Annals of Orthopedics and Musculoskeletal Disorders

Research Article
Published: 05 Sep, 2023

Investigation of the Effect of Low Intensity Ultrasound (LIPUS) Therapy on Healing in Achilles Tendinopathy Model Created by Type 1 Collagenase

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Abstract

Aim: There is a significant rate of outpatient consultations for tendinopathy symptoms nowadays. With the increase in daily pace, conservative treatments that can expedite recuperation have become more important. In the present study, we examined how Low-Intensity Pulsed Ultrasound (LIPUS) affected healing in rats in a model of tendinopathy induced by type 1 collagenase.

Method: The research was conducted on 69 Wistar albino female rats with bilateral Achilles tendinopathy. Following modeling, LIPUS therapy was initiated in eight groups at 1, 7, and 15 days. Treatment was extended for one and two weeks. Achilles tendons were removed from the treatment and control groups on the 15th, 21st, 30th, and 45th days for biomechanical and pathologic examination. PRISMA guidelines were adhered to during the planning stages of our study.

Results: In comparison to the control groups, LIPUS treatment administered in the first days of the proliferation phase increased tensile strength by approximately 30%, modulus of elasticity by approximately 53%, a fibrillar appearance by approximately 53%, and inflammation by approximately 53% to 33% in a lesser time. It was also demonstrated that starting treatment in the first days of the proliferation phase resulted in comparable success even with 1-week treatment compared to 2-week treatment.

Conclusion: The use of LIPUS in the treatment of tendinopathy yielded positive results. Its capacity to shorten recuperation time has piqued the interest of conservative treatment approaches. As a result, more clinical research is required.

Keywords: Low-intensity pulsed ultrasound; Rat; Collagenase type 1; Tendinopathy

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Received Date: *08 Aug 2023*
Accepted Date: *30 Aug 2023*
Published Date: *05 Sep 2023*

Citation:
Kurtulmuş T, Çelebi ME, Bektaş E, Arıcan CD, Küçükyıldırım BO, Demirkol M. Investigation of the Effect of Low Intensity Ultrasound (LIPUS) Therapy on Healing in Achilles Tendinopathy Model Created by Type 1 Collagenase. Ann Orthop Musculoskelet Disord. 2023; 5(1): 1036.

Introduction

Tendinopathy accounts for 45% of musculoskeletal disorders and is a significant source of work in clinics [1]. Tendon injuries can range from transitory pain and inflammation to conditions that can lead to major ruptures [1,2]. Pathologic changes in tendons begin long before the rupture and have a deleterious impact on the healing phases throughout time. Most people are unaware of these illnesses that do not generate symptoms.

For more than 50 years, ultrasound has been utilized to treat tendon diseases, joint capsule pathologies, bursitis, and skeletal muscle pain [3]. According to the scientific literature, the diffusion rate and membrane permeability of fibroblasts may vary as a result of acoustic flow, which is one of the non-thermal effects of ultrasonic applications, and the uptake of Ca^{+2} by cells may increase, resulting in increased collagen synthesis [4,5].

Although there has yet to be established an optimal animal model capable of exhibiting all characteristics of human tendinopathy, rats are the most common species for modeling Achilles tendinopathy due to their surgical approach, practical size for tissue harvesting, and ease of handling. The experimental groups were subjected to the LIPUS treatment parameters that had been highlighted in prior *in vivo* experiments [6]. Tendinopathy caused by collagenase type 1 was modeled [7].

The present study aimed to investigate whether LIPUS is successful in the treatment of tendinopathy and, if so, to determine the ideal starting time and duration of treatment. The results

were obtained through biomechanical and pathologic examinations.

Materials and Method

Ethics

Our study was approved by the Animal Experiments Local Ethics Committee at Acıbadem Mehmet Ali Aydınlar University (permission no: 2021/48). The University of Health Sciences Scientific Research Projects Unit (approval no: 2022/009) provided funding for our investigation. Animals were obtained from the Animal Experiments Laboratory at Acıbadem Mehmet Ali Aydınlar University, which holds an "AAALAC International" (American Association for Accreditation of Laboratory Animal Care) certificate in conformity with the Ministry of Food, Agriculture, and Livestock regulations (13.12.11 number: 28141). Subjects were kept in a conventional laboratory environment (12 h of day *vs.* 12 h of night lighting, 20°-22° room temperature, 50%-60% humidity) and fed food and water as needed. A certified veterinarian monitored the entire procedure on a regular basis, and all anesthesia and surgical procedures were carried out using methods specified in the literature. PRISMA guidelines were adhered to during the planning stages of our study.

