

Original Article

STUDY THE HEALING EFFECT OF COLLAGEN HYDROLYSATE FOR THE TREATMENT OF BONE TAIL FRACTURE IN MICE

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ABSTRACT

Objective: This work was done to evaluate the efficacy of oral Collagen hydrolysate in the treatment of joint fracture represented by tail fracture in mice. Different doses were tried to select the best effective strength by following the healing rates.

Methods: Thirty two mice with manual tail fracture were divided into four groups. Group I, II, III were treated orally with 10, 20 and 30 mg/kg/day doses (respectively) of collagen hydrolysate. The fourth group was the control. Efficacy parameters, included cyanosis, oedema and hematoma, were assessed daily. Urine samples were collected from the four groups for the quantitative determination of the collagen biomarker, hydroxyproline.

Results: The results indicated that the lowest dose (10 mg/kg/day) was the most effective with fastest healing rate (6 weeks). Cyanosis was found to disappear two weeks after commencing treatment in group I and after three weeks in group II and III. Edema disappeared completely after four weeks of treatment in group I, five weeks in group II and six weeks in group III. Hematoma was healed after seven weeks of treatment in group I and nine weeks in groups II and III animals. No healing was obtained in all animals in the control group. Excretion of hydroxyproline in urine was found to be consistent with the potency of the dose administered.

Conclusion: In conclusion, the oral administration of the lowest dose of collagen hydrolysate was found to be the most effective for the treatment of bone tail fracture in mice. This might suggest the possibility of using the endogenous collagen to mend the injured tissues. Besides, the dose dependent increase in the excretion of hydroxyproline indicates a stimulatory effect of the endogenous biosynthesis of collagen by the orally administered collagen hydrolysate.

Keyword: Collagen hydrolysate, Dose of collagen hydrolysate, Joint fracture.

INTRODUCTION

Collagen is the main component of connective tissue [1,2], and is the most abundant protein in mammals, making up about 25% to 35% of the whole-body protein content. Collagen, is mostly found in fibrous tissues such as tendon, ligament and skin, and is also abundant in cartilage, bone, blood vessels, and muscle tissue [3].

Collagen hydrolysate is made out of collagenous tissue from animal sources such as bone, hide, and hide split. It is a product that is obtained when these raw materials are subjected to technical processes including extraction, enzymatic hydrolysis, purification, concentration, sterilization, and drying [4].

Oesser estimated roughly that approximately 90 % of the orally administered collagen hydrolysate would be digested and absorbed within six hours from the gastro-intestinal tract [5].

In a series of preclinical studies, demonstrated that collagen hydrolysate passes across the mucosal barrier in the small bowel as a complete peptide that is no longer subjected to enzymatic cleavage, accumulates in cartilage tissue, and stimulates production of type II collagen (the major protein in articular cartilage) [6].

Many studies confirmed the beneficial effect of collagen hydrolysate in the treatment of joint degenerative diseases. Though, clinical trials have yielded mixed results on the use of collagen in the treatment of rheumatoid arthritis. Patients in a double-blind, placebo-controlled trial suffering of rheumatoid arthritis responded well to the oral administration of collagen hydrolysate with no reported side effects [7]. Which was then confirmed by other literatures [8, 9, 10]. One study claimed that oral collagen only improved symptoms in a minority of patients and reported a side effect of nausea [11]. Another study reported no improvement in disease activity in patients with rheumatoid arthritis [12]. While another study claimed that this treatment may actually cause an exacerbation of the symptoms [13]. However, other group of workers mentioned a statistically significant reduction in pain, a

decrease in the consumption of analgesics and improved mobility in patients with hip or joint osteoarthritis who had received a daily dose of collagen hydrolysate over a period of at least 3 months [14, 15], confirmed by another study which showed significant enhancement in daily activities suggesting an improvement in their quality of life with patients suffering of knee osteoarthritis [16].

Comparable results were obtained with a number of observational studies on athletes suffering from painful joints whose joints were subjected to increased mechanical stress. In these cases too the administration of collagen hydrolysate resulted in substantial pain reduction and improved mobility. Recently these results could be confirmed in a prospective, randomized, placebo-controlled, double blind study conducted at Penn State University in University Park, Pennsylvania on 147 athletes [17]. Collagen hydrolysate has demonstrated to be efficient in improving clinical status in knee osteoarthritis patients over glucosamine sulphate, with significant improvement in pain scores, functional joint status and a better quality of life [14].

