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ORIGINAL ARTICLE

The effect of liquid nitrogen exposure on the proliferative phase of Achilles tendon healing in *Rattus norvegicus* rats

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ABSTRACT

Aim Tendon healing involves a crucial proliferative phase, during which fibroblasts and fibrocytes orchestrate collagen deposition. The use of liquid nitrogen (LN) in orthopaedic oncology may inadvertently affect adjacent tendon tissues. This study aimed to evaluate the impact of LN exposure on the histological features of tendon healing.

Methods This experimental study employed a randomized post-test-only control group design involving 24 males *Rattus norvegicus*, randomly divided into four groups: control (no LN exposure) and three treatment groups exposed to LN for 1, 5, and 10 minutes, respectively, following Achilles tendon transection and repair. After a 21-day healing period, histological analysis was performed to assess the counts of fibroblasts, fibrocytes, and collagen content. Statistical analyses included one-way ANOVA, Post-hoc Tukey, and Pearson correlation ($p<0.05$ was considered significant).

Results LN exposure significantly reduced fibroblast, fibrocyte, and collagen levels compared to controls ($p<0.05$). The 10-minute group showed the lowest counts. A significant negative correlation was found between LN immersion duration and the number of fibroblasts ($r= -0.87$), fibrocytes ($r= -0.829$), and collagen content ($r= -0.83$) ($p<0.05$).

Conclusion Liquid nitrogen (LN) impairs tendon healing in a dose-dependent manner, likely due to cryo-induced cell death and disruption of blood flow. This results in an acellular and avascular tendon matrix, hindering the repair process. LN exposure negatively impacts the proliferative phase of tendon healing in rats, suggesting the need for caution in clinical use to prevent damage to surrounding tendinous tissues.

Keywords: Achilles tendon, cryosurgery, fibroblasts, tendon injuries, wound healing

INTRODUCTION

Tendon injuries heal through a coordinated process of inflammation, proliferation, and remodelling (1). In the proliferative phase of tendon healing, which occurs in the weeks following injury, fibroblasts actively proliferate and synthesize collagen to form the reparative scar tissue bridging the tendon ends. These fibroblasts eventually mature into fibrocytes as the tissue organizes and remodels. This cellular activity is crucial for the proper repair and restoration of tendon function (2).

A challenge in tendon healing arises when cryotherapy is used, particularly liquid nitrogen (LN) exposure, which is commonly employed in orthopaedic oncology to treat bone tumours (3). The LN, with its temperature reaching -196 °C, is used to induce deep tissue freezing and tumour cell necrosis. While effective in destroying cancerous tissue, LN exposure is not selective to tumour cells and can also damage surrounding healthy tissues, including tendons (4). A prior study demonstrated that LN exposure to bone causes total devitalization of bone cells (osteocytes, osteoblasts, osteoclasts) (5). However, the mineralized matrix remains to serve as a scaffold for new cell ingrowth. In bone, this allows a gradual regenerative process known as creeping substitution to restore the tissue despite the initial cell loss. Tendon tissue, on the other hand, is less mineralized and more avascular, raising the concern that LN exposure could severely impair its intrinsic healing capacity (6).

Tendon healing may be particularly vulnerable to cryo-induced cell loss. If LN eradicates tendon cells (tenocytes/fibroblasts), healing would have to rely entirely on extrinsic cell infiltration from surrounding tissues, a slower and less effective mechanism (7). This raises the question of whether LN exposure

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leads to complete tendon devitalization, or if some tendon cells can survive cryotherapy and contribute to healing. Despite its clinical applications in orthopaedic oncology, the unintended effects of LN on tendon healing have mainly been understudied. To date, few studies have specifically explored the impact of cryosurgery on tendon structure and its healing process (8). The aim of this study was to evaluate the effects of liquid nitrogen exposure on the proliferative phase of tendon healing, focusing on the Achilles tendon in a rat model, as well as to assess three key parameters of healing tissue quality: fibroblast count, fibrocyte count, and collagen content. Through this investigation, we aim to understand how LN affects tendon repair and potentially guide future cryosurgical practices to minimize unintended tendon damage.

