

Published in final edited form as:

*J Orthop Trauma*. 2010 September ; 24(9): 571–576. doi:10.1097/BOT.0b013e3181ec4712.

## Protective effect of P188 in the Model of Acute Trauma to Human Ankle Cartilage: the Mechanism of Action

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### Abstract

**Objective**—Since P188 poloxamer is effective in promoting cell survival in models of acute trauma the objectives were to understand the mechanism of its action focusing on GSK3 activation, IL-6 and p38 signaling.

**Design**—Sixteen normal human tali were impacted using 4mm diameter indenter with an impulse of 1Ns. 8mm cartilage plugs containing the 4mm impacted core and 4mm adjacent non-impacted ring were removed and cultured with or without P188. Cell lysates were analyzed using Western blots with antibodies against total and phosphorylated ERK, JNK, p38, ATF-2, GSK3, Stat1 and Stat3. Additional tests were performed with the p38 inhibitor (p38i) SB203580.

**Results**—Studied pathways were activated after impaction with the peak of activity at 1hour. P188 completely attenuated phosphorylation of Stat1 and ATF-2 and inhibited p38, Stat3, JNK, ERK, and GSK3. The p38i partially offset phosphorylation of Stat3, GSK3, and ERK suggesting a role of p38 in these three pathways. Additionally, the p38i improved cell survival ( $p=0.053$ ) and reduced apoptosis (by about 20%,  $p=0.046$ , vs almost 40% by P188) thus confirming that P188 acts (at least in part) via p38 pathway.

**Conclusion**—Our results report a novel mechanism through which P188 exerts its protective effects on cartilage in the model of acute injury. In addition to its effect on cellular membrane, P188 affects stress-related p38 signaling, apoptosis-related GSK3, and inflammation-related IL-6

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### Conflict of interest statement

None of the authors have financial or personal relationships with other people or organizations that could inappropriately influence (bias) this work.

signaling. Taken together these findings suggest that P188 alone or in combination with pro-anabolic agents may have a therapeutic potential in preventing progressive cartilage degeneration and the development of post-traumatic osteoarthritis.

## Keywords

chondrocyte viability; apoptosis; histology; impact; ankle cartilage

## Introduction

While joint injury is a major factor in the development and progression of secondary osteoarthritis (OA), the mechanisms that leads from injury to OA remain unknown<sup>1</sup>. A recent study reported that 13 to 18% of total hip or knee patients had an identifiable acute trauma to the joint<sup>2</sup>. Furthermore, about 12% of the overall prevalence of OA could be attributed to post-traumatic OA in the joints of lower extremities<sup>3</sup>. Although its end-stage is indistinguishable from idiopathic OA, patients with post-traumatic OA can be up to 2 decades younger and they have a well-defined precipitating insult. These patients have no effective early interventions since the immediate cellular responses to acute trauma are far from being understood.

Recent studies have shown that acute cartilage injury induces cell death that may contribute to the subsequent development of post-traumatic OA<sup>3,4</sup>, treatment for which remains one of the main challenges faced by orthopedists. Poor intrinsic regenerative capacity of hyaline cartilage as well as inconsistent results with existing treatments have stimulated efforts to better understand cellular responses to acute trauma in order to develop novel strategies that may protect cartilage from degradation and prevent or delay the development of secondary OA.

It has been reported that mechanical injury to cartilage can result in progressive cartilage degradation accompanied by chondrocyte necrosis, apoptosis and increased release of matrix molecules<sup>3,4</sup>. The belief is that the activation of caspases<sup>5</sup> precedes apoptosis caused by injurious impaction. In addition, impaction induced injury damages the integrity of cellular membrane resulting in an influx of calcium and leakage of intracellular material, causing damage to the surrounding matrix and neighboring cells. Acute trauma also up-regulates stress-induced MAPK pathways, extracellular signal-regulated protein kinase (ERK), c-Jun NH(2)-terminal kinase (JNK), and p38 MAPK<sup>6</sup>.

