

# Lower Ratio of Collagen's Fibre Type III/Type I and Higher Tensile Strength in Injured Rabbit's Achilles Tendon Treated with Astaxanthin

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**Abstract**—Tendon injuries vary from minor to total tear that requires surgery. The mechanical disturbance is the most complication. Inflammatory process and formation of free radicals can worsen tendon healing. Astaxanthin can be used to help tendon healing. This study aims to prove ratio of collagen type III/type I was lower and the tensile strength was higher on the administration of astaxanthin. This study is an experimental research using post-test only control group design with rabbit as subject. A total of 32 research subjects which meet inclusion were randomly divided into treatment and control groups. The treatment group is the group that was given astaxanthin after Achilles tendon was sharply cut and sewn, while the control group was not given astaxanthin. Statistical analysis using independent t-test found significant difference between treatment and control groups. The ratio of collagen type III/type I of the rabbit Achilles tendon which receive treatment of astaxanthin is smaller than the untreated group  $p = 0.000$  ( $p < 0.05$ ). The tensile strength is greater in treatment group than the non-treated group with  $p = 0.044$  ( $p < 0.05$ ). The result showed that the ratio of collagen type III/type I was lower when treated with astaxanthin thus can help improve the tensile strength.

**Index Terms**—astaxanthin, rabbit's Achilles tendon injuries, ratio of collagen type III/I, tensile strength

## I. INTRODUCTION

Tendon as an organ which connects muscle to bone and enable power transmission generated by the muscle to the bone resulting in movement of the joint. Most tendon injuries occur in areas near the joints, such as shoulders, elbows, knees, and ankles. Injury to the joints accompanied by partial or total tear of the tendon occurs as much as 45% of all musculoskeletal injuries each year. [1], [2] Injuries to the tendon are quite often, occurs in 30% - 50% of all injuries. These injuries mainly occur due to accidents, traffic accidents or sports injuries [3].

Complications that often occur as a result of this tendon injuries are disruption of flexibility, stiffness and reduced strength of the tendon itself. Thus the function of a tendon as a buffer as well as the nature viscoelasticity to reduce muscle damage will be decreased so that the movement and function of protection are limited [1], [3], [4]. The main purpose of tendon healing is to restore the mechanical properties of the tendon (gliding function). Tendons have a poor spontaneous regenerative capacity after injury, so that the pre injury biological and biomechanical function are hard to achieve [4]. This is due to the formation of adhesions and scar tissue which inhibit the regenerative process of the tendon.

In the process of tendon healing, change in the type and distribution of collagen tissue occur. Increase in ratio of collagen type III/I. The synthesis of type I collagen in tendon injury grade III will decline and will be replaced by increase in the synthesis of collagen type III in line with the expansion of granulation tissue and remodelling process so that the ratio of collagen type III/I increases and substituted by scar tissue and fibrosis. The formation of scar tissue in the tendon can cause adhesion and decrease of tensile strength up to 20% [5]-[7].

In the phase of healing tendon, many hormones and molecules that come into play. Injuries that occur in the tendon can trigger the formation of free radicals such as hydrogen peroxide and release of inflammatory mediators such as  $\text{TNF-}\alpha$ , IL-1, IL-6 and IL-10. The free radicals can activate metalloproteinase enzymes, and increased the expression of PGE2 which degrade tendon collagen matrix. This inflammatory process can also trigger the release of growth factors such as  $\text{TGF-}\beta$  which induce a dramatic tenocyte and fibroblast infiltration, causing extensive reorganization of collagen. The proportion of type III collagen formation is higher than type I due to the inhibitory effect of PGE-2 on the synthesis of collagen I mRNA and increased expression of collagen type III [8].

Various methods have been carried out to restore the function of the tendon after injury. The choice of therapy

can be non-operative and operative with or without graft followed by immobilization or controlled mobilization. Some of these methods fail to regain normal function after injury, so that the researchers focused on the quality of therapeutic modalities and acceleration of tendon healing after injury. Modalities developed include usage of growth factors or substances that can affect the expression of growth factors, genes transfer or cell therapies [8], [9]. Antioxidant supplementation is one of the agents that have strong anti-inflammatory abilities, useful in healing process of tendon injuries.

Oxidative stress can interfere with the healing process. Fibroblasts tendon will form unorganized collagen components. The proliferation of collagen type III will increased, and the maturation of cross-linked collagen fibres become slow. The collagen components will become fibrotic, stiff, has a low modulus and uneven distribution of load bearing [10], [11]. These events cause a decrease in tendon mechanical components up to 45.2%, making it vulnerable to rupture again [10], [11].