MATERIALS AND METHODS

Materials and study design

This experimental study employed a randomized post-test-only control group design to assess the effects of liquid nitrogen (LN) exposure on Achilles tendon healing in *Rattus norvegicus*.

Six-month-old *Rattus norvegicus*, weighing approximately 500 grams, with no disabilities in their extremities and actively moving, were included in the study. They were chosen to ensure that the animals were in their adult stage, thus providing mature tendon tissue for evaluation, which closely mimics human tissue in orthopaedic research. Rats with pre-existing infections, disabilities in the extremities, and those experiencing compartment syndrome were excluded from the study. The rats were housed under controlled laboratory conditions with a 12-hour light/dark cycle and free access to food and water.

The sample size was determined using Federer's formula (9) for calculating the required sample size for each group as follows:

$(n - 1)(t - 1) \geq 15$

where: n = required sample size per group, t = number of treatment groups

$$(n-1)(4-1) \geq 15$$

$$(n-1)(3) \geq 15$$

$$3n - 3 \geq 15$$

$$3n \geq 18$$

$$n \geq \frac{18}{3}$$

$$n \geq 6$$

The number of treatment groups was four:

- Control Group: Rats underwent tendon transection, followed by suturing. Tissue samples were then collected for histopathological analysis after 21 days.
- Treatment Group 1 (labelled as P1): Rat tendons were transected, followed by immersion in liquid nitrogen for 1 minute. The tendons were then sutured back, and a cast was applied. Tissue samples were collected for histopathological analysis after 21 days.
- Treatment Group 2 (labelled as P2): Rat tendons were transected, followed by immersion in liquid nitrogen for 5 minutes. The tendons were then sutured back, and a cast was applied. Tissue samples were collected for histopathological analysis after 21 days.
- Treatment Group 3 (labelled as P3): Rat tendons were transected, followed by immersion in liquid nitrogen for 10 minutes. The tendons were then sutured back, and a cast was applied. Tissue samples were collected for histopathological analysis after 21 days.

The equation solving yields a required sample size of at least six rats per group: 24 male *Rattus norvegicus* were involved in the study.

Methods

The animals were acclimatized for 7 days in a controlled environment with ad libitum access to food and water. Anaesthesia was induced by administering ketamine (2 mg/kg body weight) intraperitoneally, followed by prophylactic antibiotics (cefazolin, 0.1 mg/kg) intramuscularly. The surgical site was shaved, disinfected with betadine, 70% alcohol, and povidone-iodine, and covered with a sterile dressing. A 3 cm incision was made along the skin to expose the Achilles tendon, which was fully transected 3 cm above its insertion point on the calcaneus. The tendon was then immersed in liquid nitrogen for 1 minute (P1), 5 minutes (P2), and 10 minutes (P3) for the respective experimental groups. One group was not exposed to liquid nitrogen and served as the control group. After freezing, the tendon was sutured back into position using 6/0 monofilament and a Kessler technique (10), followed by skin closure with 4/0 monofilament sutures. The animals were immobilized with a plaster cast and allowed to heal for 3 weeks. At the end of this period, tendon samples were collected for histopathological examination.

After a 21-day healing period, the tendon tissues were harvested and immediately fixed in 10% formaldehyde. The samples were then processed for histological evaluation, with a focus on fibroblast, fibrocyte, and collagen content. The tissues were stained using Hematoxylin and Eosin (H&E) to assess general tissue structure, and Masson's Trichrome staining to evaluate collagen content. For each tendon sample, 20 high-power fields (1000× magnification) were examined using an Olympus BX-51 microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) to compare differences between the experimental groups. ANOVA was used to determine if there were any statistically significant differences in fibroblast count, fibrocyte count, and collagen content among the groups exposed to different durations of liquid nitrogen. If significant differences were found, post-hoc Tukey tests were performed to identify which specific groups differed from one another. Pearson correlation was used to assess the relationships between the duration of LN exposure and the measured healing parameters, providing insights into how the length of exposure affected the healing process. A significance level of $p<0.05$ was considered statistically significant for all analyses.