Study plan

The study was conducted on three separate groups at three distinct stages (early-mid-late acute phase) to find the best time to begin LIPUS therapy in acute tendinopathy. Based on the ultrasound mechanism of action proposed in the literature [4], it was discovered that the differential effect of treatment time difference may be noticeable in groups that began in the proliferative phase [8]. As a result, the groups with mid-acute phase commencement of treatment were separated into three subgroups with treatment durations ranging from 7 to 14 days.

The study was designed to be conducted with 144 Achilles tendons to be extracted from 72 Wistar albino female rats with typical activity and weighing 300 gm to 350 gm. To produce a model of bilateral Achilles tendinopathy, type 1 collagenase was injected into each rat. According to the time of sampling, 4 groups served as the control group, while 8 groups received treatment at various periods and for varying periods of time (Figure 1).

Achilles tendinopathy was induced by accessing the tendon under general anesthesia through a medial skin incision and injecting 3 mg/ml type 1 collagenase enzyme (Clostridium histolyticum 205 IU/mg) dissolved in balanced sterile saline solution [7]. The surgical field was closed with 4-0 silk sutures after the operation. The rats' movements were not restricted. The rats were sacrificed by decapitation on the 15th, 21st, 30th, and 45th days, and bilateral Achilles tendons were extracted. Tendons were kept in 10% neutral formalin solution until a pathological evaluation of all right Achilles tendons and a biomechanical evaluation of all left Achilles tendons. At the time of specimen collection, no infection or abscess formation was seen in any individual.

LIPUS therapy

The animals were sedated daily for 20 min with 100% isoflurane LIPUS therapy was initiated with a 5% isoflurane flow rate under 400 ml/min oxygen flow and thereafter maintained with a 2% to 3% flow rate. In this process, no treatment was provided to the control groups.

Biomechanical evaluation

Tendons were stored at -15°C before being thawed to room temperature. Prior to the tensile tests, the cross-sectional dimensions of each tendon were measured and the cross-sectional areas of each were calculated to determine the tensile strength. Tensile tests were carried out on a universal mechanical testing machine with Class 1 calibration. Tensile tests were carried out at a strain rate of 10 mm/min for better observation of the mechanical behavior of the tendons. Tensile strength was calculated by dividing maximum force that corresponding tendon can endure, to the cross-sectional area of that tendon. Modulus of elasticity is calculated from the slope of the elastic region of the stress-strain curve obtained during the tensile testing. Toughness values were calculated by integration of the stress-strain curve, which gives the area under the curve. These values are evaluated for each tendon, to interpret applied recovery treatments to each tendon biomechanically.

Pathological examination

Achilles tendons were put in a 10% formaldehyde solution for 24 h before being fixed with a tissue monitoring device. The tissues were then fixed in paraffin blocks and sectioned with a microtome into 0.5 mm pieces on slides. The slides were stained with H&E stain and prepared for examination using Nikon light microscopy. The tissue alterations were assessed at (x10) and (x40) magnifications. For degenerative alterations of the tendon and paratenon, staining affinity, nuclear appearance, fibrillar appearance and fibrosis, capillary changes, and inflammation were assessed. They were graded using a semiquantitative system [9,10].

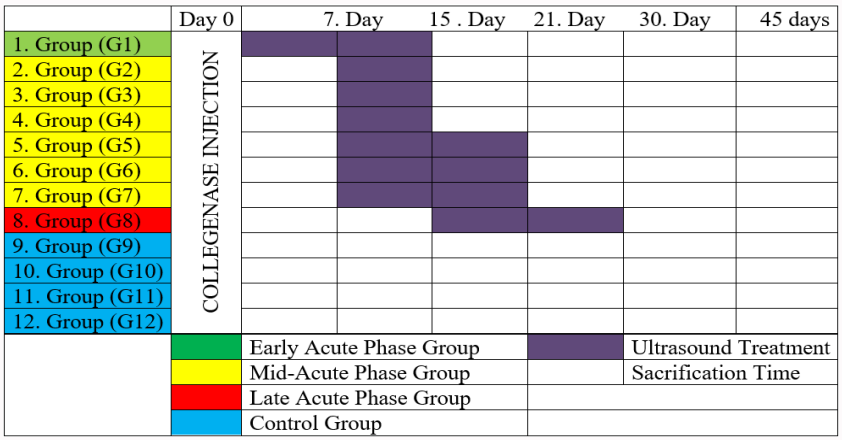

Figure 1: Study plan.

