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Nano Chitosan Oligosaccharide vs Dextrose Prolotherapy: Effects on Platelet-Derived Growth Factor (PDGF) Expression in Rat Ligament Injury – a randomized controlled experimental study

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ABSTRACT

Introductions: Ligament injury is a musculoskeletal condition that requires effective regenerative therapy owing to its slow healing process. Dextrose-based prolotherapy has been widely used, while nano chitosan oligosaccharide (NCO) has emerged as a promising alternative with anti-inflammatory and antioxidant properties. This study compared the effects of 0.4% NCO prolotherapy and 12.5% dextrose on Platelet-Derived Growth Factor (PDGF) in a Wistar rat model of patellar ligament injury. Material and Methods: A randomized post-test only control group design was used with 31 rats randomly divided into control, dextrose, and NCO groups. Prolotherapy injections were administered seven days post-injury. PDGF levels were measured by Enzyme-Linked Immunosorbent Assay (ELISA) on days 8, 14, and 21 post-injury. Results: No significant differences in PDGF expression among the groups ($p > 0.05$). However, distinct fluctuation patterns were noted. Dextrose was associated with an increased PDGF level during the mid-healing phase, possibly due to inflammatory stimulation. Conversely, the NCO group showed PDGF dynamics comparable to controls, suggestive of balanced modulation of inflammation and fibroblast proliferation. Conclusion: Administration of either 0.4% NCO or 12.5% dextrose did not significantly affect PDGF expression compared to controls. Further studies with larger samples, varied dosing, and additional healing parameters are recommended to further investigate the therapeutic potential of NCO prolotherapy in ligament healing.

KEYWORDS: prolotherapy, nano chitosan oligosaccharide, dextrose, PDGF, ligament injury

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INTRODUCTION

Ligaments are dense connective tissues integral for joint stability, yet they are highly susceptible to injury, especially in weight-bearing joints such as the knee. The healing of such injuries is protracted, often spanning months to years due to limited vascularity and complex tissue architecture.1,2 The slow and incomplete natural healing of ligaments often leads to chronic pain and joint instability, which are frequent causes of long-term disability.3 Current therapeutic approaches include surgery and non-operative methods, among which prolotherapy has gained popularity.4 Prolotherapy utilises proliferative agents, such as hypertonic dextrose, sodium morrhuate, phenol-glycerine-glucose (P2G), platelet-rich plasma (PRP), and stem cells, to stimulate tissue regeneration.3–7 Among these agents, hypertonic dextrose is the most widely used for its availability, affordability, and proven ability to trigger a mild inflammatory response that stimulates fibroblast proliferation and collagen synthesis.8,9 However, these agents may provoke inflammatory reactions causing patient discomfort and variable clinical outcomes,4,10,11 prompting the exploration of alternative biological agents with better safety profiles.

Nano chitosan oligosaccharide (NCO), derived from chitin, is a biopolymer possesses unique biological activities, including anti-inflammatory, antioxidant, and cell-stimulating properties.12–16 Chitin occurs primarily in the exoskeletons of crustaceans and insects as well as fungal cell walls. Chitosan is derived from the partial deacetylation of chitin. While chitosan has been useful in many fields including foods and biomedicine, its high molecular weight and viscosity may limit their use in vivo. Chitosan oligosaccharide (COS) is a short chain of N-acetylglucosamine units from chitin and chitosan produced via enzymatic, chemical, or physical methods, has lower molecular weight, degree of deacetylation, and polymerization, better water solubility, low viscosity, and high biocompatibility.12–14 Previous studies have demonstrated its ability to accelerate epithelial and connective tissue repair while maintaining biocompatibility.12–16 The application of NCO as a prolotherapy agent could represent an advancement in regenerative pain therapy, offering effective healing with minimal discomfort.

Platelet-Derived Growth Factor (PDGF) is a key mediator of ligament regeneration, facilitating phases of inflammation, cell proliferation, and matrix remodelling during healing process. PDGF acts as a potent chemotactic agent that stimulates mesenchymal and progenitor cell activity, supports fibroblast division, and promotes tissue repair and regeneration.17,18 This study investigates the comparative effects of NCO and dextrose as proliferative agents on PDGF expression in a rat patellar ligament injury model, postulating that NCO may exert a favourable modulatory effect on PDGF expression.