RESULTS

Tendon samples exposed to liquid nitrogen demonstrated markedly impaired healing parameters compared to controls. All LN-treated groups had significantly lower fibroblast counts, fi-

Table 1. Fibroblast, fibrocyte, and collagen counts in tendon samples exposed to liquid nitrogen for varying durations

Parameter		N	Mean (SD)
Fibroblast	Control	7	138.14 (30.03)
	P1	7	105.43 (22.48)
	P2	7	67.57 (12.26)
	P3	7	51.57 (25.69)
Fibrocytes	Control	7	174.14 (36.70)
	P1	7	125.14 (24.46)
	P2	7	73.14 (15.00)
	P3	7	60.43 (14.79)
Collagen	Control	7	157.00 (13.49)
	P1	7	130.62 (11.79)
	P2	7	122.30 (11.54)
	P3	7	108.96 (8.69)

SD, standard deviation; N, number of the sample.

brocyte counts, and collagen content than the uninjured control tendons $p<0.05$ for each). In the control group, histological analysis showed an average of approximately 138 fibroblasts and 174 fibrocytes per 20 high-power fields. In contrast, the group exposed to LN for 10 minutes showed a drastic reduction, with only approximately 51 fibroblasts and 60 fibrocytes remaining. The 1-minute and 5-minute LN exposure groups had intermediate cell counts (approximately 105 and 67 fibroblasts; 125 and 73 fibrocytes, respectively), indicating a progressive decline in cellularity with longer freeze duration (Table 1).

Collagen content in the tendon tissue also decreased correspondingly as the LN exposure time increased (Figures 1, 2). Statistical analysis confirmed that fibroblast count, fibrocyte count, and collagen content differences were significant ($p<0.05$). Post-hoc Tukey tests (Table 2) revealed significantly lower fibroblast and fibrocyte counts, as well as smaller collagen areas in the LN-exposed groups (1, 5, and 10 minutes) compared to the control group ($p<0.05$ for all comparisons).

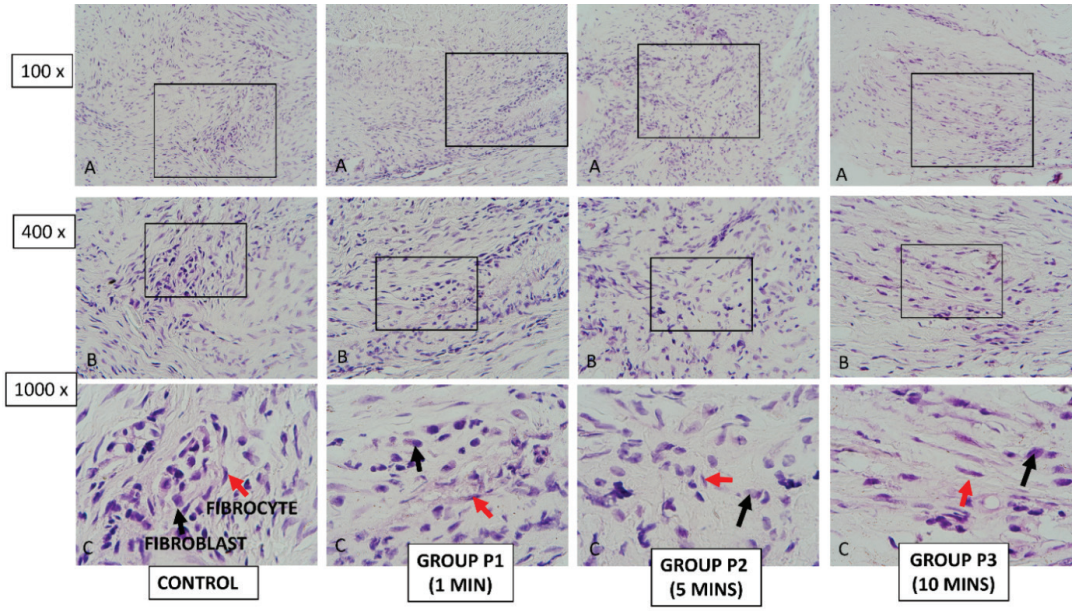

Figure 1. Fibroblast (black arrow) and fibrocytes (red arrow) counts under 1000x magnification (Faculty of Medicine, Universitas Brawijaya, 2025)