Tendon, paratenon, and total pathologic scores were determined for each specimen based on the sum of all pathologic alterations. Tendon and paratenon scores ranged from 0 to 9, while the total histopathologic score ranged from 0 to 18. A higher score suggested that all parts experienced degeneration and inflammation.

Statistical analysis

IBM® SPSS® 26 and GraphPad Prism 8.3.0 software were used for statistical analyses. Analytical approaches (Kolmogorov-Smirnov/ Shapiro-Wilk tests) were used to assess the variables' conformance to normal distribution. Descriptive analyses were carried out on normally distributed variables using mean ± standard deviation, and on non-normally distributed variables using median and min-max. Because of the non-normal distribution of continuous data (biomechanical and pathologic evaluation parameters), the Mann-Whitney U test was employed to compare paired groups, and the Kruskal-Walli's analysis was utilized for comparisons between more than two groups. Furthermore, Dunn's test was employed in post hoc comparisons. Statistical significance was defined as p-values under 0.05.

The effect size for the a priori hypotheses was computed as 2.1 in the study using G*Power 3.1.9, the error amount α=0.05, the targeted power of test 1-β=0.85, and the sample size required for statistical analysis was found to be 6 [7,8].

Results

In conclusion, 138 Achilles tendons from 69 rats have been collected. During the experiments, one rat from each of the first, tenth, and twelfth groups perished. Six separate criteria were used to analyze the histology of 69 right Achilles tendons. Biomechanical testing was performed on 69 left Achilles tendons using four different criteria.

We used the comparison plan we devised to conduct our statistical analysis on a total of 690 different data sets. First, we compared the control groups to one another. Although the control groups improved over time in the defined criteria, we chose to focus on the criteria where we could show a statistically significant change in order to compare them to the treatment groups.

Tensile strength (p=0.014) and toughness (p=0.042) values for G11 demonstrated a statistically significant difference between control groups. Modulus of elasticity (p=0.036), fibrillar appearance (p=0.018), and inflammation (p=0.044) in G12 were statistically significantly different from the control groups (Table 1, 2).

In terms of the tensile strength (p=0.135) and toughness (p=0.163), G3 and G6, which were sampled at the same time as G11 (day 30) and subjected to various treatment models, did not exhibit any statistically significant differences. There was no statistically significant difference in the tensile strength (p=0.204) and toughness (p=0.318) outcomes of G2 who received the same 1-week LIPUS therapy as G3 (Table 3). When compared to the tensile strength (p=0.268) and toughness (p=0.716) outcomes of G5 who received comparable 2-week LIPUS therapy as G6, no statistically significant difference was observed (Table 4).

When the samples were analyzed on the 21st day, no statistically significant difference was discovered between the tensile strength and toughness values obtained by the groups receiving 1 or 2 weeks of treatment and the groups whose tendons were extracted on the 30th day. In other words, regardless of treatment duration, the tensile strength and toughness findings obtained on the 30th day without treatment were obtained on the 21st day by the groups receiving therapy in the mid-acute phase.

G4 and G7 were sampled at the same time (day 45) as G12 and were subjected to different treatment models, but there was no statistically significant difference in modulus of elasticity (p=0.371). When the modulus of elasticity (p=0.325) results of G2, who received 1 week of LIPUS treatment comparable to G4, and G3, whose Achilles tendons were severed on day 30, were examined, no statistically significant difference was found (Table 3). There was no statistically significant difference in the modulus of elasticity (p=0.603) outcomes of G5 and G6 who received similar 2-week LIPUS therapy with G7 (Table 4).

When the findings of the modulus of elasticity attained by the groups receiving treatment for 1 or 2 weeks were compared to the groups whose samples were taken on the 45th day, no statistically significant difference could be discovered. In other words, regardless of treatment duration, the modulus of elasticity results obtained on the 45th day without treatment were obtained on the 21st day by the

Table 1: Comparison of biomechanical tests between G9, G10, G11 and G12 groups.