The amphipatic nonionic surfactant poloxamer P188 has been shown to significantly arrest cell death in the impacted area<sup>7</sup>. In our previous work with human ankle cartilage, we showed that P188 was effective in preventing cell death, reducing apoptosis, inhibiting the spread of cell death and preventing radial progression of apoptosis longitudinally and horizontally in to non-impacted areas, which all together resulted in the protection of cartilage from degenerative changes<sup>8</sup>. In the present continuing study we used the same model of acute trauma to human ankle cartilage in order to understand the mechanism of action of P188 on key signaling pathways thought to govern cellular responses after injury.

## Material and Methods

### Specimen Preparation and culture

Fresh human tali were removed *en bloc* from 16 organ donors with no documented history of joint diseases within 24 to 48 hours of death through the Gift of Hope-Organ & Tissue Donor Network (Elmhurst, IL). Only normal joints of Collins grade 0–1 (1949)<sup>14</sup> were used. Impaction and handling of specimens was done as described<sup>8</sup>. Using a pneumatically

controlled impactor, a single impact of 1 Ns was applied. The impulse generated a peak contact force of up to 600 N, initiating partial damage to the surface. Immediately after the impact, full thickness 8mm cartilage plugs consisting of impacted region (4mm diameter core) and the adjacent 4mm ring were removed from the bone and placed in serum free Dulbecco's modified Eagle medium supplemented with 100U penicillin and 100 ug/ml gentamicin at 37°C and 5% CO<sub>2</sub> atmosphere. The total of 192 full thickness explant discs were removed from the tali of 16 donors and were randomly assigned to each of the following experimental groups: 1) impacted non-treated control; 2) impacted explants treated with P188 (Pluronic F68, Sigma-Aldrich, St Louis, MO; 8mg/ml) for 20 minutes, 1 hour or 24 hours; 3) impacted explants treated with 20uM p38 inhibitor (p38i) SB 203580 (Calbiochem 559389) for 20 minutes, 1 hour or 24 hours; 4) impacted explants treated with the combination of P188 and p38i for 20 minutes, 1 hour or 24 hours.

### Western Blot Analysis

After culture, the treated tissue was immediately submerged in liquid nitrogen in order to prevent dephosphorylation of phosphatases. The frozen plug was pulverized and cell lysates were prepared using cell lysis buffer (20mM Tris HCl, pH 7.5, 150mM NaCl, 1mM Na<sub>2</sub>EDTA, 1mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1mM glycerophosphate, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin), and 10 μg/ml of Phosphatase Inhibitor (Sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole). Western blot analysis was done with rabbit polyclonal antibodies against phosphorylated MAP Kinases (p38, JNK, ERK), Stat1, Stat3, ATF-2, Elk-1, and GSK3; immunoreactivity was determined using SuperSignal West Pico chemiluminescent substrate (ThermoScientific, 34080, Rockford, IL). All primary antibodies were from Cell Signaling (Danvers, MA) except for phospho JNK, which was from Invitrogen (Carlsbad, CA). Antibody to total βactin used as control was purchased from Abcam (Cambridge, MA). As a secondary antibody, goat anti-rabbit IgG from Cell Signaling was used. Explants were divided into two halves ensuring bisection of the impacted core for histology and TUNEL stains and for cell survival by live/dead assay.

**Live/Dead Assay**—Cell survival was measured as previously described in detail<sup>8</sup> using calcein AM and ethidium bromide homodimer-1 (Molecular Probes, Eugene, OR). Two regions of interest were chosen for image analysis, superficial and middle/deep zones. The superficial zone was defined as the first 100 μm of the tissue from the articular surface; while the rest of the tissue was designated as the middle/deep zone. For a given region of interest, the percentage of live cells was determined using an image processing program, ImageJ<sup>9</sup>.

### Assessment of interleukin-6 (IL-6) by Multiplex ELISA and

**immunohistochemistry**—Aliquots of media were collected every other day in order to detect catabolic cytokines released as a result of injury. To quantify the levels of IL-6, samples were examined with a multiplex bead-based assay (Bio-Plex Cytokine Assay kit; Bio-Rad Laboratories) according to the manufacturer instructions. Data points for each group were evaluated via ANOVA and Fisher's PLSD post-hoc. IL-6 protein was analyzed by immunohistochemistry on paraffin sections with rabbit anti-human IL-6 antibody from Abcam.

