield, P. Morley, G.E. Willick **The control of bone growth by parathyroid hormone, leptin, & statins** Crit Rev Eukaryot Gene Expr, 12 (2002)

R.R. Gonzalez, S. Cherfils, M. Escobar, *et al.* **Leptin signaling promotes the growth of mammary tumors and increases the expression of vascular endothelial growth factor (VEGF) and its receptor type two (VEGF-R2)** J Biol Chem, 281 (2006), pp. 26320-26328

Liu Y., Fan Y., Cao D., Zhang J., Wu Z., Qiu G. **Calcitonin enhanced lumbar spinal fusion in a New Zealand rabbit model: a study with morphologic and molecular analysis** Spine, 37 (2012), pp. E139-46

S.D. Boden, J.H. Schimandle, W.C. Hutton **An experimental lumbar intertransverse process spinal fusion model. Radiographic, histologic, and biomechanical healing characteristics** Spine, 20 (1995), pp. 412-420

S.D. Boden, J.H. Schimandle, W.C. Hutton **1995 Volvo Award in basic sciences. The use of an osteoinductive growth factor for lumbar spinal fusion. Part II: study of dose, carrier, and species** Spine, 20 (1995), pp. 2633-2644

S.D. Daffner, S. Waugh, T.L. Norman, N. Mukherjee, J.C. France **Effect of serum nicotine level on posterior spinal fusion in an in vivo rabbit model** Spine J, 15 (2015), pp. 1402-1408

M. Kanayama, B.W. Cunningham, J.C. Sefter, *et al.* **Does spinal instrumentation influence the healing process of posterolateral spinal fusion? An in vivo animal model** Spine, 24 (1999), pp. 1058-1065

J. Louis-Ugbo, H. Murakami, H.S. Kim, A. Minamide, S.D. Boden **Evidence of osteoinduction by Grafton demineralized bone matrix in nonhuman primate spinal fusion** Spine, 29 (2004), pp. 360-366
discussion Z1

M.J. Mermer, M.C. Gupta, D.L. Wheeler, *et al.* **Efficacy of osteogenic protein-1 in a challenging multilevel fusion model** Spine, 29 (2004), pp. 249-256

A.M. Riordan, R. Rangarajan, J.W. Balts, W.K. Hsu, P.A. Anderson **Reliability of the rabbit postero-lateral spinal fusion model: a meta-analysis** J Orthop Res, 31 (2013), pp. 1261-1269

U.M. Ayturk, C.M. Jacobsen, D.C. Christodoulou, *et al.* **An RNA-seq protocol to identify mRNA expression changes in mouse diaphyseal bone: applications in mice with bone property altering Lrp5 mutations** J Bone Miner Res, 28 (2013), pp. 2081-2093

D. Seol, H. Choe, Zheng H., *et al.* **Selection of reference genes for normalization of quantitative real-time PCR in organ culture of the rat and rabbit intervertebral disc** BMC Res Notes, 4 (2011), p. 162

Li N., C. Whitaker, Xu Z., M. Heggeness, Yang S.Y. **Therapeutic effects of naringin on degenerative human nucleus pulposus cells for discogenic low back pain** Spine J, 16 (2016), pp. 1231-1237

T.D. Schmittgen, K.J. Livak **Analyzing real-time PCR data by the comparative CT method** Nat Protoc, 3 (2008), pp. 1101-1108

C.L. Duvall, W.R. Taylor, D. Weiss, A.M. Wojtowicz, R.E. Guldberg **Impaired angiogenesis, early callus formation, and late stage remodeling in fracture healing of osteopontin-deficient mice** J Bone Miner Res, 22 (2007), pp. 286-297

A. Schindeler, M.M. McDonald, P. Bokko, D.G. Little **Bone remodeling during fracture repair: the cellular picture** Semin Cell Dev Biol, 19 (2008), pp. 459-466

J. Upadhyay, O.M. Farr, C.S. Mantzoros **The role of leptin in regulating bone metabolism** Metabolism, 64 (2015), pp. 105-113

T. Rőszer, T. Józsa, É.D. Kiss-Tóth, N. De Clerck, L. Balogh **Leptin receptor deficient diabetic (db/db) mice are compromised in postnatal bone regeneration** Cell Tissue Res, 356 (2014), pp. 195-206

G.A. Williams, K.E. Callon, M. Watson, *et al.* **Skeletal phenotype of the leptin receptor-deficient db/db mouse** J Bone Miner Res, 26 (2011), pp. 1698-1709

K.N. Ealey, D. Fonseca, M.C. Archer, W.E. Ward **Bone abnormalities in adolescent leptin-deficient mice** Regul Pept, 136 (2006), pp. 9-13

R. De la Garza-Ramos, M. Bydon, N.B. Abt, *et al.* **The impact of obesity on short- and long-term outcomes after lumbar fusion** Spine, 40 (2015), pp. 56-61

D.C. Sing, Yue J.K., L.N. Metz, *et al.* **Obesity is an independent risk factor of early complications after revision spine Surgery** Spine, 41 (2016), pp. E632-40

J.J. McClendon, T.R. Smith, S.E. Thompson, *et al.* **The impact of body mass index on hospital stay and complications after spinal fusion** Neurosurgery, 74 (2014), pp. 42-50

B. Beamer, C. Hettrich, J. Lane **Vascular endothelial growth factor: an essential component of angiogenesis and fracture healing** HSS J, 6 (2010), pp. 85-94

K. Sarahrudi, A. Thomas, T. Braunsteiner, H. Wolf, V. Vecsei, S. Aharinejad **VEGF serum concentrations in patients with long bone fractures: a comparison between impaired and normal fracture healing** J Orthop Res, 27 (2009), pp. 1293-1297

J. Street, Bao M., L. deGuzman, *et al.* **Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover** Proc Natl Acad Sci USA, 99 (2002), pp. 9656-9661

K.N. Ealey, J. Kaludjerovic, M.C. Archer, W.E. Ward

Adiponectin is a negative regulator of bone mineral and bone strength in growing mice

Exp Biol Med (Maywood), 233 (2008), pp. 1546-1553 I. Kanazawa **Adiponectin in metabolic bone disease** Curr Med Chem, 19 (2012), pp. 5481-5492

I. Kanazawa, T. Yamaguchi, M. Yamamoto, M. Yamauchi, S. Yano, T. Sugimoto **Relationships between serum adiponectin levels versus bone mineral density, bone metabolic markers, and vertebral fractures in type 2 diabetes mellitus** Eur J Endocrinol, 160 (2009), pp. 265-273

A. Lubkowska, A. Dobek, J. Mieszkowski, W. Garczynski, D. Chlubek **Adiponectin as a biomarker of osteoporosis in postmenopausal women: controversies** Dis Markers, 2014 (2014)

Y. Shinoda, M. Yamaguchi, N. Ogata, *et al.* **Regulation of bone formation by adiponectin through autocrine/paracrine and endocrine pathways** J Cell Biochem, 99 (2006), pp. 196-208

C. Tricarico, P. Pinzani, S. Bianchi, *et al.* **Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies** Anal Biochem, 309 (2002), pp. 293-300

R.D. Barber, D.W. Harmer, R.A. Coleman, B.J. Clark **GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues** Physiol Genomics, 21 (2005), pp. 389-395