The use of potent antioxidants i.e. Astaxanthin in the tendon injury may help reduce oxidative stress due to the formation of ROS. It has a suppressive effect on the expression of MMP, PGE2 as well as growth factors such as TGF- $\beta$  so that the fibrosis process in the healing of tendon injuries can be reduced [8], [11]-[13].

## II. MATERIALS AND METHODS

The experiment was conducted from December 2015 to January 2016 at the faculty of Veterinary Medicine Udayana University Bali. The aim of this study is to prove administration of Astaxanthin can reduce the ratio of collagen fibres type III/I and increase tensile strength of tendon healing. This study is an experimental research design using post-test only control group design with the subject rabbits from eligible subject population with randomized sampling.

Sixteen adult male New-Zealand White rabbits of the same age (12 weeks) weighing 2 – 3kg were used in this experiment. All the animal was given adequate food and water in the laboratory. These rabbits were divided randomly into control and treatment groups. The control group was not given Astaxanthin, while the treatment group was given Astaxanthin after Achilles tendon had been cut sharply and sewn with the non-absorbable nylon thread (monofilament 4-0) with a 4 strand modified Kessler technique. Followed with immobilization of the affected limb with a cast.

The Astaxanthin was given 4mg (AstaREAL 200mg) ad libitum with dose 0.106mg/kgbw/day for about 3 weeks. After that, the rabbits were sacrificed and the Achilles tendon was isolated for further examination.

The rabbit tendon tissues were examined for tensile strength in the vertical direction in which the tendon is stretched until load failure with universal testing machine. The immunohistochemistry examination is then performed to know the ratio of collagen type III/I. This procedure is carried out with rabbit tendon tissue fixation using 10% neutral buffered formalin and processed for routine histological preparations with anti-mouse

collagen III/I antibody and secondary antibody anti-mouse IgG/biotin which has been conjugated with horseradish peroxidase. The calculation of the amount of collagen tendon using the Image J. Network processing.

The obtained data are presented as mean  $\pm$  SD of number of rabbits (n) used in the experiment. The mean difference was analysed with independent t-test for statistical significant of examined variables i.e. the tensile strength and the ratio of collagen III/I. The difference was assumed to be significant at  $p < 0.05$ .

## III. RESULTS

The distribution of the research subjects are as many as 32 subjects. The treatment group with the administration of Astaxanthin is as much as 16 or 50.00% of the total subjects and the control group without the administration of Astaxanthin as many as 16 or 50.00%.

The mean of tensile strength in the treatment group was  $92.56 \text{ N} \pm 4.3384$  while the average tensile strength in the control group was  $88.53 \text{ N} \pm 6.3248$ . The highest tensile strength was  $98.74 \text{ N} \pm 4.3385$  in the treatment group and the minimum tensile strength was  $80.05 \text{ N} \pm 6.3248$  found in the control group. The mean percentage of type III collagen in the treatment group was  $14.80\% \pm 2.0559$ , while the control group was  $29.70\% \pm 6.4301$ . The mean of collagen type I in the treatment group was  $86.25\% \pm 0.9402$ , while in the control group was  $73.07\% \pm 4.7582$ . The ratio of collagen III/I in the treatment group was  $0.172 \pm 0.0242$  compared with the control group  $0.408 \pm 0.0919$ . The smallest ratio found in the treatment group with ratio  $0.133 \pm 0.0242$  while the highest ratio found in the control group with ratio  $0.570 \pm 0.0919$ .

TABLE I. THE MEAN OF TENSILE STRENGTH AND COLLAGEN TYPE III/I RATIO

| Variables               | Groups  |  |
|-------------------------|---|--|
|                         | Treatment with Astaxanthin (n=16) (Mean $\pm$ SD) | Control without Astaxanthin (n=16) (Mean $\pm$ SD) |
| Collagen Type III (%)   | $14,80 \pm 2,0559$                                | $29,70 \pm 6,4301$                                 |
| Collagen Type I (%)     | $86,25 \pm 0,9402$                                | $73,07 \pm 4,7582$                                 |
| Ratio of Collagen III/I | $0,172 \pm 0,0242$                                | $0,408 \pm 0,0919$                                 |
| Tensile Strength (N)    | $92,56 \pm 4,3384$                                | $88,53 \pm 6,3248$                                 |

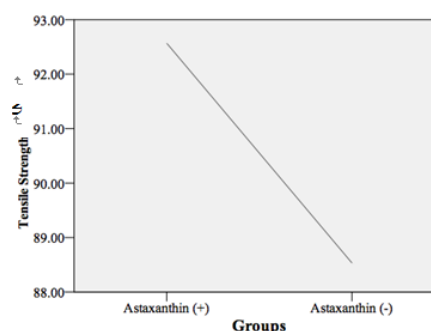


Figure 1. Tensile strength distribution among groups

Table I and Fig. 1 showed that tensile strength is higher in the treatment group compared with the control group, and the mean difference between treatment and control group was statistically significant with  $p=0.044$  ( $p<0.05$ ).