**Tunel stain for chondrocyte apoptosis**—Apoptotic cells were labeled *in situ* using commercial Tunel assay (ApopTag™ Plus peroxidase detection kit, #S7101 Chemicon International, Temecula, CA) as described<sup>8</sup>. Light microscopy was used to determine the percentage of apoptotic cells. Brown nuclei indicated apoptotic cells and blue nuclei indicated viable cells.

**Histological Assessment with Safranin O staining**—Paraffin-embedded sections adjacent to those used for Tunel assay were utilized for histology with Safranin O/fast green staining<sup>10</sup>. Histological grading was conducted based on modified criteria originally established by Mankin et al<sup>11</sup>. Specimens were analyzed primarily for abnormalities in cellularity, Safranin O stain distribution, and surface fibrillation; cracks resulting from impaction were not graded since their random appearance was greatly dependent upon characteristics of cartilage and donor's age.

## Results

### Detection of IL-6 protein

Using ELISA we determined the release of various mediators after acute trauma; with IL-6 demonstrating one of the most pronounced patterns associated with trauma-induced cellular response (Fig. 1). It was up-regulated initially within the first 24–48 hours (Fig. 1A) indicating perhaps an acute inflammatory phase. Then, its expression was decreased to a baseline level of undamaged control and activated again later in culture (day 12–14), during perhaps the remodeling phase. IL-6 was identified not only in the media, but also intracellular and extracellular in the impacted cartilage matrix. The strongest staining was found within the first 4 days of culture in the areas immediately adjacent to the impact (Fig. 1B).

### Signaling studies with Western blotting

To understand the mechanism of P188 activity, we performed a panel of signaling studies based on the facts that P188 inhibits apoptosis and the elevation of IL-6 was one of the earliest cellular responses. With regard to apoptosis, the focus was on phosphorylation of glycogen synthase kinase-3 (GSK3), a kinase that controls cellular responses to damaged DNA and phosphorylates  $\beta$ -catenin in the Wnt signaling pathway<sup>12</sup>. With regard to IL-6, the focus was on key mediators and transcription factors: Stat1 and Stat3, stress-associated kinase p38 and its downstream partners ATF-2 and Elk-1, as well as ERK and JNK. Immediately after impaction, all tested signaling proteins were activated in the non-treated control (Fig. 2A) with peak activity level between 20 to 60 min. P188 completely blocked phosphorylation of Stat1 after 20 min and this effect persisted for at least another 24 hours. Stat3 phosphorylation was only partially reduced with pronounced effect after 1 hour of culture with P188. In addition to IL-6 pathway, P188 also affected MAP kinases. It partially inhibited phosphorylation of p38, ERK, and JNK. As with mediators of IL-6 signaling, the effect was already noticed after 20 min of treatment (Fig. 2A), while the strongest inhibition of p38 was detected at 1 hour of culture with P188. The level of ERK and JNK inhibition was similar at both time points. P188 treatment also affected a downstream mediator of p38 signaling, ATF-2, for which phosphorylation was completely attenuated by the surfactant at 20 min and 1 hour; however, Elk-1 activity (another downstream mediator of p38 signaling) was not influenced at all (data not shown). Phosphorylation of GSK3 was only partially blocked at every tested time point (Fig. 2A). Total  $\beta$ -actin protein remained at the same level in all experimental groups.

### Role of p38 in trauma-induced responses. Verification of P188 effects

To verify the role of p38 in trauma-induced cellular responses, we performed series of studies with impacted explants cultured in the presence of p38i (Fig. 2B). We found that p38i in addition to its direct inhibitory effect on p38 phosphorylation also partially impeded phosphorylation of Stat3, GSK3 and ERK (Fig. 2B) suggesting an up-stream role of p38 kinase in the regulation of these three pathways. P38i had no effect on JNK phosphorylation. Addition of P188 to p38i cultures further inhibited activation of p38 and ERK and inhibited JNK phosphorylation, which was unaffected by p38i (data not shown).