MATERIAL AND METHODS

This study used a true experimental randomized post-test only control group design. A total of 40 male Wistar rats acquired from Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, subjected to induced patellar ligament injury. Inclusion criteria were healthy male Rattus norvegicus Wistar rats aged between 16 to 24 weeks, weighing 200-350 grams. Exclusion criteria included rats presented any physical abnormalities. Dropout criteria comprised death during the process or exhibited signs of infection at the treatment site.

All rats underwent a one-week acclimatization period to the environment of the experimental animal facility. Following adaptation and exclusion of 9 dropouts, 31 rats remained randomly divided into three main groups: Control Group (n = 8), Dextrose Group (n = 11), and NCO Group (n = 12), using a simple random allocation technique, with the minimum size of 27 samples. The Control Group (K0) consisted of subjects that received patellar ligament injury but no prolotherapy injection. The Dextrose Group (K1) consisted of subjects that received patellar ligament injury followed by injection with 12.5% hypertonic dextrose solution. The NCO Group (K2) consisted of subjects that received patellar ligament injury followed by injection with 0.4% NCO solution.

All rats underwent a standardized surgical procedure to induce patellar ligament injury. The rat ligament injury model was adapted from the protocol proposed by Akamatsu et al.,19 with minor modifications. Rats were anaesthetised intramuscularly using a solution containing ketamine 2.5% and xylazine 1% at a dose of 0.1 ml per 100 grams of body weight. The patellar ligament transection procedure began by shaving the fur around the right hind knee, followed by a 1 cm vertical incision on the skin and subcutaneous tissue from the patella to the tibial tuberosity to expose the patellar ligament. A horizontal incision was then performed on the patellar ligament, across 30% of its diameter, as determined using a micrometre calliper. The wound was then sutured, treated with 2% topical mupirocin, and subsequently covered with a dressing as Figure 1.

(a) (b) (c)

Figure 1. Preparation of ligament injury model: a) vertical incision on the skin and subcutaneous tissue to expose the patellar ligament; b) micrometre calliper was used to determine the length of the patellar before the horizontal incision was made across 30% of its diameter; c) the sutured wound subsequently covered with dressing.

Dextrose and NCO solutions were prepared under sterile conditions to the specified concentrations. The 12.5% dextrose solution was prepared by diluting standard 40% dextrose with sterile distilled water at a dilution ratio of 40% dextrose solution to solvent of 1 : 2.2. The NCO was produced at the Mineral and Material Processing Laboratory, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia, using shrimp shell as the raw material. The process involved microwave pyrolysis without chemical additives, resulting in weight average molecular weight (Mw) of 449 Da, particle size of 60.73 nm, with 98.99% degree of deacetylation. The 0.4% NCO solution was prepared by dissolving 400 mg of NCO salt in sterile distilled water to a final volume of 100 ml.

The prolotherapy injection was administered on day 7 following the surgical injury. This timing was chosen to coincide with the shift from the inflammatory phase to the proliferative phase of healing, aiming to maximize the stimulus for regenerative response. The injection was administered in a volume of 0.1 ml directly into the injured area of the patellar ligaments using a fine-gauge needle (Figure 2). The Dextrose Group received 12.5% dextrose solution, the OKN group received 0.4% NCO solution, and the Control Group received no injection, serving as a baseline for the natural healing process.

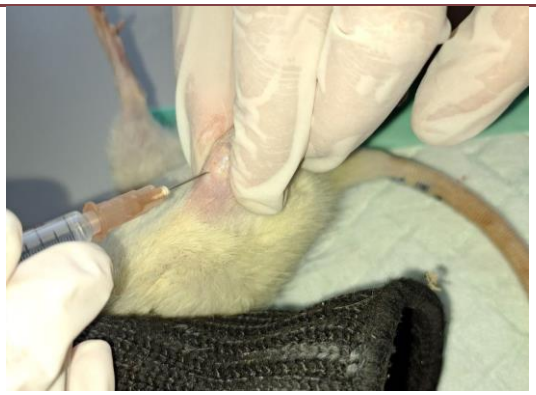

Figure 2. Prolotherapy administration: the proliferative agents were injected directly into the injured area of the patellar ligaments using a fine-gauge needle.

