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Article

Effect of Fucoidan on Anterior Cruciate Ligament Transection and Medial Meniscectomy Induced Osteoarthritis in High-Fat Diet-Induced Obese Rats

Sabri Sudirman [1], Alan Darmasaputra Ong [1], Heng-Wei Chang [1] and Zwe-Ling Kong [1,*]

[1] Department of Food Science, National Taiwan Ocean University, 202 Keelung, Taiwan
* Correspondence: kongzl@mail.ntou.edu.tw; Tel.: +886-24622192 #5130

Abstract: Osteoarthritis (OA) has become one of the most common disabilities among elders, especially in female. Obesity and mechanical injury causing OA are attributed to joint loading, cartilage disintegration, bone loss and inflammation as well. Several strategies used for treatment OA including non-pharmacological and pharmacological. Fucoidan possesses several bioactivities such as antitumor, antiviral, anticoagulation, anti-obesity, and immunomodulation. This study aims to investigate the effect of fucoidan in surgery-induced OA on diet-induced obesity rats. OA was induced by anterior cruciate ligament transection and partial medial meniscectomy (ACLT+MMx). Male SD rats were fed high-fat diet (HFD) for 4 weeks to induce obesity before ACLT+MMx to induce OA. OA rats were administered with intragastric water or fucoidan in three different concentrations (32 mg/kg, 64 mg/kg, and 320 mg/kg) after the surgeries for 40 days with HFD. We observed that the swelling in knee joint was alleviated and hind paw weight distribution was rectified after feeding fucoidan, with no significant effect on weight gain and feed intake. Fucoidan administration indicated no significant variation on HDL-Cholesterol level, but reduced plasma triglycerides and LDL-Cholesterol level. In addition, weight-bearing tests showed improvement in the fucoidan-treated group. Our results suggested that fucoidan may improve meniscal/ligamentous injury and obesity-induced OA.

Keywords: Anterior cruciate ligament; fucoidan; osteoarthritis.

1. Introduction

Osteoarthritis (OA) is the most common form of arthritis [1]. OA is one of the most common chronic health conditions and a leading cause of pain and disability among adults [2]. OA has an inflammatory component affecting the synovium and cartilage, which leads to subchondral bone tissue breakdown, resulting in pain, stiffness, and joint failure [3-5]. Several studies have suggested that OA joint degeneration results from a combination of mechanical stresses and biochemical factors [4,6]. Chondrocytes, as well as synovial cells, of OA patients produce increased levels of inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, which in turn increase matrix metalloproteinase (MMPs) and other inflammatory mediators such as IL-8, IL-6, prostaglandin E2, and nitric oxide [5].

A torn anterior cruciate ligament (ACL) rarely heals into its anatomic or physiologic position. It is commonly associated with damage to the menisci, other ligaments, articular cartilage, and subchondral or cancellous bone [7-9]. Approximately 50% of ACL tears are believed to be accompanied by meniscal injury at the time of the acute injury, while in the chronic ACL-deficient knee, meniscal tears have been observed in as high as 80% of the patient population [7,10]. Meniscectomy might be the most important risk factor for developing knee osteoarthritis after an ACL injury [11].

Obesity is considered as a worldwide health problem with low-grade inflammatory status [12]. Obesity have long been recognized as potent risk factors for OA, especially knee OA [13]. It is

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primarily accepted that excess body weight may leads to cartilage degeneration by increasing the mechanical forces across weight-bearing joints [14,15]. Other causes are associated with inflammation and lipid metabolism disorder in obesity [16]. Recent studies reported inflammatory cytokines such as leptin, adiponectin, and IL-1β were involved in obesity-associated OA progression [17,18].

Various strategies used for management of OA such as non-pharmacological and pharmacological. Pharmacological treatments include analgesics or anti-inflammatory agents such as acetaminophen, glucosamine/chondroitin sulfates, non-selective non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase (COX)-2 inhibitors, and intra-articular (IA) corticosteroids. NSAIDs are associated with an increased risk of serious gastrointestinal (GI), cardiovascular (CV), and renal injury [19-21]. For this reason, many studies have focused on functional foods, which may promote cartilage health and safety, even after long-term use.