Studies regarding skin care revealed that oral administration of collagen hydrolysate resulted in a pronounced improvement in the moisture content of the stratum corneum [18]. In addition to the reported efficacy of CH on joint health, it has been also indicated a positive effect on bones as it demonstrated a significant improvement in the healing process of bone tail fracture in mice. Besides using a drug formulation containing in addition to collagen, vitamin C, calcium, and phosphate, (Collagen Tabs®) showed better healing rate than using a formulation containing collagen alone (Genacol®), as healing was achieved within four weeks for the former and eight weeks for the later drug [19]. Reports were variable regarding the dose of collagen hydrolysate. Oral treatment of rheumatoid arthritis patients with chicken collagen II at 0.25mg/day for 6 months was found ineffective and resulted in only small and inconsistent benefits [13], while doses as low as 20 - 2,500 microg/day were reported effective in another literature [7].

The present study was undertaken to test the efficacy of 3 different dosages of orally administered collagen hydrolysate in a form of Collagen Tabs® in mice with bone tail fracture. Hydroxyproline, a major component of collagen, comprises around 13.5% of its amino acid composition. Due to its highly restricted distribution in collagen, the hydroxyproline content accurately reflects the amount of collagen in the samples [20, 21, 22]. Therefore, quantitative analysis of hydroxyproline has been utilized for evaluating tissue fibrosis or collagen deposition.

MATERIALS AND METHODS

Drugs

Collagen Tabs® : Each tablet contains; collagen hydrolysate 700 mg, vitamin C as ascorbic acid 3 mg, calcium as dicalcium phosphate 30 mg and phosphorus as dicalcium phosphate 25 mg. (Batch number: 111432), manufactured by international agencies, USA.

Animals

Albino mice (25 ± 5 g) were housed under controlled condition ($25 \pm 2^\circ\text{C}$ with a 14-hr Light and 10-hr dark cycle). Using an isolator caging system and with access to food and water *ad libitum*, maintained in groups of six in a cage.

Treatment Protocol:

A total of 32 mice were anaesthetized with chloroform, the tail of each mouse was fractured manually. Animals were then divided randomly into 4 groups each consisted of six mice:

Group I: administered a dose of 10mg/kg/day

Group II: administered a dose of 20mg/kg/day

Group III: administered a dose of 30mg/kg/day

Group IV: Control group received distilled water only.

Collagen Tabs® were finely powdered and the required amount of the powder was then weighed and dissolved in 0.2 ml distilled water and given to each animal orally, via an oro-gastric tube [23], once daily and continuously until complete healing was achieved. Clinical efficacy parameters such as cyanosis, oedema, and hematoma at the affected joint were monitored for a period of nine weeks. The intensity of each was assessed by observation using numeric rating scale [24, 25].

Quantitative Determination of Hydroxyproline in Urine:

Urine samples were obtained from each group of animals (1 ml from each group) [26]. Hydroxyproline was quantified according to the method done by Leach AA [27].

Statistical Analysis

All results are expressed as mean values \pm SD. The significance of differences in values was assessed by the SPSS Ver. 15, the following statistical procedures were used; ANOVA, and ANCOVA with an accompanying LSD's *post hoc* test performing intergroup comparisons. Differences with $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered significant.

RESULTS

Oral administration of graded doses of Collagen hydrolysate was found to be effective in the treatment of tail fracture in mice with differences in healing rate between groups. It was found that the healing rate was faster in group I animals given a dose of 10 mg/kg/day than group II and III animals which were on 20 and 30 mg/kg/day doses respectively. Group I was completely healed after six weeks of treatment, while group II and III recovered after eight weeks of treatment.

As for the disappearance of the signs of tail bone fracture in mice, cyanosis was found to disappear two weeks after commencing treatment in group I and after three weeks in group II and III. While the control group animals that received distilled water only, the sign of cyanosis did not disappear even after nine weeks, figure 1.

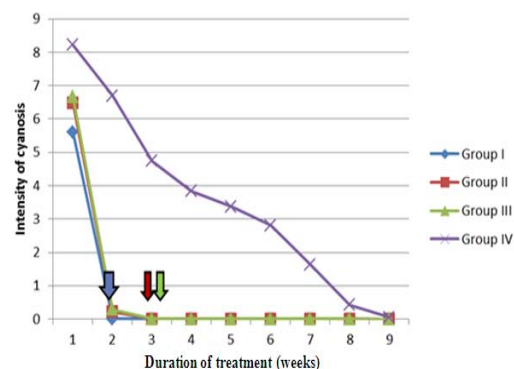


Fig. 1: The healing rate of cyanosis in the fractured vertebra of mice after oral administration of 10, 20 and 30 mg/kg/day of collagen hydrolysate to groups I, II, and III (respectively). Group IV was the control group.