The primary outcome was the expression level of Platelet-Derived Growth Factor (PDGF) in the ligament tissue. Tissue samples of the patellar ligament were harvested from the three groups at three different time points post-injury: day 8 (inflammatory phase, early post-intervention), day 14 (mid-proliferative phase), and day 21 (late-proliferative/early-remodelling phase). The animals were euthanized using a humane procedure before tissue harvest. The excised ligament tissue was immediately processed for PDGF quantification.

Tissue PDGF levels were measured using the Enzyme-Linked Immunosorbent Assay (ELISA) method. This technique involves homogenizing the tissue sample, preparing the supernatant, and reacting it with specific antibodies to PDGF, allowing for the quantitative measurement of the PDGF concentration. Optical density values were measured at 450 nm and converted into concentration units in nanograms per millilitre (ng/ml). All assays were performed in duplicate according to the manufacturer's protocol to ensure accuracy and minimize variability.

Statistical analyses were performed using SPSS version 23, with the significance level set at $p < 0.05$. Normality and homogeneity of variances tests were performed using Shapiro-Wilk and Levene's tests respectively. Statistical comparisons between groups were conducted using one-way ANOVA for normally distributed data. For data that were not normally distributed, the Kruskal-Wallis test was applied.

RESULTS

The average body weight of the rats in this study was 250.65 ± 29.5 grams, ranging from 208 to 308 grams. As show in Table 1, body weight data in each sample group were normally distributed ($p > 0.05$). Levene's test confirmed homogeneity of variance across groups ($p = 0.933$), and ANOVA analysis indicated no significant differences in mean body weight between groups ($p > 0.05$). These results suggest that pre-treatment variables did not introduce bias into the treatment outcomes.

Table 1. Body Weight Distribution by Sample Groups

	K_0 (n = 8)	K_1 (n = 11)	K_2 (n = 12)	p-value*	Total (N = 31)
Weight (g) Mean ± SD	241.75 ± 27.56	265.82 ± 29.73	242.67 ± 27.00	0.113	250.65 ± 29.5
p-value**	0.273	0.722	0.714		0.0316

N: total sample size; n: sample size in each group; SD: standard deviation; K0: Control Group; K1: Dextrose Group; K2: NCO Group; *) ANOVA test, significant if $p < 0.05$; **) Shapiro-Wilk test, data normally distributed if $p > 0.05$

Table 2 presents the comparison of PDGF expression between groups. On day 8 post-injury, the highest mean PDGF level was observed in the Control Group, followed by the NCO Group, with the Dextrose Group showing the lowest value. One-way ANOVA indicated no statistically significant difference between groups at this time point ($p = 0.581$).

The Dextrose Group exhibited the highest average PDGF levels compared to both the Control and NCO groups on day 14. The NCO and Control groups showed lower levels, with the NCO Group maintaining a stable level similar to that of the control. However, Levene's test revealed non-homogeneous data ($p = 0.003$), so the Brown-Forsythe test was performed, confirming that the differences were not statistically significant ($p = 0.118$).

By day 21, the Control Group had the highest mean PDGF level, although the Kruskal-Wallis test showed no significant difference was found between groups ($p = 0.137$).