Fucoidan is a sulfated polysaccharide that contain *L*-fucose and sulfate ester groups, mainly found in various species of brown seaweed such as *Sargassum binderi* [22], *Undaria pinnatifida* [23], *Fucus vesiculosus* [24], *Laminaria japonica,* and *Hizikia fusiforme* [6]. In 1913, Kylin first time isolated the fucoidan from brown algae and later on according to the International Union of Pure and Applied Chemistry (IUPAC) rules these polysaccharides were named as fucoidin [25] and later on known as fucoidan [26]. Fucoidan have gained significant attraction because of their pharmacological properties such as antioxidant, anti-tumor, anti-inflammation, anti-diabetic, and anti-obesity [22,23,27]. Recent studies indicated fucoidan has potential to suppress inflammation in collagen-induced arthritis [28]. This study aims to investigated the hypolipidemic and anti-inflammatory properties of fucoidan. Furthermore, it also determined the effects of fucoidan on high-fat diet (HFD) fed rat with anterior cruciate ligament transection (ACLT) and medial meniscectomy (MMx) surgery induced OA.

2. Materials and Methods

2.1. Fucoidan

Fucoidans (low-molecular weight (MW) ~ 5,000 Daltons) were prepared from *Cladosiphon okamuranus* by hot water extraction and degraded by enzymatic hydrolysis.

2.2. Animal Model

Five-week-old male Sprague Dawley (SD) rats were purchased from BioLASCO Taiwan Co., Ltd. (Yilan, Taiwan). Rats were housed one in each cage in an animal room with a 12 h light/dark cycle at a temperature of 25 ± 2°C and 55% humidity. All procedures were followed the standard of Institutional Animal Care and Use Committee National Taiwan Ocean University.

During the experiment, diets and water were provided *ad libitum*. During acclimatization phase, all rats were given standard diet. After acclimatization, rats were divided into 2 main groups, Sham and Obese group. The obese group was given high-fat diet (HFD) for 4 weeks. Following HFD induction, obese rats were divided into obese sham (OBSham) group and OA (OBOA) group. Anterior Cruciate Ligament Transection and Medial Meniscectomy (ACLT + MMx) were performed to induce OA. For this purpose, the rats were anesthetized with Zoletil 50 (25 mg/kg, intraperitoneal (i.p.)), and the hair on the right knee was clipped. An incision was made in the medial aspect of the joint capsule (anterior to the medial collateral ligament), the ACL was transected, and the medial meniscus was removed. Following surgery, the joint was irrigated with normal saline, the capsule was sutured with 4–0 chromic catgut, and the skin was closed with 4–0 silk braided sutures. In sham-operated rats, incisions were made in the medial aspect of the joint capsule to expose the ACL, but neither the ACL was not transected nor the medial meniscus was not removed. The rats were supplied with supplemental heat and were monitored until recovery from anesthesia. The rats were also checked daily regarding their general health and for pain, discomfort and infection in the post-operative period, and cefazolin (20 mg/kg i.p.) was injected after the surgery to prevent infection. Following the surgery, the rats were intragastric treated with different doses of fucoidan, 32 mg/kg body weight (F1), 64 mg/kg (F2), or 320 mg/kg (F10) daily for 40 days. Body weights were measured

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weekly with digital balance and the width of the knee joint was measured using digital calipers before the surgery and every week for 40 days after the surgery. Additionally, Incapacitance tests were performed weekly before and every week after the surgery within 40 days. The animals were sacrificed at the age of 15 weeks, blood samples were collected and operated knees were dissected after all tests were completed.