To further prove the role of p38 kinase in early cellular responses to injury, impacted cartilage explants were cultured in the presence of p38i for 24 hours and processed for live/dead assay and Tunel stain (Fig. 3). Quantitative analysis was performed as described<sup>8</sup>. Within 24 hours of treatment p38i reduced the number of dead cells in the superficial layer by 6% ( $p=0.053$ ) and the number of apoptotic cells by 24% ( $p=0.046$ ). When Tunel-positive cells were assessed through the entire tissue section, an overall inhibition constituted about 15% ( $p=0.07$ ).

## Discussion

This study explored for the first time the mechanism of action of P188 poloxamer in human articular cartilage beyond its function as a surfactant on plasma membrane. Protective short- and long-term effects of a single injection of P188 on cell survival have been shown previously *in vitro* and *in vivo* in a rabbit cartilage<sup>7</sup>. Our laboratory expanded these findings by documenting that P188 inhibited cell death and apoptosis not only in the areas that were subjected to the impaction, but also in the areas immediately adjacent to it. Critically, P188 arrested longitudinal and horizontal spread of apoptosis, which resulted in the protection of cartilage from degenerative changes<sup>8</sup>. Of note, our previous study was the only one that went beyond cell viability in exploring the effects induced by P188.

To better understand immediate cellular responses to acute trauma and to further investigate direct and indirect effects triggered by the application of P188 here we hypothesized that P188 by sealing a cell membrane might be able to counteract signaling pathways involved in injury-mediated reactions. In this study we focused on three signaling pathways: 1) IL-6, since we found that IL-6 was immediately up-regulated in response to injury; 2) MAP kinases, those that are activated by stress and pro-inflammatory mediators, and 3) GSK-3, as a kinase that promotes cell death caused by the mitochondrial intrinsic apoptotic pathway activated by cell damage<sup>6,12</sup>. IL-6 belongs to a cytokine subfamily and acts on the cell through the Janus Kinase (JAK) signal transducer and activates the transcription of Stat signaling pathway. Stat activation promotes down regulation of type II collagen, leading to impaired matrix formation and resulting in chondrocyte disruption<sup>13</sup>. In the current study, impaction to cartilage induced an elevation and release of IL-6 accompanied by phosphorylation of Stat-1 and -3. Interestingly, an up-regulation of IL-6 expression had two peaks: one was subsequent to injury and was found within 48 hours after the insult; while a second, a weaker response, appeared later, towards the end of the culture. Apparently two phases of IL-6 activation are responsible for two different events: 1) pro-inflammatory/pro-catabolic, that drives cells death, apoptosis, acute inflammation, and matrix breakdown and 2) remodeling responses that could be viewed as pro-anabolic reaction. Treatment with P188 inhibited Stat1 and Stat3 phosphorylation suggesting a potential role of IL-6/Stat signaling in an immediate cellular responses induced by injury. Besides being one of the key transducers of IL-6 signaling, Stat3 was also linked to p38 pathway, which controls cellular responses after mechanical stress and injury<sup>6,13</sup>. P188 treatment inhibited activation of both, p38 and Stat3, along with ATF-2, a downstream target for p38 activity. A critical role of p38 kinase that might be viewed as one of the central players in the post-traumatic responses was verified with its specific inhibitor. To our surprise, in addition to expected effect on p38 it also inhibited Stat3 and GSK3 phosphorylation suggesting that p38 acts upstream of these two mediators and, perhaps, controls both inflammatory and apoptotic responses to acute injury. This was further supported by our findings that a short treatment of cartilage explants with p38i promoted cell survival and reduced apoptosis. Interestingly, inhibition of p38 by P188 was more pronounced than that by p38i suggesting that other pathways contribute to activation of p38 kinase and that P188 regulates multiple pathways, for example, ERK and JNK as was confirmed by combined treatments of P188 and p38i. Proposed mechanism of P188 action is summarized in Fig. 4.



In conclusion, our studies unraveled novel effects/mechanisms exhibited by P188 surfactant in the model of acute cartilage trauma. An ability of P188 to not only seal cell membrane resulting in improved cell survival and cartilage integrity, but also to control inflammatory responses expands its potential as a therapeutic application in post-traumatic OA.