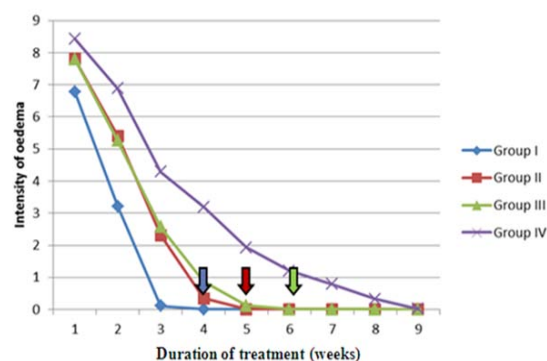


Fig. 2: The healing rate of edema in the fractured vertebra of mice after oral administration of 10, 20 and 30 mg/kg/day of collagen hydrolysate to groups I, II, and III (respectively). Group IV was the control group.

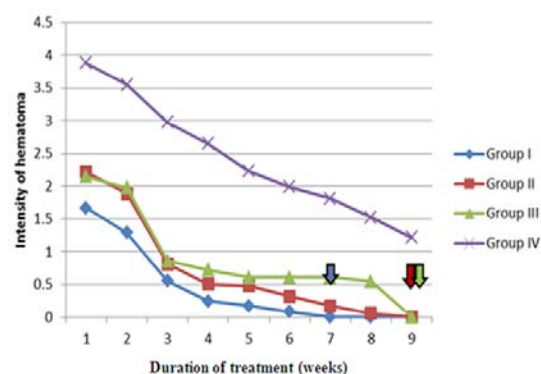


Fig. 3: The healing rate of hematoma in the fractured vertebra of mice after oral administration of 10, 20 and 30 mg/kg/day of collagen hydrolysate to groups I, II, and III (respectively). Group IV was the control group.

Figure 2 shows that edema disappeared completely after four weeks of treatment in group I, five weeks in group II and six weeks in group III, however mice in the control group did not show any healing even after nine weeks which was the observation period.

Hematoma was healed after seven weeks of treatment in group I and nine weeks in groups II and III animals but not maintained in the control, figure 3. Our results revealed that the lowest dose of collagen hydrolysate, 10 mg/kg/day, produced a better healing rate than higher doses. Data were analyzed via a one-

way ANOVA with an accompanying LSD's post hoc test performing intergroup comparisons. Table 1 shows that the difference in the healing of cyanosis, edema and hematoma of the fractured tails exerted by collagen hydrolysate administration by each test group (group I, II, and III) compared to the control mice (group IV) was significant.

Quantitative Determination of Hydroxyproline in urine

Figure 4 reveals that hydroxyproline concentration in urine was consistent with the potency of the dose administered of collagen hydrolysate. Besides, as long as treatment continues, hydroxyproline excretion was increased with time.

Table 1: Shows a comparison between the effect of Collagen hydrolysate on the healing parameters (cyanosis, oedema and hematoma) of tail fracture in mice of each group. Collagen hydrolysate was given in doses of 10, 20 and 30 mg/kg/day to groups I, II, and III (respectively). Group IV was the control group.

Cyanosis	Group I	Group II	Group III	Group IV
Week 1	5.59±0.11b	6.48±0.12b	6.65±0.53b	8.21±0.08
Week 2	0.01±0.00b	0.22±0.17b	0.29±0.24b	6.70±0.28
Week 3	0.01±0.00b	0.01±0.00b	0.01±0.00b	4.74±0.60
Week 4	0.01±0.00b	0.01±0.00b	0.01±0.00b	3.83±0.42
Week 5	0.01±0.00b	0.01±0.00b	0.01±0.00b	3.37±0.47
Week 6	0.01±0.00b	0.01±0.00b	0.01±0.00b	2.81±0.62
Week 7	0.001±0.00b	0.01±0.00b	0.01±0.00b	1.64±0.56
Week 8	0.001±0.00b	0.01±0.00b	0.01±0.00b	0.42±0.34
Week 9	0.001±0.00b	0.001±0.00b	0.001±0.00a	0.04±0.04
Oedema				
Week 1	6.78±0.2b	7.81±0.29a	7.80±0.43a	8.42±0.00
Week 2	3.21±0.55b	5.40±0.53b	5.26±0.57b	6.88±0.40
Week 3	0.10±0.11b	2.31±0.82b	2.57±0.70b	4.28±0.13
Week 4	0.01±0.00b	0.34±0.43b	0.86±0.37b	3.19±0.63
Week 5	0.01±0.00b	0.01±0.00b	0.12±0.25b	1.92±0.76
Week 6	0.01±0.00b	0.01±0.00b	0.01±0.00b	1.20±0.72
Week 7	0.001±0.00b	0.01±0.00b	0.01±0.00b	0.79±0.47
Week 8	0.001±0.00a	0.01±0.00a	0.01±0.00a	0.34±0.28
Week 9	0.001±0.00b	0.001±0.00b	0.001±0.00a	0.02±0.02
Hematoma				
Week 1	1.67±0.72b	2.21±0.42a	2.14±0.67a	3.88±0.88
Week 2	1.29±0.44b	1.88±0.60a	1.97±0.52a	3.55±1.04
Week 3	0.55±0.65b	0.81±0.70b	0.86±0.41b	2.98±1.23
Week 4	0.25±0.58b	0.50±0.54b	0.72±0.69a	2.65±1.23
Week 5	0.17±0.40b	0.48±0.52b	0.60±0.54a	2.23±1.07
Week 6	0.08±0.17b	0.32±0.48b	0.60±0.54a	1.99±1.00
Week 7	0.001±0.00b	0.17±0.40b	0.60±0.54a	1.81±0.87
Week 8	0.001±0.00b	0.06±0.11b	0.55±0.50a	1.52±0.71
Week 9	0.001±0.00b	0.001±0.00b	0.001±0.00b	1.21±0.70