2.3. Measurement of Plasma Biochemical Parameters

Whole blood samples were centrifuged after collected and blood plasma were separated from blood pellets. Plasma samples were preserved at -80°C and ready for use. Plasma triglycerides (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), superoxide dismutase (SOD), and glutathione peroxidase (GPX) were measured with commercial enzymatic kits (Randox, United Kingdom). Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and adipokine (leptin) were measured with ELISA kit (Abcam, Cambridge, United Kingdom; R&D Systems, Minneapolis, U.S.A.; Novex Life Technologies, Massachusetts, U.S.A, respectively).

2.4. Weight-bearing Distribution Assessment

Weight-bearing distribution changes were measured using an Incapacitance tester (Linton Instrumentation, Norfolk, UK) to detect changes in postural balance. In particular, the rats were stood on their hind paws in an inclined plane (65° from horizontal) chamber that was placed above the incapacitance apparatus; the weight applied to each hind limb was measured independently with the apparatus. Three to five measurements, each 5-s readings, were taken for each rat, and the average was calculated after excluding the outlier. The data was expressed as the difference between the weight applied to the limb contralateral to the injury and the weight applied to the ipsilateral limb (Δ Force).

2.5 Knee Width and Joint Histopathology

The width of the knee joint was measured with digital calipers every week for 40 days after the operation and the width of the contralateral knee was used as the baseline. At day 40, after all, tests were completed, the rats were euthanized with carbon dioxide, and the knee joints were collected and fixed in 4% paraformaldehyde for 2 days. The following decalcification, embedded in paraffin, and histological sectioning (5 mm) were done by Li Pei Co. Ltd. Hematoxylin/eosin (H&E) staining and Safranin-O staining were then used to examine the morphological changes and proteoglycan loss.

2.6. Statistical Analysis

All experimental data are expressed as mean ± standard error of mean (S.E.M.). Body weight, weight-bearing difference, and knee width were analyzed with Two-way analysis of variance (Two-way ANOVA) followed by Dunnett's test. Others were analyzed with one-way ANOVA followed by Duncan's multiple comparison tests with $p< 0.05$ was defined as statistically significant.

3. Results

3.1. Reduction of Body Weight and Body Fat by Fucoidan

The body weights of HFD-induced obese rats were significantly increased compared to the sham group ($p<0.05$). After treatment with fucoidan for 40 days, body weights were lowered by 9%. The perirenal adipose tissue weight also decreased after fucoidan treatments (**Table 1**). Plasma lipids were also analyzed, TG, TC, and LDL-C level of rats fed with HFD significantly ($p<0.05$) higher than treatment with fucoidan (**Table 2**).

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Table 1. Body weight and adipose HFD-induced obese and ACLT+MMx surgery induced OA male rats.

Group	Sham	Obese	Obese + OA			
			Control	F1	F2	F10
Body weight (g)						
Initial	136.24 ± 1.58^{a}	139.15 ± 2.98^{a}	141.82 ± 4.61^{a}	137.71 ± 1.43^{a}	135.19 ± 2.34^{a}	140.93 ± 2.83^{a}
Final	385.47 ± 16.50^{c}	530.89 ± 33.53^{a}	537.94 ± 36.55^{a}	477.98 ± 19.75^{b}	489.45 ± 22.23^{b}	477.61 ± 35.41^{b}
Adipose Tissue Weight (g /100g body weight)						
Perirenal	1.54 ± 0.15^{c}	3.23 ± 0.54^{a}	2.54 ± 0.37^{b}	2.23 ± 0.35^{b}	2.17 ± 0.33^{b}	2.05 ± 0.43^{bc}
Epididymal	1.02 ± 0.09^{c}	2.21 ± 0.33^{a}	2.06 ± 0.27^{ab}	1.80 ± 0.12^{b}	1.85 ± 0.27^{ab}	1.87 ± 0.36^{ab}

Data are expressed as the mean ± S.E.M (n = 7). Values with different superscript letters (a-c) represent significant difference (p<0.05) via one-way ANOVA followed by Duncan's multiple range test.

Table 2. Plasma lipid in HFD-induced obese and ACLT+MMx surgery induced OA male rats.