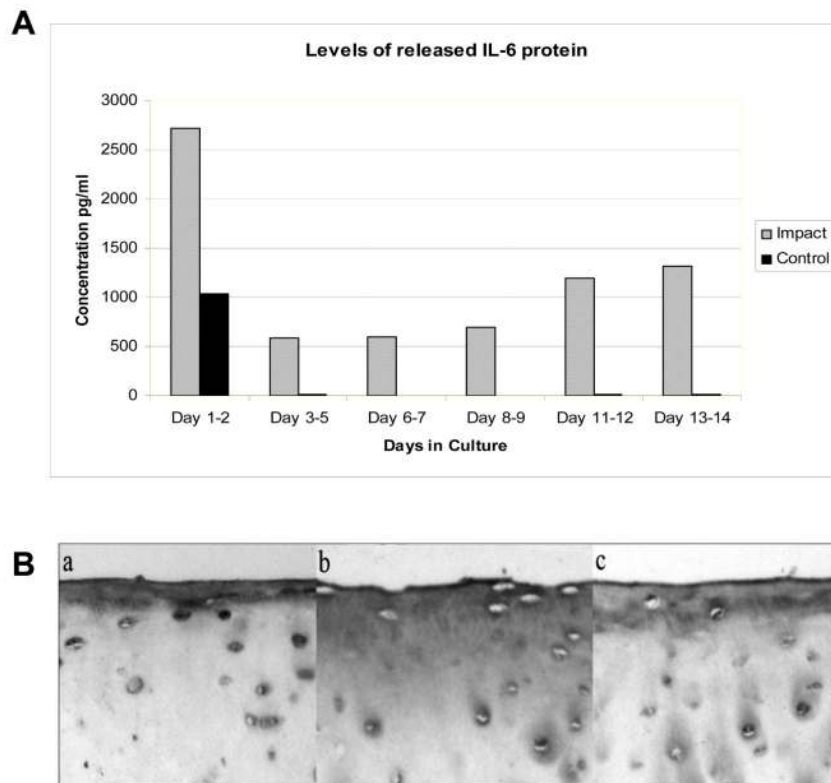
## Acknowledgments

This work was supported by the NIH SCOR grant P50 AR 39239 (TO), NFL Charity Foundation (SC), NIH T32 training grant AR052272 (fellowship for SB), Rush Medical College Dean's Fellowship (TS), and Dr. Jorge Galante's fellowship (CPG).

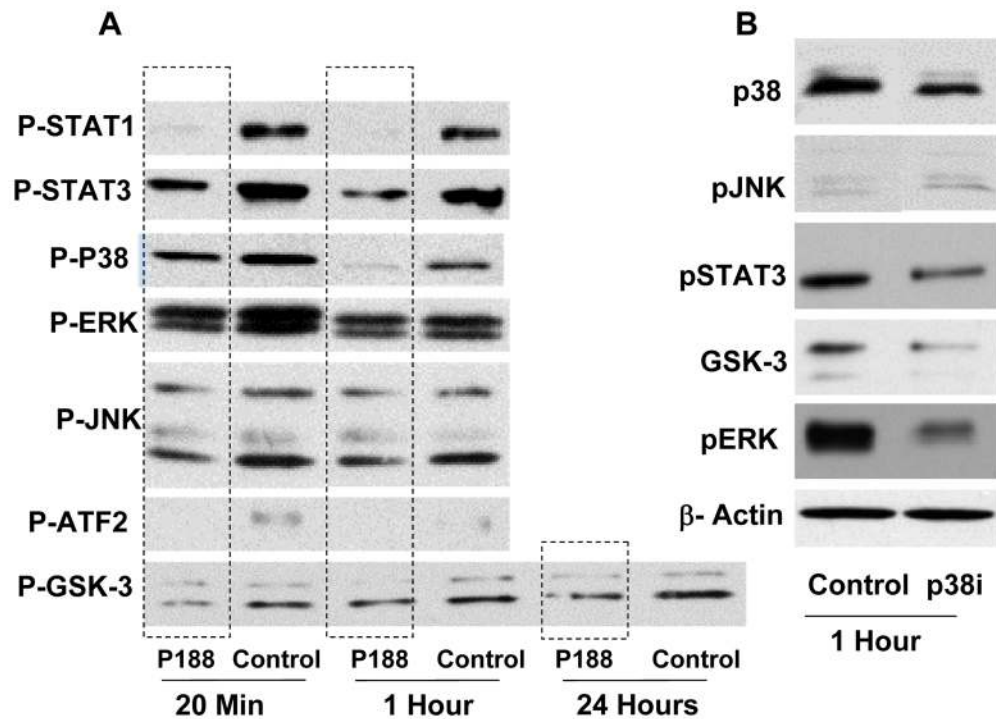
The authors would like to acknowledge the Gift of Hope Organ & Tissue Donor Network and donors' families as well as Dr. Arkady Margulis for human tissue procurement.