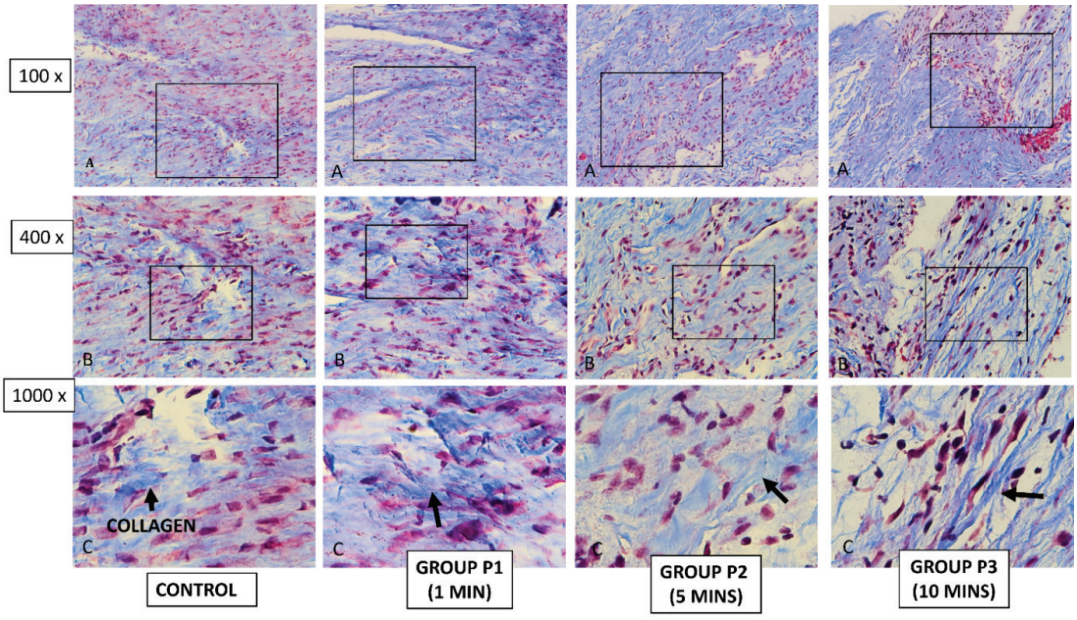

Figure 2. Collagen (black arrow) counts under 1000x magnification (Faculty of Medicine, Universitas Brawijaya, 2025)

Table 2. Post-hoc Tukey tests for fibroblast, fibrocyte, and collagen counts across experimental groups

Group comparison		Fibroblast	p*	Fibrocyte	p*	Collagen	p*
		Average difference		Average difference		Average difference	
Control	P1	32.714	0.070	49.000	**0.005**	26.378	**0.001**
	P2	70.571	**0.000**	101.000	**0.000**	34.703	**0.000**
	P3	86.571	**0.000**	113.714	**0.000**	48.037	**0.000**
P1	P2	37.857	**0.029**	52.000	**0.003**	8.325	0.540
	P3	53.857	**0.001**	64.714	**0.000**	21.658	**0.000**
P2	P3	16.000	0.589	12.714	0.766	13.333	0.161

*statistical significance in bold.