Groups	Statistics	Max Force	Tensile Strength	Modulus of Elasticity	Toughness
G9	Median	38	5.7	29.7	1.2
	Minimum	22	2.8	4.6	0.4
	Maximum	52	22.9	110.1	5.6
G10	Median	25	13.1*	97.7#	4.3#
	Minimum	16	9.3	73.6	0.4
	Maximum	118	20.1	919.2	6.1
G11	Median	64	25.6**	216.0*	10,0**
	Minimum	21	15.9	52.7	3
	Maximum	105	41.6	487.9	14.3
G12	Median	91	10.6#	305.5**	2
	Minimum	39	4.7	13.4	1,2
	Maximum	111	25.1	696.6	18.3
	p Value	0.155	0.014	0.036	0.042

Kruskal-Wallis and post hoc Dunn's test was applied and p<0.05 is significant. In group comparisons, ** >* > # shows the order of significance according to the high score

Table 2: Comparison of pathological scores between G9, G10, G11 and G12 groups.

Groups	Statistics	Staining Affinity	Nuclear Outlook	Fibrillary Appearance	Fibrosis	Capillarization	İnflammation	Total Score
G9	Median	1	0	1	2	1	2.0*	7
	Minimum	1	0	1	0	0	0	3
	Maximum	2	2	1	2	1	2	9
G10	Median	1	0	2.0*	1	1	1.0#	5
	Minimum	1	0	1	0	0	0	3
	Maximum	1	1	2	2	2	2	8
G11	Median	1	0	1	1.5	1	1.0#	5
	Minimum	1	0	0	0	0	0	4
	Maximum	1	0	1	3	1	1	7
G12	Median	1	0	1	1	0	0	3
	Minimum	1	0	1	0	0	0	3
	Maximum	1	0	1	2	1	1	5
p Value		0.446	0.284	0.018	0.394	0.131	0.044	0.078

Kruskal-Wallis and post hoc Dunn's test was applied and p<0.05 is significant

Table 3: Comparison of biomechanical tests between G2, G3 and G4 groups.

Groups	Statistics	Max Force	Tensile Strength	Modulus of elasticity	Toughness
G2	median	27	21.7	109.5	3.7
	Minimum	16	4.4	33.5	0.5
	Maximum	54	30.7	701.6	5.2
G3	median	96.0*	19.9	317.1	7.3
	Minimum	24	6.1	40.5	0.8
	Maximum	109	55.7	820.3	10.4
G4	median	60.5*	11	288.7	5
	Minimum	39	4.6	46	1.8
	Maximum	82	18.7	610.7	6.4
p value		0.03	0.205	0.325	0.318

Kruskal-Wallis and post hoc Dunn's test was applied and p<0.05 is significant

Table 4: Comparison of biomechanical tests between G5, G6 and G7 groups.

Groups	Statistics	Max Force	Tensile Strength	Modulus of elasticity	Toughness
G5	median	48	4.5	12.7	2.6
	Minimum	7	0.9	6.6	0.4
	Maximum	111	29.2	956.6	8.1
G6	median	50.5	17.9	129.9	4.6
	Minimum	12	4.2	6.6	1
	Maximum	123	41.2	1279.6	13.7
G7	median	93.5	10.1	135.1	2
	Minimum	90	7	4.8	1.4
	Maximum	99	12.4	433.7	2.6
p Value		0.416	0.268	0.603	0.716

Kruskal-Wallis and post hoc Dunn's test was applied and p<0.05 is significant

groups receiving therapy in the mid-acute phase.

G4 and G7 were sampled at the same time (day 45) as G12 and received different treatment models, but there was no statistically significant difference in fibrillar appearance (p=0.069) or inflammation (p=0.062). When the fibrillary appearance (p=0.109) and inflammation (p=0.052) findings of G2 and G3, which underwent comparable 1-week LIPUS treatment as G4, were evaluated, no statistically significant difference was found (Table 5). A statistically significant difference was observed between the fibrillary appearance (p=0.043) and inflammation (p=0.002) findings of G5 who underwent comparable 2-week LIPUS treatment as G7 (Table 6). However, there was a statistically significant difference in the fibrillary appearance results of G6, who received the same 2-week LIPUS treatment as G7, but no statistically significant change in the inflammatory findings (Table 6).