Table 2. Comparison of PDGF Expression Between Groups

Day of observation	PDGF level (ng/ml) Mean ± SD Median (Min – Max)			p-value*
	K_0 (n = 8)	K_1 (n = 11)	K_2 (n = 12)	
Day-8 (n = 11)	1.720 ± 0.798 2.130 (0.798 – 2.224) (n = 3)	0.981 ± 0.694 1.120 (0.025 – 1.659) (n = 4)	1.34 ± 1.11 1.167 (0.202 – 2.815) (n = 4)	0.581#
Day-14 (n = 11)	0.701 ± 0.033 0.701 (0.668 – 0.733) (n = 3)	1.190 ± 0.521 1.198 (0.636 – 1.722) (n = 4)	0.601 ± 0.235 0.668 (0.270 – 0.798) (n = 4)	0.118##
Day-21 (n = 9)	1.072 ± 0.204 1.072 (0.927 – 1.216) (n = 2)	0.427 ± 0.084 0.438 (0.338 – 0.504) (n = 3)	0.779 ± 0.420 0.668 (0.405 – 1.375) (n = 4)	0.137###
p-value*	0.077###	0.220#	0.470#	

n: sample size; SD: standard deviation; K0: Control Group; K1: Dextrose Group; K2: NCO Group; #) ANOVA test; ##) ANOVA Brown-Forsythe test; ###) Kruskal-Wallis test; *) Significant if $p < 0.05$

While no statistically significant differences were found in mean PDGF levels among the groups at any single time point, distinct patterns of PDGF fluctuation were evident across the three observation points, which are visualised using box plots and line charts (Figure 3). In the Control Group, PDGF levels peaked on day 8, declined markedly by day 14, followed by a slight increase on day 21. This pattern aligns with the natural healing process, where PDGF peaks during the inflammatory/early proliferative phase and gradually decreasing as tissue enters the remodelling phase.

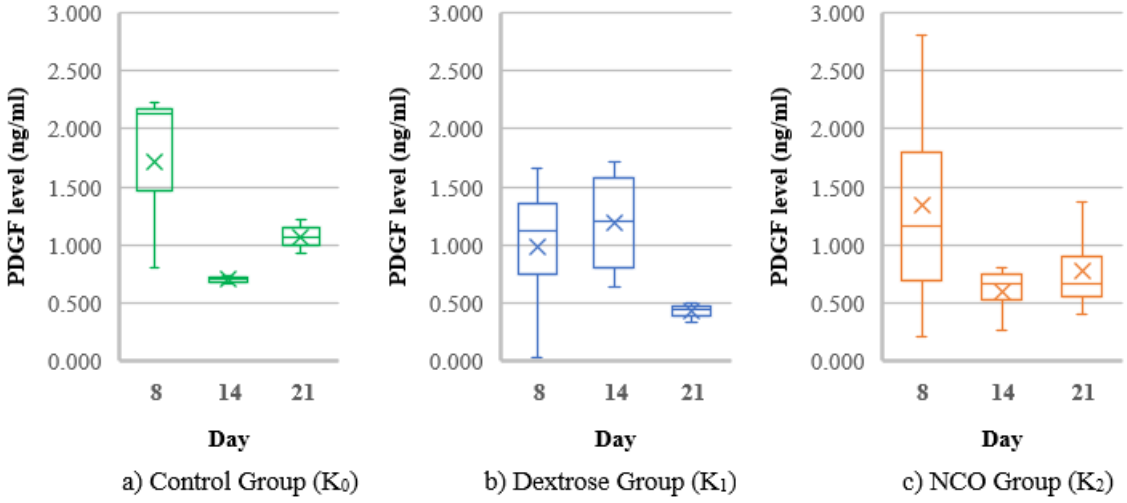

Figure 3. PDGF levels based on observation time in Control and Treatment Groups

In the Dextrose Group, PDGF levels rose from day 8 to day 14, then dropped sharply by day 21. This suggests a transient stimulatory effect during mid-healing, followed by a decline, consistent with its pro-inflammatory stimulation.

The NCO Group displayed a PDGF expression pattern closely similar to that of the Control Group, indicating steady modulation without excessive inflammatory peaks.

Although fluctuations were observed, statistical analysis revealed no significant differences in PDGF expression among the Control, 12.5% Dextrose, and 0.4% NCO groups across all three observation time points: day 8, day 14, and day 21 post injury ($p > 0.05$). These findings indicate distinct but non-significant expression patterns across treatments.

