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Title: Zinc has Insulin-mimetic Properties which Enhance Spinal Fusion in a Rat Model

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Abstract

Background Context: Previous studies have found that insulin or insulin-like growth factor treatment can stimulate fracture healing in diabetic and normal animal models, and increase fusion rates in a rat spinal fusion model. Insulin-mimetic agents, such as zinc, have demonstrated anti-diabetic effects in animal and

human studies, and these agents that mimic the effects of insulin could produce the same beneficial effects on bone regeneration and spinal fusion.

Purpose: The purpose of this study was to analyze the effects of locally applied zinc on spinal fusion in a rat model.

Study Design/Setting: IACUC approved animal study using Sprague-Dawley Rats

Methods: 30 Sprague-Dawley rats (450-500g) underwent L4-L5 posterolateral lumbar fusion (PLF). After decortication and application of approximately 0.3 g of autograft per side, one of three pellets were added to each site: high dose Zinc Calcium Sulfate (ZnCaSO4), low dose ZnCaSO4 (half of the high dose), or a control palmitic acid pellet (no Zn dose). Systemic blood glucose levels were measured 24 hours postoperatively. Rats were sacrificed after 8 weeks and the PLFs analyzed qualitatively by manual palpation and radiograph review, and quantitatively by micro-computed tomography (CT) analysis of bone volume and trabecular thickness. Statistical analyses with p-values set at 0.05 were accomplished with ANOVA, followed by post-hoc tests for quantitative data, or Mann-Whitney Rank tests for qualitative assessments. **No external funds were received in support of this work.**

Results: Compared with controls, the low dose zinc group demonstrated a significantly higher manual palpation grade ($p=0.011$), radiographic score ($p=0.045$), and bone formation on microCT (172.9 mm^3 vs. 126.7 mm^3 for controls) ($p<0.01$). The high dose zinc also demonstrated a significantly higher radiographic score ($p=0.017$) and bone formation on microCT (172.7 mm^3 vs. 126.7 mm^3) ($p<0.01$) versus controls, and was trending towards higher manual palpation scores ($p=0.058$).

Conclusions: This study demonstrates the potential benefit of a locally applied insulin-mimetic agent, such as zinc, in a rat lumbar fusion model. Previous studies

have demonstrated the benefits of local insulin application in the same model, and it appears that zinc has similar effects**.**

Introduction

Spinal fusion is a common treatment for many spinal disorders, and agents that can improve fusion rates and decrease time until fusion can help limit morbidity associated with pseudoarthrosis. Previous studies have found that insulin and insulin-like growth factor treatment can stimulate fracture healing in diabetic and normal animal models[1,2]. Our laboratory has previously demonstrated the ability of local insulin to enhance posterolateral fusion in a rat model[3]. Several metals, such as vanadium and zinc have been shown to exert insulin-mimetic effects in isolated cells, tissues and diabetic animal models[4,5]. Recently, our laboratory demonstrated that locally applied zinc accelerated healing in a rat femur fracture model[6]. We hypothesized that local administration of an insulin-mimetic agent such as zinc, would enhance spinal fusion in a rat model. To our knowledge, no in vivo evaluation of therapy on spinal fusion by local administration of an insulin-mimetic agent has been performed.

Methods:

Study Design

The protocol was approved by the Institutional Animal Care and Use Committee at the UMDNJ-New Jersey Medical School, now known as Rutgers Biomedical Health Sciences. This study was part of a larger project which investigated the effects of local insulin application to lumbar spinal fusions in a rat model[3]. A power analysis determined that to detect a 30% difference between groups, 10 animals would be needed in each group. Thirty skeletally mature Sprague-Dawley Rats (10 per group) weighing approximately 450-500g underwent L4-L5 posterolateral intertransverse lumbar fusion with iliac crest autograft. After decortication and application of approximately 0.3g of autograft per side, one of three pellets were added to each fusion site: a low dose Zinc Calcium Sulfate pellet (0.25 mg.kg per side, 0.5 mg/kg total), a high dose Zinc Calcium Sulfate pellet (0.5 mg/kg per side, 1.0 mg/kg total),

or a control of micro-recrystallized palmitic acid pellet (Linshin Canada, Inc., ON, Canada). Systemic blood glucose levels were measured at 24 hours postoperatively. Animals were sacrificed at 8 weeks and analyzed qualitatively by two blinded independent observers with radiographs and manual palpation, as well as quantitatively by microCT analysis. All outcome parameters were independently reviewed by two separate individuals in a blinded manner. For categorical variables (radiographic and manual palpation scoring), the lower of the two grades were used for analysis when there was a discrepancy.