The fibrillar appearance and inflammatory results of the 1-week

Table 5: Comparison of pathological scores between G2, G3 and G4 groups.

Groups	Statistics	Staining Affinity	Nuclear Outlook	Fibrillary Appearance	Fibrosis	Capillarization	İnflammation	Total Score
G2	Median	1	0.5	2	1	1	1	8
	Minimum	1	0	1	0	1	1	5
	Maximum	2	2	3	3	2	2	11
G3	median	1	0	2	2	2	0.5	8
	Minimum	1	0	1	0	1	0	3
	Maximum	1	1	3	3	2	1	10
G4	median	1	1	1	1	1	1	5.5
	Minimum	1	0	1	0	0	0	5
	Maximum	2	2	2	1	2	1	7
P value		0.322	0.178	0.109	0.48	0.084	0.052	0.257

Kruskal-Wallis and post hoc Dunn's test was applied and p<0.05 is significant

Table 6: Comparison of pathological scores between G5, G6 and G7 groups.

Groups	Statistics	Staining Affinity	Nuclear Outlook	Fibrillary Appearance	Fibrosis	Capillarization	İnflammation	Total Score
G5	Median	1	1	1.5*	2	1	1.5*	8.5*
	Minimum	1	1	1	0	1	1	7
	Maximum	2	2	3	3	1	2	10
G6	Median	2.0*	2.0*	1.5*	2,5	1	0.5	9.0*
	Minimum	1	1	1	0	1	0	6
	Maximum	2	2	2	3	1	1	10
G7	Median	1	0	1	1.5	1	0	4.5
	Minimum	1	0	0	0	1	0	3
	Maximum	1	1	1	3	1	0	6
p Value		0.015	0.006	0.043	0.717	1,000	0.002	0.003

Kruskal-Wallis and post hoc Dunn's test was applied and p<0.05 is significant

treatment group, whose samples were taken on the 21st day, did not indicate a statistically significant difference between the 45th-day groups. In other words, the fibrillar appearance and inflammatory outcomes obtained on day 45 without treatment were obtained by the group treated for 1 week in the mid-acute phase on day 21.

The fibrillar appearance and inflammatory outcomes of the 2-week treatment group whose samples were obtained on day 21 and the groups whose tendons were taken on day 45, however, were statistically significant. Furthermore, while the fibrillar appearance achieved by the 2-week treatment group whose samples were taken on day 30 was statistically significant, there was no statistically significant difference in the inflammation outcomes between the groups whose samples were taken on day 45. In other words, the 2-week therapy group did not achieve the fibrillar appearance and inflammation results on day 21 but did achieve the inflammatory results on day 30.

Discussion

Many studies have proved that LIPUS therapy is an excellent conservative therapeutic approach for bone and cartilage tissues [6,11,12]. Among the non-surgical therapy alternatives for tendon injuries, the use of LIPUS is one of the most explored. However, no research demonstrating the efficacy of the treatment supported by *in vivo* investigations exist. There is currently no agreement on the parameters for therapeutic ultrasound treatment, and the evidence on the clinical efficacy of therapeutic ultrasound on tendinopathy is still being finalized.

The fact that the longest examination period was determined as day 45 and the examination could not be performed at later times constituted our study's limitations. Additionally, because there were so many groups, the number of subjects per group was restricted to 6, and samples could not be taken at separate times with different treatment durations in the groups where treatment was started in the early and late acute phases.

The change in muscle temperature was monitored throughout a 10-min treatment with 1 MHz and 3 MHz therapeutic ultrasound using thermistors placed at various depths (5 cm or less). The findings demonstrate that therapeutic ultrasonic treatments at 1-MHz and 3-MHz led to a time- and dose-dependent rise in tissue temperature [13-15]. When the ultrasonic intensity was increased from 1.5 W/cm^2 to 3.0 W/cm^2 (1 MHz frequency), he discovered that skin and subcutaneous tissues warmed [16].