Significance relative to control: a: P<0.01; b: P<0.001

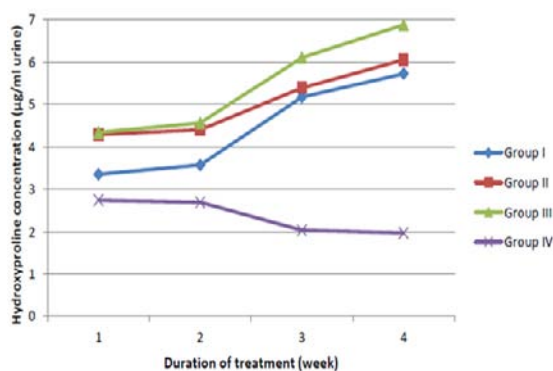


Fig. 4: The concentration of hydroxyproline in the urine of mice in group I, II, and III during treatment with 10, 20, and 30 mg/kg of collagen hydrolysate respectively, compared to the control (group IV).

DISCUSSION

Oral administration of collagen has been shown to ameliorate joint disorders in animal models. This was confirmed by different clinical studies which have been able to confirm the positive effect of CH in the treatment of degenerative joint disease. Preliminary studies have suggested that this novel therapy is clinically beneficial and safe in patients with rheumatoid arthritis, osteoarthritis of the hip or knee joint. The reported results were an increase of mobility, a decrease

of pain, and a reduction in the dependency on analgesics. Different reports mentioned the use of different dose strengths ranging from few micrograms to many grams per day. All exerted beneficial, but no side effects.

Bone fracture involved more extensive damage to the joint tissues. None of the published literatures has justified the use of collagen in the treatment of bone fractures before. However, According to a previous study done in our laboratory, collagen hydrolysate was used successfully in the healing of tail fracture in mice administered in a dose of 15 mg/kg/day. The present study was undertaken to test the efficacy of 3 different dosages (10, 20, and 30 mg/kg/day) of orally administered collagen hydrolysate in mice with tail bone fracture. The healing rate was determined on the bases of following the disappearance of the signs of inflammation characterized by cyanosis, edema, and hematoma at the site of injury.

Previous studies have shown that undenatured type-II collagen is effective in the treatment of rheumatoid arthritis [7, 9, 28, 29] in obese-arthritis dogs given 4 mg or 40 mg per day undenatured type-II collagen for 90 days, significant declines in overall pain, pain during limb manipulation and lameness after physical exertion were noted [30]. Greater improvement was observed with the 40 mg dose. In another investigation; efficacy of undenatured type-II collagen was evaluated in arthritic horses [22]. In this study, groups of horses were orally administered undenatured type-II collagen at 320, 480 or 640 mg for 150 days. All exhibited significant reduction in arthritic pain. However, horses receiving placebo did not show any improvement in arthritic condition [31]. Numerous international clinical studies have been able to confirm the positive effect of

collagen hydrolysate in the treatment of degenerative joint disease. When taken orally by patients diagnosed with osteoarthritis of the hip or knee joint, collagen hydrolysate is a nutritional supplement and the results was statistically significant ($p < 0.05$) in an increase of mobility, a decrease of pain, and a reduction in the dependency on analgesics [4, 32]. Overall, treatment of the patients involved with collagen hydrolysate over a longer period of time was regarded as being remarkably successful, with more than 75% of the patients reported improvement of symptoms. Studies of this supplement were available in the medical literature with more than 2000 patients who had experienced degenerative joint disease [33].