Surgical Procedure

After obtaining general anesthesia with intraperitoneal ketamine (40 mg/kg) and xylazine (5 mg/kg), the lumbar region of the rat was shaved and cleansed with povidone iodine-soaked gauze. A dorsal midline incision was made from L3 to the sacrum. Two paramedian incisions were made through the lumbar fascia 5 mm from the midline. Dissection was taken to the iliac crest, and approximately 0.3 g of bone was harvested with small rongeurs. The harvested autograft was measured on a sterile scale to obtain 0.3 g per side. Blunt dissection was carried down posterolaterally, reflecting the paraspinal muscles lateral to the facet joints on each side. The reflected paraspinal muscles were held in place with retractors. The transverse processes of L4–L5 were stripped of soft tissue and decorticated with a high-speed burr. The crushed autograft was then spread over and between the transverse processes at the appropriate level (L4–L5). One of the two test substances or blank was incorporated into the autograft bed. Retractors were removed and the paraspinal muscles were allowed to cover the fusion bed. The dorsal lumbar fascia was closed using a running 4-0 resorbable suture, and the skin was closed with interrupted 4-0 resorbable sutures. The surgical site was treated with an antibiotic ointment, and the rats were given a dose of enrofloxacin antibiotic (10 mg/kg). Radiographs were taken immediately after surgery.

Zinc Pellet Preparation

In order to prepare the pellets, 0.2 mL of each stock solution was mixed with 0.4 g of CaSO4 to obtain the appropriate consistency of the carrier in a 1 mL syringe. It was then injected into 2mm diameter clear Tygon laboratory tubing and allowed to harden overnight. Once set, pellets were sectioned into 7mm pieces and autoclaved (to sterilize), prior to implantation. The weight of each rat was assumed to be 450g for dosage calculation. As such, the low dose Zn group (0.5 mg/kg) received a total dose of 0.225 mg which was divided in half and applied to the left and right posterolateral fusion beds. The high dose Zn group (1.0 mg/kg) received a total dose of 0.45 mg divided in half between the left and right posterolateral fusion beds. In order to prepare the stock solution, the volume of solution in each pellet was calculated by using the volume ratio of solution to mixture.

Manual palpation

After removal of all soft tissue, two blinded independent observers manually palpated and stressed across the fusion site (L4-L5). Specimens were graded as fused (A), partially fused (B), or not fused (C). For any discrepancies between observers, the lower of the two grades was used for statistical analysis.

Radiographic analysis

Posteroanterior radiographs at 35 kV for 90 seconds were taken at 8 weeks after sacrifice and harvest. All soft tissue was removed before radiographic examination. Two blinded independent observers graded the radiographs as solid fusion mass bilaterally (A), unilateral fusion mass (B), small fusion mass bilaterally (C), and graft resorption (D) based on previously published radiographic scales [7]. For any discrepancies between observers, the lower of the two grades was used for statistical analysis.

Quantitative MicroCT analysis

Spines harvested at 8 weeks also underwent scanning by micro-CT (Bruker SkyScan 1172; Kontich, Belgium; 80 keV, 126 μA) and subsequent analysis in CTAn software

(Bruker; v.1.15) to quantitatively calculate new bone formation, including trabecular thickness (Tb.Th). L4-L5 segments were submerged in saline and scanned, one at a time, at an isotropic voxel resolution of 17 μ m. Regions of interest (ROI) were demarcated from the top of the L4 transverse process cephalad to the bottom of the L5 transverse process caudally, including any bone lateral to a vertical line connecting the pars of the involved vertebrae. The bone volume in these bilateral ROI for each specimen were quantified. CTAn 3D analysis was completed on the new bone for Tb.Th in a smaller mid-coronal ROI that was the same size for all specimens, and averaged over slices within a 1 mm thickness.