A notable finding of this study is the large variation seen in several box-plots within a single treatment group, reflecting biological heterogeneity among individual rats as in Figure 3. This represented that despite using the same animal model to control genetic and environmental factors, each rat exhibited differences in immune response, local inflammation, tissue metabolism, and rates of fibroblast and platelet proliferation.2,18

DISCUSSION

This study sought to compare the regenerative effects of two distinct prolotherapy agents—hypertonic dextrose and Nano Chitosan Oligosaccharide (NCO)—in rat models of patellar ligament injury, using Platelet-Derived Growth Factor (PDGF) expression as a key molecular marker. The principal finding that, neither 12.5% dextrose nor 0.4% NCO significantly affected the mean PDGF expression compared to the control group at three critical time points, warrants a nuanced interpretation.

The lack of a statistically significant difference does not necessarily imply a lack of biological activity, but rather suggests that the overall effect on PDGF expression, at the specific dosage and time points chosen, was insufficient to cross the statistical threshold for significance against the natural post-injury inflammatory response. It is well-documented that PDGF levels naturally surge following a severe ligament injury as part of the body's innate regenerative mechanism.1,2,17 The baseline levels of PDGF in the un-treated control group were already high, potentially obscuring a relative increase induced by the proliferative agents. This phenomenon highlights a key challenge in regenerative research, where the therapeutic signal must be robust enough to significantly elevate a factor that is already maximally or near-maximally expressed due to the injury itself.

Despite the non-significant statistical outcome, the distinct temporal expression patterns observed provide valuable biological insight into the specific mechanisms of each proliferant. In the Control Group, PDGF levels rose on day 8, declined markedly by day 14, and slightly increased again by day 21. This pattern reflects the normal course of ligament healing without pharmacological stimulation, where an early PDGF peak signifies acute inflammation and fibroblast recruitment, followed by a decline as the proliferative phase stabilises. Similar observations were reported by Wheaton and Jensen18 and Doxey et al.,20 confirming that PDGF dynamics are tightly linked to the transition from inflammation to proliferation and remodelling.

Dextrose prolotherapy displayed a different pattern, with a more pronounced PDGF rise at day 14, indicating a stronger inflammatory–proliferative response triggered by hyperosmotic irritation. Dextrose solutions cause local osmotic stress that induces aseptic inflammation, platelet degranulation, and macrophage activation, leading to the release of PDGF, Transforming Growth Factor-beta (TGF-β), and other cytokines.21 This response prolongs the inflammatory-proliferative phase and promotes fibroblast activity and collagen synthesis. Hauser et al.1 and Nair4 demonstrated that dextrose prolotherapy induces controlled inflammation leading to collagen remodelling, consistent with the PDGF elevation seen at day 14 in the present study. However, excessive inflammation may result in disorganised collagen deposition or fibrosis,1 highlighting the need for careful control of concentration and injection frequency. The elevated PDGF at day 14 likely represents the culmination of this dextrose-induced inflammatory cascade transitioning directly into the proliferative phase, maximizing fibroblast recruitment and activity before the levels normalize toward the remodelling phase. The temporal lag between the day 7 injection and the day 14 peak suggests the time required for the initial inflammatory cell recruitment, degranulation, and subsequent release of PDGF from platelets and macrophages to reach maximum concentration in the tissue matrix.

In contrast, NCO prolotherapy produced a stable PDGF expression profile resembling that was comparable to the Control Group. NCO acts through bioactive modulation rather than osmotic irritation. As a natural, biocompatible polymer with anti-inflammatory and regenerative properties, NCO enhances fibroblast proliferation and angiogenesis while limiting excessive cytokine release.12,22 Howling et al.23 demonstrated that the effect of chitosan on fibroblast proliferation correlates with its degree of deacetylation: chitosan with a higher degree of deacetylation significantly stimulates fibroblast proliferation, whereas lower deacetylation levels result in reduced activity. Furthermore, its stimulatory effect is dependent on the presence of serum in the culture medium, suggesting that chitosan may interact with serum growth factors, thereby amplifying their biological effects These mechanisms explain the absence of a sharp PDGF surge and suggest that NCO promotes a harmonised healing environment characterised by moderated inflammation and sustained fibroblast activity. The observed biological trends are consistent with previous findings; Benchamas et al.13 reported that nano-chitosan accelerated tendon and skin healing in rats without excessive inflammatory infiltration, while Prastika et al.24 found that topical chitosan improved fibroblast proliferation and collagen deposition with minimal irritation.

