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Short communication

Design of experiments confirms optimization of lithium administration parameters for enhanced fracture healing

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ABSTRACT

Fracture healing is a lengthy process which fails in 5–10% of cases. Lithium, a low-cost therapeutic used in psychiatric medicine, up-regulates the canonical Wingless pathway crucial for osteoblastic mineralization in fracture healing. A design-of-experiments (DOE) methodology was used to optimize lithium administration parameters (dose, onset time and treatment duration) to enhance healing in a rat femoral fracture model. In the previously completed first stage (screening), onset time was found to significantly impact healing, with later (day 7 vs. day 3 post-fracture) treatment yielding improved maximum yield torque. The greatest strength was found in healing femurs treated at day 7 post fracture, with a low lithium dose (20 mg/kg) for 2 weeks duration. This paper describes the findings of the second (optimization) and third (verification) stages of the DOE investigation. Closed traumatic diaphyseal femur fractures were induced in 3-month old rats. Healing was evaluated on day 28 post fracture by CT-based morphometry and torsional loading. In optimization, later onset times of day 10 and 14 did not perform as well as day 7 onset. As such, efficacy of the best regimen (20 mg/kg dose given at day 7 onset for 2 weeks duration) was reassessed in a distinct cohort of animals to complete the DOE verification. A significant 44% higher maximum yield torque (primary outcome) was seen with optimized lithium treatment vs. controls, which paralleled the 46% improvement seen in the screening stage. Successful completion of this robustly designed preclinical DOE study delineates the optimal lithium regimen for enhancing preclinical long-bone fracture healing.

1. Introduction

Skeletal fractures affect 2% of the population annually. The regenerative process of fracture repair is complex with predicted healing failing in 5–10% of cases (Hoeppner et al., 2009; MacKenzie et al., 2006). Despite the identification of potential therapeutic targets, cost effective treatments are lacking which clinically accelerate healing. Endochondral fracture healing involves well-orchestrated progression through the stages of cartilaginous callus formation, callus transition from chondrocytic to osteoblastic type and mineralized woven bone formation. The canonical Wingless (Wnt) pathway is a key modulator in this process- it signals for chondrocyte hypertrophy and up-regulates osteoblastic activity until woven callus develops (Dong et al., 2006; Fig. 1). Lithium, a long standing psychotropic medicine, inhibits glycogen synthase kinase-3β (GSK-3β) in the Wnt pathway promoting β-catenin-induced osteoblastic gene expression (Hedgepeth et al., 1997). Lithium has been shown to positively impact bone formation as documented in patients with bipolar disorder on therapeutic doses of lithium (Vestergaard et al., 2005; Zamani et al., 2009). To optimize lithium administration in the context of fracture healing requires delineation of treatment dose, onset time and duration. A design-of-experiments (DOE) approach was utilized to simultaneously evaluate these three parameters, enabling quantification of the impact of each parameter and their potential interactions, and minimizing animal use.

In this, a preclinical study was previously performed to carry out the first of three stages (screening) (Bernick et al., 2014). Using a rat femoral fracture model, eight combinations of low and high level lithium dose, onset and duration along with a ninth group at middle parameter levels (to account for possible nonlinear responses) were evaluated and compared to controls (no treatment and saline-only treatment) (Fig. 2). After 28 days, biomechanical testing (primary outcome of maximum yield torque) and μCT-based 3D morphometry of the femurs were evaluated. Onset time was found to be the only significant parameter with 21% improvement in torque

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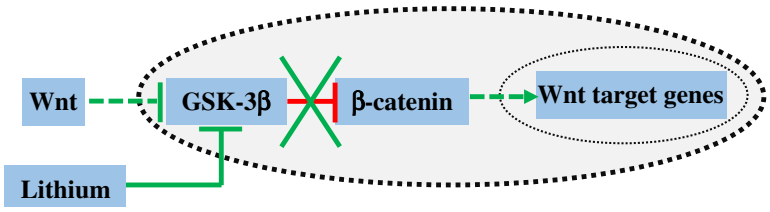