Statistical Analysis

Mann-Whitney Rank tests were performed for the analysis of radiographs and manual palpation. Analysis of variance (ANOVA) was completed for MicroCT mean bone volumes and trabecular thickness of control and treatment groups and to compare blood glucose levels at 24 hours. Statistical analysis was performed using SigmaPlot 12.5 software (Systat Software, Inc., San Jose, CA). Statistical significance was assumed at $p<0.05$.

No external funds were received in support of this work. Several of the authors are listed as inventors on a related patent application for which notification of recordation was issued November 9, 2015. Some of the authors are co-founders of CreOsso, LLC., an entity formed to license related intellectual property from the investigators' affiliated university.

Results

One of the control group rats died on postoperative day one, likely due to anaesthesia. The mean systemic blood glucose levels at 24 hours were not significantly different between groups (Table 1).

Radiographic analysis

Based on radiographs, 2 of 9 controls had a solid fusion mass bilaterally, 3 of 9 had unilateral fusion mass, 1 of 9 had small fusion mass bilaterally, and 3 of 9 had graft resorption. The high dose zinc group had 7 of 10 solid fusion masses bilaterally, 3 of 10 had unilateral fusion, 0 of 10 had small fusion masses bilaterally, and 0 of 10 had graft resorption (p=0.017). The low dose zinc group had 7 of 10 solid fusion mass bilaterally, 1 of 10 had unilateral fusion, 2 of 10 had small fusion mass bilaterally, and 0 of 10 had graft resorption (p=0.045). While both zinc groups were significantly better than the control group, there was no significant difference between the high and low dose zinc groups (p=0.815). (Figure 1 Table 2, Figures 2-4)

Manual palpation test

Based on manual palpation, none of the controls were graded as a solid fusion, 1 of 9 was partially fused, and 8 of 9 were not fused. In the high dose Zinc group, 4 of 10 had solid fusion, 1 of 10 had partially fused, and 5 of 10 were not fused (p=0.058). In the low dose Zinc group, 3 of 10 had solid fusion, 4 of 10 had partially fused, and 3 of 10 were not fused (p=0.011). There was no significant difference between the high and low dose zinc groups (p=0.809). (Table 3, Figure 5)

Quantitative Micro-CT analysis

Based on MicroCT analysis, the mean bone volume of the L4/L5 transverse processes and fusion mass for controls was 126.7mm^3. The high dose Zinc group had a mean of 172.7mm^3, and the low dose Zinc group had a mean of 172.9mm^3. Both the high dose (p=0.002) and low dose zinc groups (p=0.003) were significantly higher than control. (Table 4) The mean trabecular thickness for the control group was 0.144mm. The high dose Zinc group had a mean trabecular thickness of 0.142mm (p= 0.988) and the low dose Zinc group had a mean of 0.164mm (p=0.056) (Table 4)(Figure**s** 6**,7**).

Discussion

Pseudarthrosis following spinal fusion procedures is an undesirable outcome, and local adjuncts to help prevent this complication are of significant interest. Our laboratory previously reported that locally applied insulin enhanced posterolateral lumbar fusion in this same rat model[3]. This study demonstrates the potential benefit of a local insulin-mimetic agent applied to the fusion bed in a rat posterolateral intertransverse lumbar fusion model. To our knowledge this is the first study to examine the effects of local zinc on lumbar spinal fusion. Potential advantages of insulin mimetics over insulin for this application include avoiding incompatibility between drug and delivery systems as seen in protein therapeutics, enhanced stability, decreased manufacturing costs, and decreased potential for hypoglycemia.