Overall, the temporal PDGF variations observed among groups represent two principal mechanisms of prolotherapy action: dextrose as an inductive agent that enhances inflammation-driven proliferation,10,11,21 and NCO as a modulatory agent that supports regeneration through anti-inflammatory signalling.12,15,22–24 Although the quantitative results were statistically insignificant, these qualitative trends have physiological and clinical relevance. Dextrose may be more effective in chronic or degenerative conditions requiring strong stimulation, while NCO may be preferable in acute injuries or postoperative recovery, where controlled regeneration and minimal pain are desirable.

The study's limitation includes the focus on single biomarker, PDGF alone. Evaluation of other growth factors, histological assessments, and biomechanical analyses would provide a more comprehensive understanding of the healing process. If NCO achieves comparable or superior tissue regeneration, as measured by other outcomes like biomechanical strength, with a less pro-inflammatory profile indicated by the stable PDGF expression, it would indicate a potential advantage for NCO as a regenerative, biocompatible alternative to dextrose, offering similar healing benefits with reduced inflammatory intensity—an appealing feature for clinical applications in pain and rehabilitation medicine. Additionally, the animal model limits direct extrapolation to human clinical conditions. Nevertheless, these findings establish a valuable foundation for future translational research.

CONCLUSIONS

Administration of prolotherapy with 0.4% Nano Chitosan Oligosaccharide (NCO) or 12.5% hypertonic dextrose did not result in a statistically significant difference in PDGF expression compared to the control group across post-injury days 8, 14, and 21 in

this experimental Wistar rat model of patellar ligament injury.

The distinct PDGF fluctuation patterns observed with a tendency for a mid-phase increase with dextrose versus a stable profile with NCO suggest different mechanisms of action: dextrose relies on a pro-inflammatory stimulus, while NCO may promote controlled regeneration with balanced inflammatory modulation.

Future research should utilize a broader set of parameters, including evaluation of other growth factors, inflammatory markers, biomechanical strength and histological scoring, as well as an exploration of varied doses, to fully determine the therapeutic potential of NCO as a novel, well-tolerated prolotherapy agent for ligament repair.

Take Home Message

- Neither 0.4% Nano Chitosan Oligosaccharide (NCO) nor 12.5% dextrose significantly increased PDGF expression compared to the control group across days 8, 14, and 21 after ligament injury ($p > 0.05$).
- Dextrose prolotherapy tended to elevate PDGF levels at day 14, indicating a stronger inflammatory–proliferative response typical of hyperosmotic stimulation.
- NCO demonstrated a stable PDGF expression pattern similar to natural healing, suggesting balanced inflammatory modulation and regeneration without excessive inflammatory peaks.
- Different mechanisms of action were implied: dextrose stimulates healing through controlled inflammation, while NCO may support regeneration with anti-inflammatory and antioxidant properties.
- Future research is recommended using larger sample sizes, multiple dosing strategies, additional growth factors, and biomechanical or histological outcomes to fully determine the therapeutic potential of NCO as an alternative prolotherapy agent.

Abbreviations

NCO	Nano Chitosan Oligosaccharide
PDGF	Platelet-Derived Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
P2G	phenol-glycerine-glucose
PRP	platelet-rich plasma
COS	Chitosan Oligosaccharide
TGF-β	Transforming Growth Factor-beta
ACUC	Animal Care and Use Committee

DECLARATIONS

Ethical Consideration

The study protocol, including the use of animal models and surgical procedures, received ethical approval by the Animal Care and Use Committee (ACUC) of Universitas Airlangga, Surabaya, Indonesia, prior to the commencement of the study.

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Conflict of Interest

The authors of this work declare that they have no conflicts of interest.

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