Table 3. Pearson correlation analysis between liquid nitrogen exposure duration and healing parameters (fibroblast, fibrocyte, and collagen)

Variables Relationship	r	p*
Exposure duration – Fibroblast	-0.829	**0.000**
Exposure duration – Fibrocyte	-0.871	**0.000**
Exposure duration – Collagen	-0.830	**0.001**

*statistical significance in bold;
r, correlation coefficient;

Pearson correlation analysis (Table 3) demonstrated strong negative correlations between LN exposure duration and healing parameter: fibroblast count ($r = -0.87$; $p<0.05$), fibrocyte count ($r = -0.829$; $p<0.05$), and collagen content ($r = -0.83$; $p<0.05$), indicating that longer LN exposure was associated with progressively fewer tendon cells and lower collagen deposition in the healing tissue.

DISCUSSION

The significant reduction in fibroblast and fibrocyte counts observed in LN-exposed tendons indicates that liquid nitrogen exposure severely impairs the proliferative phase of tendon healing (11). Fibroblasts, responsible for synthesizing collagen and forming the reparative matrix, are vital for tendon repair. A drastic decrease in fibroblast numbers suggests that much less collagen is produced, halting the normal healing process (12). The parallel reduction in fibrocytes further indicates that the overall cellularity of the healing tissue is diminished, which likely leads to the formation of a weaker repair scar with inferior biomechanical properties. This impairment could lead to poor functional outcomes, as tendon repairs typically result in fibrotic scar tissue that never achieves the strength of uninjured tendon tissue (13-15).

The biophysical injury mechanisms of extreme cold on biological tissues can explain the deleterious effect of liquid nitrogen on tendon healing. At –196 °C, LN causes almost instantaneous freezing of tissue fluid. Ice crystals form inside and outside cells, rupturing cell membranes and organelles, which leads to immediate cell death when a critical temperature threshold is passed. Experimental cryobiology studies have noted that cooling cells below roughly –40 °C results in irreversible cell injury for nearly all cell types (16). In our context, immersion of the Achilles tendon in LN would have rapidly brought the tissue well below –40 °C, ensuring that the majority of resident tendon cells (fibroblasts, tenocytes, and vascular cells) were irreparably damaged. In addition to this direct cryogenic cell destruction, the freeze-thaw process induces secondary ischemic injury. As the frozen tendon thaws, microvascular circulation may remain stagnant (vascular stasis) for some time, leading to hypoxia and further cell death due to nutrient deprivation (17,18). These processes likely explain the significantly reduced population of fibroblasts and fibrocytes observed in the LN-treated tendons.

From a clinical perspective, these results highlight significant risks associated with LN exposure during cryosurgery. While LN is effective for tumour ablation, its use in proximity to tendons and other critical soft tissues should be approached with caution (19). Unintended exposure of tendon tissue to LN during procedures, such as tumour removal where tendons are nearby, could lead to postoperative tendon damage and compromised healing. Surgeons should consider protective measures (e.g., insulating tendons with gauze or limiting the spread of local anaesthesia) and use the minimal effective freeze duration to minimize collateral damage. Previous clinical observations have reported complications, including non-union or pathological fractures, when LN cryotherapy is used aggressively on bone, highlighting the need to balance tumour control with preservation of healthy tissue (20,21). This study provides quantitative evidence that tendon tissue is highly susceptible to LN-induced freeze injury. Future research should focus on optimizing cryosurgery protocols, such as adjusting freeze times and developing strategies to protect tendons, ensuring tumour destruction without compromising tendon healing.

In conclusion, liquid nitrogen (LN) exposure impairs the proliferative phase of tendon healing in a dose-dependent manner. A significant decrease in fibroblast, fibrocyte, and collagen content was observed with longer LN exposure times. These findings underscore the importance of minimizing exposure to adjacent tendons during cryotherapy to prevent significant tendon damage. Surgeons should carefully consider reperfusion intervals and protective measures to limit the risks of ischemic reperfusion injury and promote better functional outcomes following orthopaedic procedures. Future research is needed to assess biomechanical strength post-cryotherapy, as well as the long-term effects of prolonged LN exposure, particularly regarding its impact on tendon healing beyond the Achilles tendon.

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TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.

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