In the first clinical trial of 24-weeks duration to show improvement of joint pain in athletes who were treated with the dietary supplement collagen hydrolysate 10 g. When data from all subjects ($n = 97$) were evaluated, six parameters showed statistically significant changes with the dietary supplement collagen hydrolysate compared with placebo. Despite the study's size and limitations, the results suggest that athletes consuming collagen hydrolysate can reduce parameters (such as pain) that have a negative impact on athletic performance [4].

In addition, in a parallel double-blind, randomised clinical trial comparing Colatech® versus Glucosamine sulphate, all patients underwent a treatment period of three months (Colatech® 10 g every day vs. Glucosamine sulphate 1.5 g every day). Clear improvement was observed in both joint pain and symptoms in patients with Knee Osteoarthritis treated with Enzymatic Hydrolysate Collagen (Colatech®) and significant differences were observed [14].

While a higher dose was used in comparative, double-blind, randomized, multicentre study, in parallel groups, including 200 patients of both genders of at least 50 years old with joint pain assessed as ≥ 30 mm on a visual analogical scale (VAS). After 6 months of treatment with 1200 mg Genacol® (collagen hydrolysate), the proportion of clinical responders to the treatment, according to VAS scores, was significantly higher in the collagen hydrolysate group 51.6%, compared to the placebo group 36.5% ($p < 0.05$) [34]. The current study indicated that all treatments reduced the efficacy parameters of inflammation scores resulted from tail fracture which measures cyanosis, edema, and hematoma at the affected site. Healing rates after administration of either of the doses (10, 20, or 30 mg/kg/day) were found to be significantly different than the control group.

However it has been found that the lowest dose (10 mg/kg/day) was the most effective with the fastest healing rate than the other higher doses (20, 30 mg/kg/day). Disappearance of the signs of cyanosis was within 2 weeks on administration of 10 mg dose, 3 weeks with the higher doses compared to 9 weeks in the control group.

Edema healing was inversely related to the strength of the dose, being 4, 5, and 6 weeks on administration of 10, 20, and 30 mg/kg collagen hydrolysate respectively. As for hematoma, healing was obtained after 7 weeks with the 10 mg/kg and 9 weeks with the higher doses (20 and 30 mg/kg) but not achieved in the control during the observation time. Best healing rate of tail fracture in mice was observed with collagen at the lowest dose tested (10 mg/kg). This is in agreement with a previous Results of a multicenter, double-blind, placebo-controlled trial where patients with active rheumatoid arthritis were enrolled at 6 different sites and randomized to receive placebo or 1 of 4 dosages (20, 100, 500, or 2,500 $\mu\text{g}/\text{day}$) of oral collagen for 24 weeks. A statistically significant increase ($P = 0.035$) in response rate for the 20 $\mu\text{g}/\text{day}$ group versus placebo was detected using only the Paulus criteria. This was explained by detecting the presence of serum antibodies to collagen at baseline which was significantly associated with an increased likelihood of responding to treatment [7].

The efficacy seen with the lowest dosage is consistent with the findings of a recent animal studies when 1, 3 and 10 mg kg⁻¹ collagen were daily administered using a rat osteoarthritis model. Treated animals showed a reduced postural unbalance and a general improvement of motor activity. The efficacy of collagen in reducing pain was as higher as the dose was lowered [35].

However, the urinary excretion of hydroxyproline, the collagen biomarker, was found to be dose and time related. As shown in figure 4 that increasing the dose of collagen hydrolysate was proportionally related to the loss of hydroxyproline via urine which was obvious after the 2nd week of treatment. This means that there is no accumulation of the externally administered collagen in the body and that the excess is excreted readily. While for the control group, the excretion of hydroxyproline was less and steady in the first 2 weeks after injury (tail fracture) after which followed by a marked decrease. The decrease of hydroxyproline in urine suggests the possibility of using the endogenous collagen to mend the injured tissues. Besides, the dose dependant increase in excretion indicates a stimulatory effect of the endogenous biosynthesis of collagen by the orally administered collagen hydrolysate.

The higher efficacy of the lower dose in this study might suggest a possible feedback mechanism for the regulation of collagen turnover in the injured tissue. This is in consistence with the previous work which investigated the effect of degraded collagen on the formation of type II collagen in a cell culture model [36].

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