Group	Sham	Obese	Obese + OA			
			Control	F1	F2	F10
TG	70.83 ± 3.37^{b}	81.59 ± 4.61^{ab}	95.26 ± 8.04^{a}	79.94 ± 5.68^{ab}	77.08 ± 7.19^{a}	70.30 ± 3.57^{a}
TC	89.43 ± 7.83^{b}	120.13 ± 9.92^{a}	125.81 ± 7.08^{a}	98.29 ± 4.91^{b}	91.52 ± 3.45^{b}	88.57 ± 3.16^{b}
HDL-C	39.23 ± 1.66^{a}	41.44 ± 2.98^{a}	39.69 ± 2.02^{a}	41.86 ± 2.52^{a}	38.65 ± 2.51^{a}	38.60 ± 3.16^{a}
LDL-C	36.03 ± 7.70^{b}	62.38 ± 9.84^{a}	67.07 ± 6.90^{a}	40.45 ± 6.76^{b}	37.46 ± 4.17^{b}	35.91 ± 2.23^{b}

Triglycerides (TG), Total cholesterol (TC), High density lipoprotein-cholesterol (HDL-C), Low density lipoprotein-cholesterol (LDL-C). Data are expressed as the mean ± S.E.M (n = 7). Values with different superscript letters (a-b) represent significant difference (p<0.05) via one-way ANOVA followed by Duncan's multiple range test.

3.2. Effect of Fucoidan on Antioxidant Properties and Anti-inflammatory

Antioxidant activity of SOD and GPx were decreased and plasma MDA increased in HFD fed groups. Treatment with fucoidan restore the activities of SOD and GPx and reduce plasma MDA (**Figure 1**). Chronic systemic inflammation introduced by obesity increased pro-inflammatory cytokine synthesis. In rats fed with HFD, plasma inflammatory cytokine was increased, especially TNF-α and leptin (**Figure 2**). Treatment with fucoidan reduces inflammatory cytokines in plasma compared to HFD fed untreated groups.

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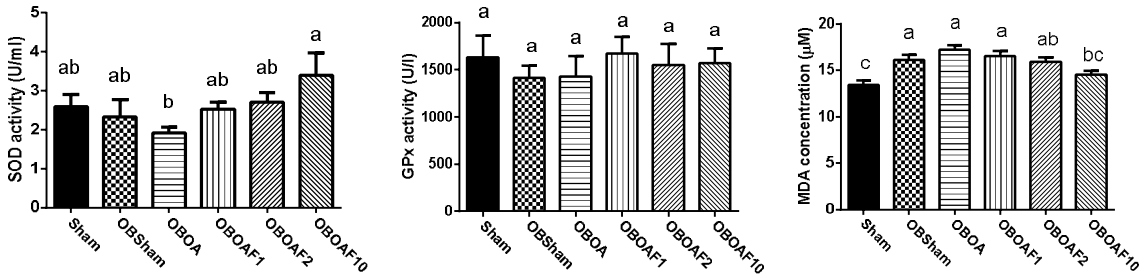
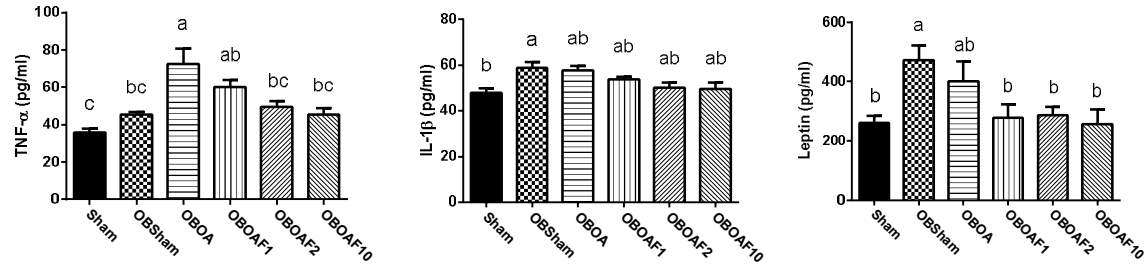


(**a**) (**b**) (**c**)

Figure 1. Effect of fucoidan treatment on antioxidant activities in HFD-induced obese and ACLT+MMx surgery induced OA male rats: **(a)** Superoxide dismutase, SOD. **(b)** Glutathione peroxidase, GPx. **(c)** Malondialdehyde, MDA. Data are the activity of each enzymeand concentration of plasma reactive oxygen species, expressed as the mean ± S.E.M (n = 7). Values with different superscript letters (a-c) represent significant difference (p<0.05) via one-way ANOVA followed by Duncan's multiple range test.