Zinc, in the form of zinc chloride, has been recognized to be insulin-mimetic in its ability to stimulate lipogenesis in rat adipocytes[7], and numerous studies have been done demonstrating its relation to diabetes[8]. Vardatsikos et al recently performed an in depth review of the insulin-mimetic and anti-diabetic effects of zinc[9]. Multiple in vitro studies have demonstrated beneficial effects of zinc on bone formation[10-13]. Our laboratory recently demonstrated that local administration of zinc accelerates bone formation without a systemic effect on blood glucose[6]. In this study, utilizing a rat model, femur fractures treated with zinc demonstrated increased mechanical properties, more cortical bridging, and increased mineralized tissue compared to controls. Cell proliferation was increased in both the subperiosteal and gap callus regions. Additionally, vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) levels within the fracture callus were increased by local zinc administration. Zinc has also been studied as an addition to hydroxyapatite, where it demonstrated an increase in the growth of human adipose-derived mesenchymal stem cells and bone cell differentiation markers in vitro[14,15]. The mechanism by which zinc exerts insulin-like effects includes activation of insulin signaling pathways including extracellular signal-regulated kinase 1/2, and phosphatidylinositol 3-kinase/protein kinase B/Akt pathways[9]. While our study did

not investigate the mechanism of action of zinc, it may be similar to these previously established pathways.

We recognize limitations to this study. As part of a larger study exploring the effects of local insulin in the same model[3], we concluded the control surgeries and harvests before some of the presented experimental groups due to logistical issues. The surgical team and technique were identical but this block allotment resulted in unblinding of the surgical team. The evaluators, however, were blinded to the various groups at the time of the manual palpation and radiographic scoring. We used a palmitic acid pellet as a control because of similar characteristics to the implant used to deliver locally applied insulin. We did not include an additional control group with a blank calcium sulfate pellet because we desired to limit the total number of animals utilized and because we believe that neither a blank calcium sulfate nor palmitic acid pellet alone would enhance fusion in this setting. At the time of harvest, some of the pellets in both control and experimental groups had not completely dissolved. The clinical effects of this observation are unknown, and future studies will determine the optimal carrier and dosage. We did note that the manual palpation results did not always match the radiographic and MicroCT analyses. It is possible that in some cases more pronounced bone formation seen on image analyses did not uniformly result in solid arthrodesis.

Biomechanical tests were not performed in this study, which could have added to our findings. In our previous studies, we performed biomechanical testing in a femur fracture model, which is a torsional test. This three point bending test needs a relatively long specimen length to thickness ratio, or the experiment will be testing shear and bending[16]. In the spinal fusion small animal model, a 4-point bending test has recently been described as the most representative for small animal spine experiments[16-18]. The description of the 4-point bending model was only recently published, therefore we were not aware at the time of our experiment. However in their study, Robinson et al. found results of the 4-point bending model to be consistent with their preliminary grading according to manual palpation[16] Other

studies have also found that the results of manual palpation correlate with quantitative results obtained from biomechanical testing[17,19]. Future studies however, will include biomechanical testing as well as histological analysis of the fusion and surrounding tissues.

While some studies have also presented a ratio of bone volume/total volume (BV/TV), others have suggested that this measurement may be inappropriate in models using iliac crest autograft [17]. At the typical sacrifice time frame, the fusion beds contain both new bone and residual mineralized graft, which cannot be reliably distinguished using microCT. This differs from studies looking at demineralized bone matrix (DBM) products or bone morphogenetic protein (BMP) on a collagen sponge, where any bone seen between the transverse processes is new bone, while residual DBM or collagen sponge will have different density on the microCT.

Based on this study, zinc demonstrated beneficial effects compared to autograft controls at both dosages tested. While the fusion rate of our control group was low, this is comparable to other autograft fusion rates in a rat model[20-23]. Radiographically, both zinc groups had significantly higher fusion rates. In an effort to eliminate some of the subjective nature of radiographic and manual palpation scoring, MicroCT was used to quantitatively determine new bone formation. Each test group scored significantly higher than the control group.