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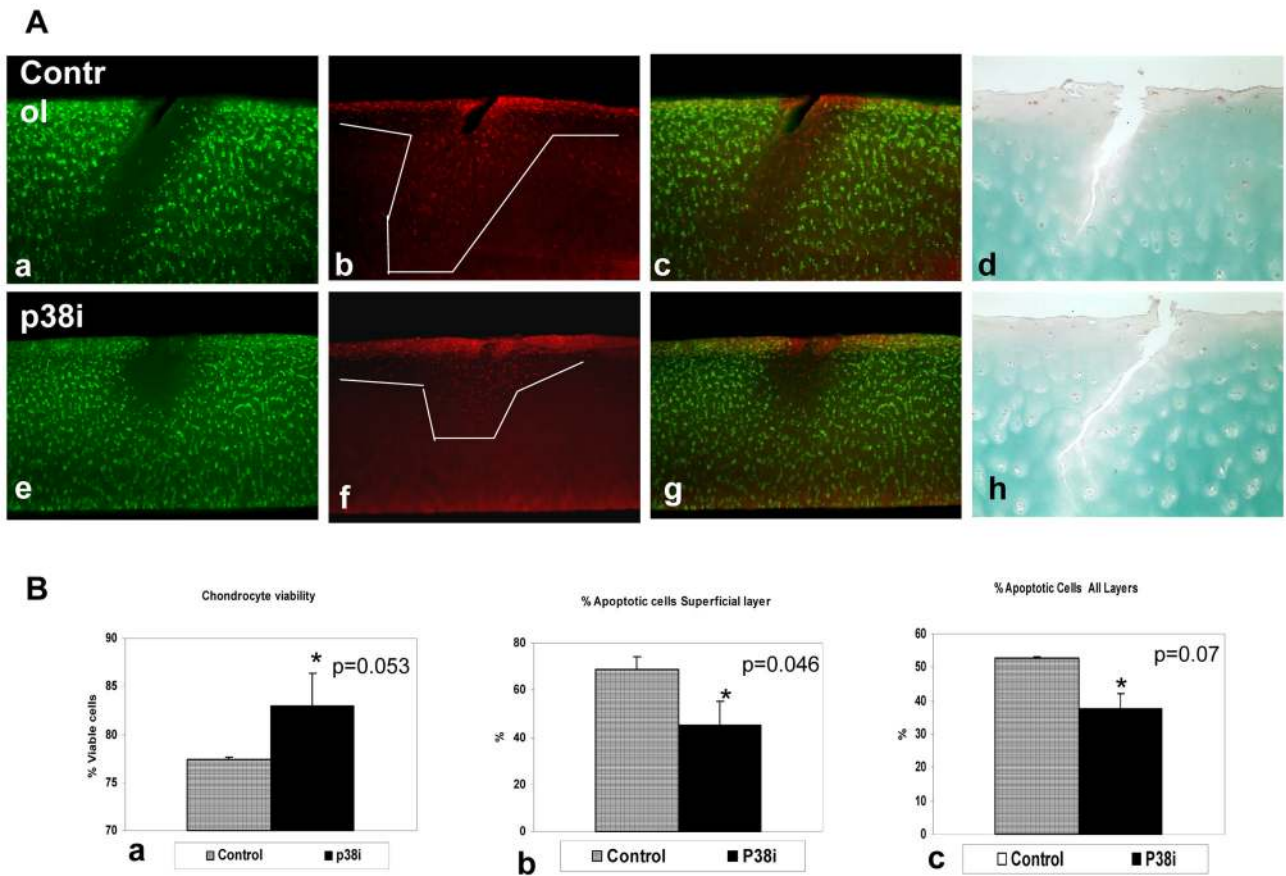


**Figure 1.** Elevation of IL-6 protein after acute impact. A, IL-6 ELISA data obtained from the media samples collected every 48 hours. B, Immunohistochemistry of cartilage sections adjacent to the impact area stained with anti-IL-6 antibody. a, Day 0 immediately after the impact; b, day 2 of culture; c, day 4 of culture.