In modelling, they used therapeutic ultrasound for 5 weeks, 4 min per day, three days a week, at a frequency of 3 MHz, with an intensity of 1 W/cm^2. On the seventh day following surgery, they began the temporal planning of the treatment, and three weeks later, the immobilization with a plaster cast came to an end. At the end of this research, they discovered that even at high doses and frequencies, they could not get favorable results [17].

In modeling made to measure the effect of continuous therapeutic ultrasound on Achilles tendinopathy in rabbits, it was discovered that the tendon's tensile strength and capacity to absorb energy had significantly improved after treatment with 3 MHz continuous therapeutic ultrasound at 1 W/cm^2 for 5 min [18]. Another study

demonstrated that identical outcomes for tendon treatment may be obtained with a lower dosage of 0.5 W/cm^2 [19].

In a different study, Achilles tendons were completely severed sutured, and then, following immobilization, ultrasound therapy was administered. The tensile strength of completely severed and healed rat Achilles tendons was shown to be higher in the group that received therapeutic ultrasounds nine days following the operation [18,19]. In a study, therapeutic ultrasound was used at a modest dose (0.5 W/cm^2) for 5 min each day to simulate post-rupture treatment in rat Achilles tendons. They acquired healing characteristics that seemed to be significantly higher in the low-dose therapeutic ultrasound group compared to the control group in light of biochemical and biomechanical findings [20]. There was no evidence of increased tendon strength in rabbits treated with pulsed ultrasound for six weeks at a dosage of 0.8 W/cm^2 [21].

Another study found that treatment partly ruptured Achilles tendons with low-dose (1.0 W/cm^2) and high-dose (2.0 W/cm^2) therapeutic ultrasound led to higher mean power in the high-dose group compared to the low-dose group [22]. The findings of their modeling, which involved continuously treating the tendon with therapeutic ultrasound at a power density of 1 W/cm^2 for four minutes per treatment session, demonstrated that ultrasound treatment accelerated the rate at which rats' damaged Achilles tendons were repaired. These outcomes also support the hypothesis that tendon restoration leads to greater stretch due to increased collagen synthesis [23].

In our study, we opted to use a device (IGEA SpA, Carpi, Italy) that generates low-intensity pulsed ultrasound with parameters for the LIPUS technique that include ultrasound frequency of 1.5 MHz, pulse width of 200 μsec, repetition rate of 1 kHz, and SATA (Spatial Average-Temporal Average) acoustic intensity of 30 mW/cm^2. In order to conveniently examine the phases taking place in the healing process, we adopted the Achilles tendinopathy model published in the literature, which is built with a single dosage of 3 mg/ml type 1 collagenase injection [7].

According to our research, the end of the inflammatory phase of healing and the start of the proliferation phase is when LIPUS treatment in the tendinopathy model produces the best outcomes. One week of treatment is adequate compared to two weeks to have the same effects. Compared to pathology results, successful biomechanical outcomes of LIPUS therapy are seen earlier. With LIPUS therapy, it takes about 53% less time to reach the biomechanically statistically significant modulus of elasticity level and about 30% less time to attain the tensile strength and toughness values. The degree of inflammation is attained roughly 53% faster and the pathologically relevant fibrillar appearance is reached around 33% faster with LIPUS treatment.

Based on the results, it is concluded that samples should be given time for pathological examination in future research since cellular changes cannot occur as quickly as biomechanical changes and that the effect on pathology results is seen 1 week after the completion of the therapy.

When we initially planned the groups for our experiment, we anticipated that LIPUS treatment in the early and late acute phases would provide less successful results than the start of treatment in the mid-acute phase group. From this perspective, the timing of the start of treatment was the most important variable in the speed of recovery.

Conclusion

Even a single week of timely LIPUS treatment speeds up recovery. It has been a promising and conservative treatment approach, considering the rapid pace of life and the expectations of patients. We think that the positive outcomes of our study will serve as a model for subsequent clinical research.

Authors Contribution

Tuhan Kurtulmuş and Mehmet Emin Çelebi contributed to every stage of the study. Tuhan Kurtulmuş contributed to the planning and writing of the study. Ebubekir Bektaş contributed to the realization of the experiment. Çiğdem Dicle Arıcan contributed to the pathological evaluation of grafts from rats. Bedri Onur Küçükyıldırım and Metehan Demirkol contributed to the biomechanical evaluation of grafts taken from rats. All authors read and approved the final version of the manuscript.

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