The rat posterolateral lumbar fusion model has been used repeatedly for preliminary investigations of spinal fusion due to its cost-effectiveness, reproducibility, and the rat's resistance to infection[21]. The model has been proven to be predictive of human clinical results. In fact, some clinical practices, such as avoidance of nonsteroidal anti-inflammatory drugs (NSAIDS) after spinal fusion are based on preliminary studies using the rat model[20]. Many of the preliminary studies of BMP-2 and BMP-7 utilized the rat model, which eventually demonstrated similar results in clinical trials[23-26]. It is unknown how zinc would affect fusion in humans, however our results from the rat model in the beginning stages of "burden of proof"

[27] are promising and have instigated further study with animal models that more closely mimic human conditions.

This study is the first to examine the effects of a locally applied insulin-mimetic, such as zinc, in a rat spinal fusion model. The results are promising, and future work will focus on the optimal dosage and carrier, as well as examining the mechanism by which insulin-mimetics affect spinal fusion. Theoretically zinc could supplement either autograft or allograft in posterolateral lumbar fusion in humans, however further animal studies need to be performed before considering a human trial.

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Figures Legend

Figure 1: Radiographic Scoring

Figure 2: AP Radiograph of a Control Spine

Figure 3: AP Radiograph of a low-dose Zinc treated Spine

Figure 4: AP Radiograph of a high-dose Zinc treated Spine

Figure 5: Manual Palpation Results

Figure 6: 3D Reconstruction of a low-dose Zinc treated Spine

Figure 7: Sequential axial micro-CT images for Control (A-B), low-dose Zinc (C-D), and high-dose Zinc (E-F) samples