Post-fracture Day (D)	Healing Event at Fracture Site	Canonical Wnts	β-catenin	Wnt target genes
D 0-3	Hematoma formation, inflammation by leukocytes	-	-	-
D 3-5	Intramembranous ossification begins away from gap MSCs differentiate to chondrocytes Chondrogenesis begins at the gap	↑	↑	↑
D 6-10	Cartilaginous callus evident, chondrocytes mature MSCs differentiate to osteoblasts Endochondral ossification begins ~day 8	↑↑↑	↑↑↑	↑↑↑
D 11-15	Chondrocytes hypertrophy and resorbed Osteoblasts proliferate and callus mineralizes	↑↑	↑↑↑	↑↑↑
D 16-20	Osteoblasts proliferate; mineralization continues Callus remodeling begins	↑	↑↑	↑↑
D 21-25	Woven bone callus formation completes Remodeling continues	↑	↑	↑

Fig. 1. A simplified schematic of the canonical Wnt pathway (top). In this signaling cascade, extracellular expression of canonical Wnts (Wnt 1, 2, 3b, 4, 8 and 10b) leads to intracellular inhibition of GSK-3β and its phosphorylating action on β-catenin. Subsequently, β-catenin accumulates and translocates into the nucleus, thereby inducing transcription of Wnt target (osteogenic) genes. With lithium treatment, the pathway activity is enhanced as lithium competitively binds to the Mg^{2+} site on GSK-3β molecule and further inhibits β-catenin phosphorylation. Stages of endochondral fracture healing and associated activity of Wnt/β-catenin pathway in rats (bottom). Cell recruitment begins immediately post fracture. Away from the gap, periosteal cells activate and osteoblasts initiate hard callus formation on intact bone surface via intramembranous ossification. At the gap, mesenchymal stem cells (MSC) commit to chondrocytic lineage and cartilaginous plug callus begins to form as the canonical Wnt activity remains low. At ~day 5, Wnt signaling increases leading to elevation in β-catenin expression. Consequently, chondrocytic differentiation halts and MSC differentiation is diverted to osteoblastic lineage. The ensuing proliferation of osteoblastic cells and mineral deposition marks the transition in callus composition, first evident at ~day 8. Osteoblastic activity peaks around day 10 and remains elevated until day 20. Callus ossification then slows down and canonical Wnt signaling drops. Remodelling of the woven bone becomes the predominant activity at the fracture site.

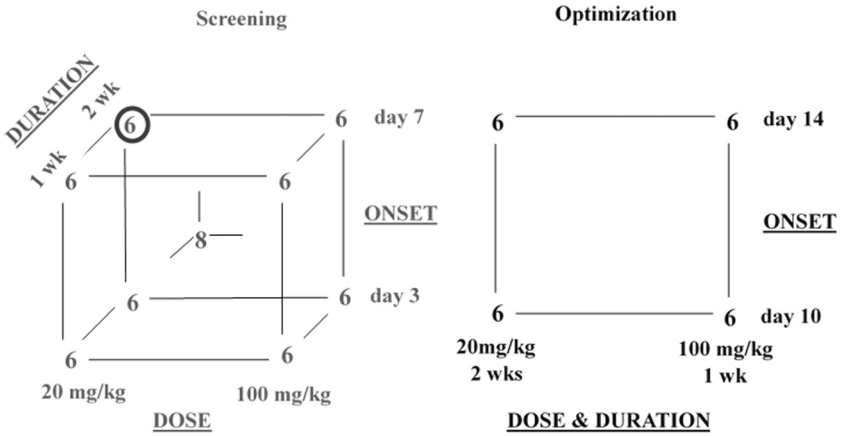

Fig. 2. Design-of-experiments model for stage 2 optimization (right, black), based on the previously completed stage 1 screening (left, grey). The corners denote the treatment combinations and the numbers represent their respective group size. The screening stage evaluated lithium dose, onset and duration at their respective low and high values. An additional group was treated at the mid-values of the three parameters to evaluate for non-linear interactions. Saline (n=6) and no treatment control (n=6) groups are not shown. Onset time was the only significant parameter identified in the screening stage, thus onset time was the focus of the optimization stage. Low dose was combined with high duration and vice versa as combination of opposing levels of dose and duration in screening had resulted in highest and lowest outcomes, respectively. The optimal treatment based on the screening and optimization results (circled: 7 day onset, 20mg/kg dose, 2 week duration) was re-evaluated in the verification stage against saline-treated controls (n=10/group).