(**a**) (**b**) (**c**)

Figure 2. Effect of fucoidan treatment on plasma cytokines in HFD-induced obese and ACLT+MMx surgery induced OA male rats: **(a)** Plasma tumor necrosis factor (TNF)-α; **(b)** Interleukin (IL)-1β. **(c)** Leptin. Data are the concentration of each cytokine, expressed as the mean ± S.E.M (n = 7). Values with different superscript letters (a-b) represent significant difference (p<0.05) via one-way ANOVA followed by Duncan's multiple range tests.

3.3. Fucoidan Attenuate OA Caused Pain and Damage

Oral administration of fucoidan helps alleviate the pain induced by OA, as shown by the diminishing of hind limb force differences (**Figure 3(a)**). Post-surgery of OA results in swelling of joints. By the measurement of knee width, the joint underwent surgeries will have joint swelling after surgery and recover after 2 weeks. Due to inflammation caused by OA, the joint underwent ACLT+MMx had their joint swelling for a longer period. Treatment with fucoidan helps alleviate the swelling as the knee width differences between both hind limbs diminishing over time (**Figure 3(b)**).

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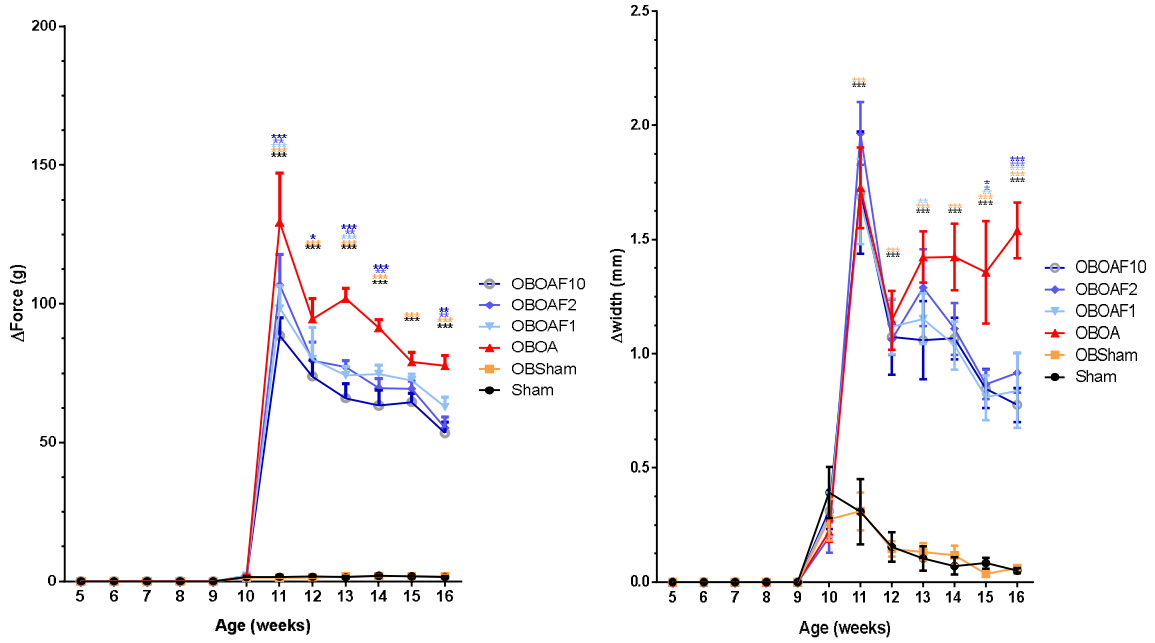