Figure 1

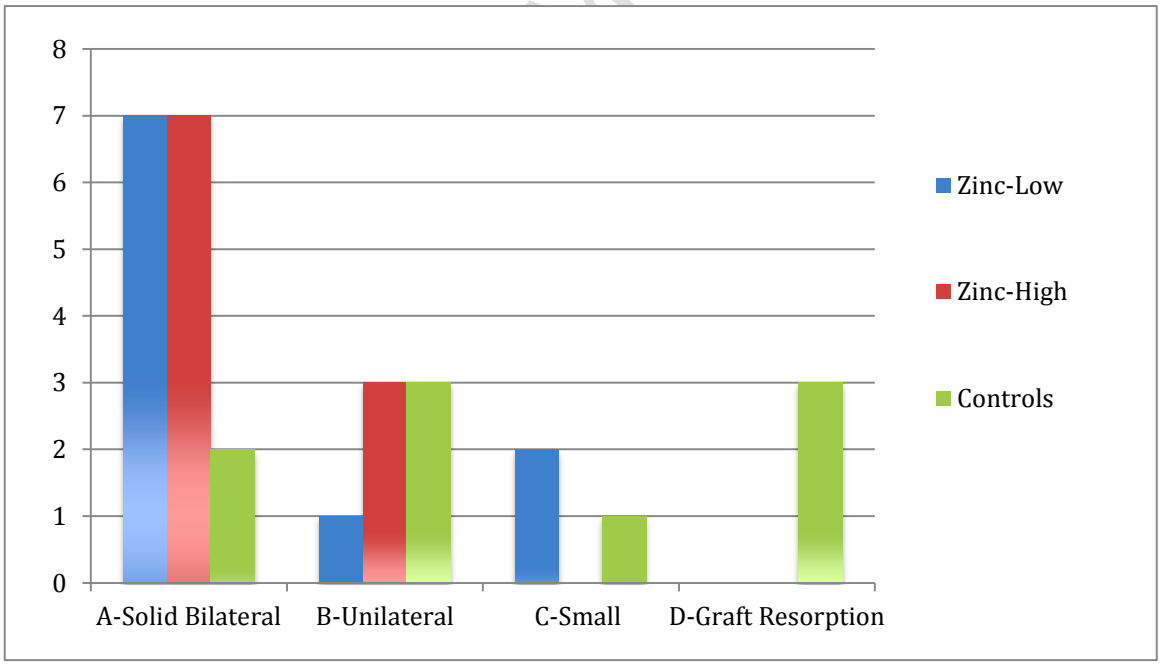

Figure 5

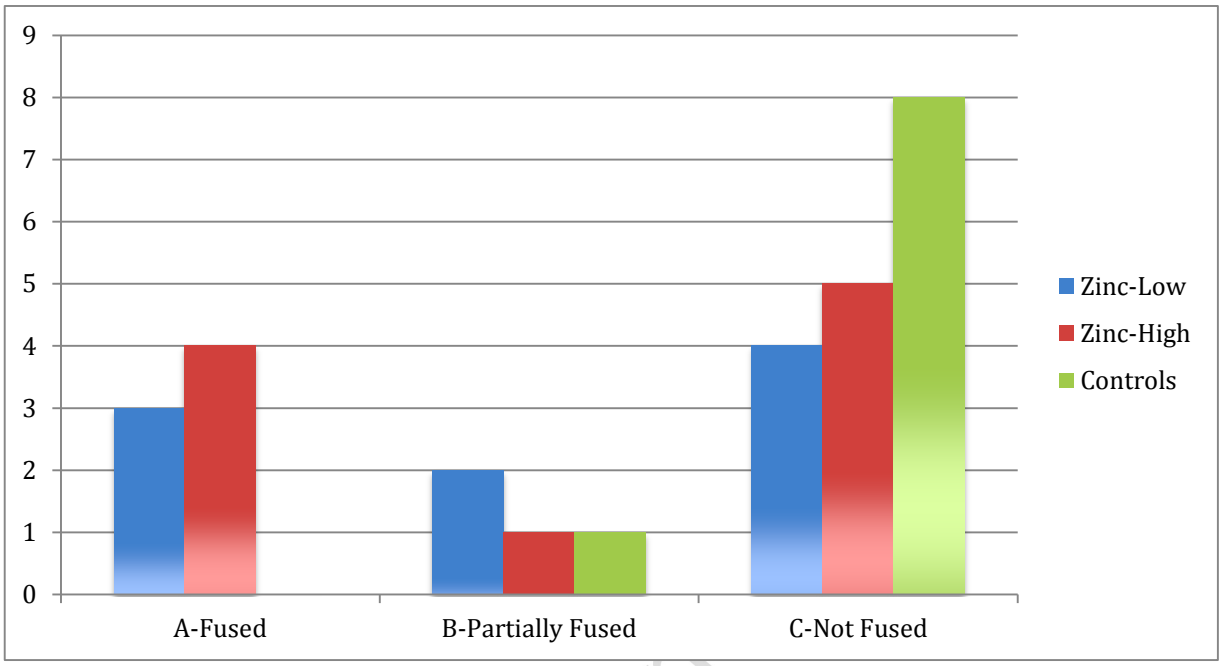

Table 1: Systemic Blood Glucose Levels at 24 hours

Group	Mean Systemic Blood Glucose at 24 hours (mg/dL) (Standard deviation)
Controls(n=9)	91.4 (+/- 12.20)
Zn-low(n=10)	101.8 (+/- 34.01)
Zn-high(n=10)	89.0 (+/- 13.92)

NS differences between groups, P=0.421

Table 2: Radiographic scoring

Group	Bilateral Fusion	Unilateral Fusion	Small Fusion Mass	Graft Resorption	P Value (vs control)
Controls (n=9)	2	3	1	3	
Zn-low (n=10)	7	1	2	0	0.045
Zn-high (n=10)	7	3	0	0	0.017

NS difference between low and high dose zinc groups, p=0.815

Table 3: Manual palpation results

Group	Fused	Partially Fused	Not Fused	P Value (vs control)
Controls (n=9)	0	1	8	
Zn-low (n=10)	3	4	3	0.011
Zn-high (n=10)	4	1	5	0.058

NS difference between low and high dose zinc groups, p=0.809

Table 4: Mean Bone Volume (mm^3) and Mean Trabecular Thickness (mm) on MicroCT

Group	Mean Bone Volume mm^3	Std Dev	P value (vs control)	Mean Trabecular Thickness mm	Std Dev	P value (vs control)
Controls (n=9)	126.7	26.3		0.144	0.0188	
Zn low dose (n=10)	172.9	31.6	0.003	0.164	0.0238	0.056
Zn high dose (n=10)	172.7	26.4	0.002	0.142	0.0374	0.988