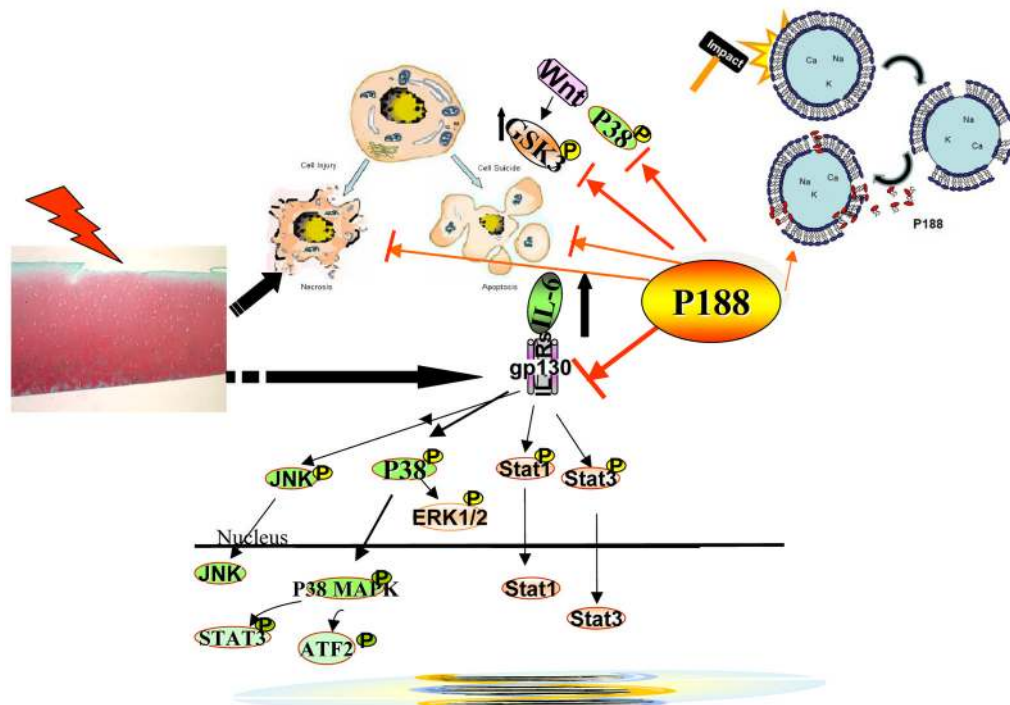
**Figure 2.** Western blot analysis of cell lysates from the impacted cartilage blotted with antibodies to the corresponding phosphorylated proteins. A, lysates from the non-treated control or explants treated with P188 for 20min, 1hour, 24 hours. B, Cell lysates from the non-treated control or explants treated for 1 hour with p38 inhibitor. Exposure time was the same for all gels.





**Figure 3.**

Effect of p38 inhibitor on cell viability and apoptosis. A, Representative images of impacted non-treated control (upper panel), or impacted explants treated with p38 inhibitor (lower panel) for 24 hours. a & e, images of live cells; b & f, images of dead cells; c & g, overlaid images of live/dead cells; d & h, tunel stain. (original magnification 100x). B, a, Quantitative analysis of cell viability in the superficial layer; b, number of TUNEL-positive cells in the superficial layer; c, number of TUNEL-positive cells in the entire section.



**Figure 4.** Proposed mechanism of P188 effect. Impact to cartilage induces matrix disruption and cell death, which translates on the cellular level to the activation of IL-6, p38, and GSK3 signaling. In addition to sealing cell membrane, P188 inhibits necrosis, apoptosis, IL-6, p38, and GSK3 pathways.