While the ratio of collagen type III/I in the treatment group is smaller than the control group, and the mean difference between treatment and control group was statistically significant with  $p=0.000$  ( $p<0.05$ ) as seen on Fig. 2.

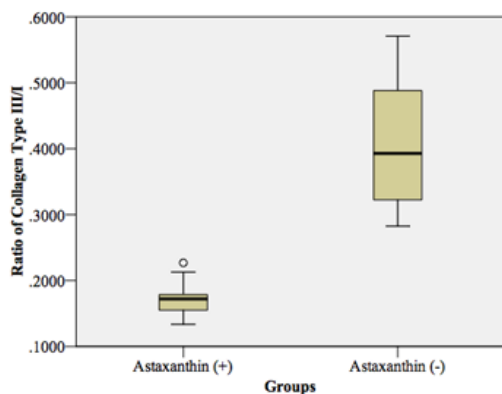


Figure 2. The ratio of collagen type III/I distribution among groups

#### IV. DISCUSSION

##### A. Effects of Astaxanthin on the Ratio of Collagen III/I

The results of the study are consistent with previous study by Bauge and Mizuta that mentioning the proportion of type I collagen synthesis and expression will be improved whereas the type III will be suppressed so that the ratio of mRNA expression of collagen III/I will fall in the injured tendon that supplemented with antioxidants such as Astaxanthin and spirulina (TOL19-001) [8], [11]. This is due to the activity of Astaxanthin inhibits the formation of ROS, IL-1 $\beta$  and PGE2 so that the inflammatory process can be less extensive and apoptosis is inhibited. Another study by Kishimoto and Song showed that Astaxanthin can inhibit several MMP expression and performance including MMP-1, MMP-2, MMP-8, MMP-13 and MMP-14 collagenase activity by suppressing ROS generation and IL -1. This process will prevent fibrogenesis and degradation of collagen fibrils type I [14], [15]. The collagen fibrils type I on the treatment group is thicker and regularly arranged as seen on Fig. 3 compared with control, whereas the type III is less composed than type I as seen on Fig. 4.

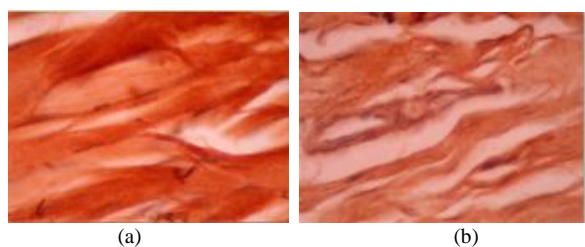


Figure 3. The histopathology image of collagen type I of the Achilles tendon on the treatment group (a) compared with the control group (b)

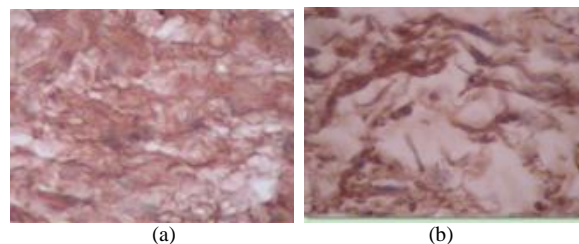


Figure 4. The histopathology image of collagen type III of the Achilles tendon on the treatment group (a) compared with the control group (b)

##### B. Effect of Astaxanthin on Tendon Tensile Strength

The results of the study are consistent with previous research by Woo and Aro which demonstrated that tendon with dominant composition of type I collagen cross-link will increase its tensile properties [7], [9]. Antioxidants such as Spirulina (TOL19-001) can help improve cross-linking, tensile strength, elasticity and structure of the tendon [8]. Those findings were inline with this study where the tensile strength of tendon in the group treated with Astaxanthin was significantly higher than the control group. The mean tensile strength tendon after treatment is still in the normal range of tensile strength in rabbits, but the maximum strength after loading decreased compared with normal tendon. The biomechanical component of tendon after a grade III injury still inferior compared with normal tendon [7].

#### V. CONCLUSION

Our study investigated the effect of Astaxanthin as an antioxidant on ratio of collagen type III/I and tensile strength of Achilles tendon after injury. The results showed that Astaxanthin significantly lowering the ratio of collagen type III/I and thus increasing the tensile strength. We suggest further experiment over the role of antioxidant on musculoskeletal events.

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