(**a**) (**b**)

Figure 3. Effect of fucoidan treatment in HFD-induced obese and ACLT+MMx surgery induced OA male rats: **(a)** on the weight-bearing distribution of the hind limbs. **(b)** Knee joint width. Data are the difference between the weights applied to the contralateral and ipsilateral limbs, expressed as the mean ± S.E.M. Two-way ANOVA and Dunnett's multiple comparisons test were used to analyze the data. *p<0.05, **p<0.01, ***p<0.001, when compared to the OA group.

In the end of experiments, rats were euthanized and knee joint specimens were collected. Joint sections were stained with hematoxylin & eosin stain to observe the morphological changes by surgery-induced OA. Result showed reduction of cartilage thickness in OBOA group where improvements observed in fucoidan-treated groups (**Figure 4**). Other joint sections were stained with Safranin-O and fast green to observe proteoglycan loss by OA. In OBOA group, joint histology showed major loss of proteoglycan in the cartilage matrix. Treatment of fucoidan prevent further proteoglycan loss (**Figure 5**).

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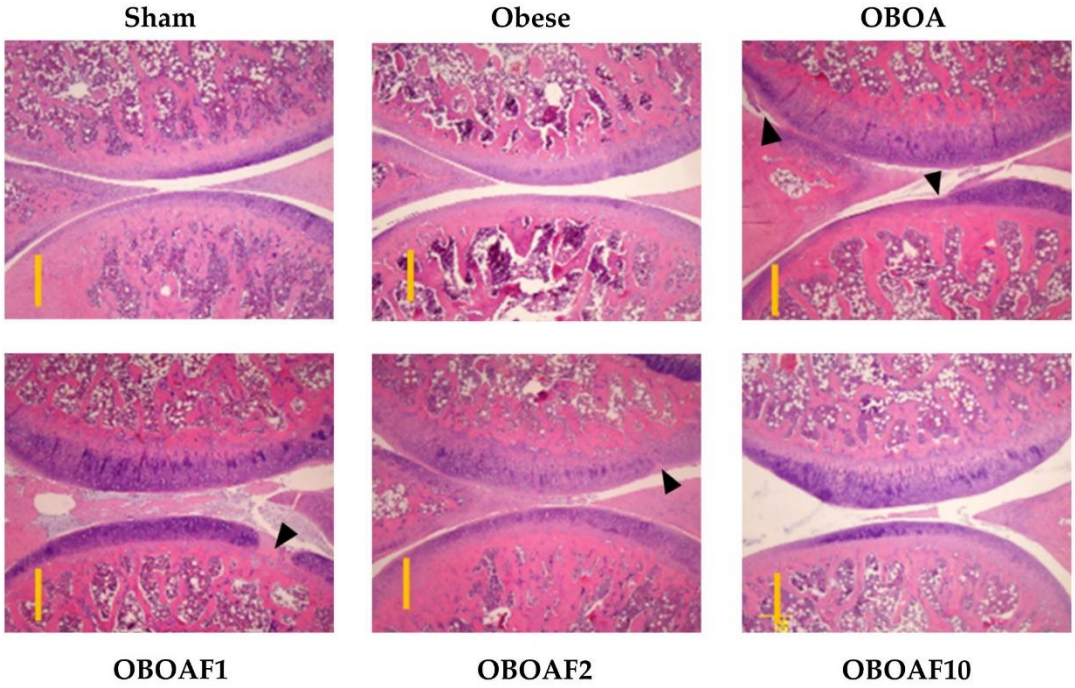

Figure 4. The histopathological difference between the knee joints in HFD-induced obese and ACLT+MMx surgery induced OA male rats. Representative cartilage sections from right medial condyle of femur and tibia were stained with Hematoxylin and Eosin. Specimens were observed with 40× magnification. Scale bar length is 500 μm.

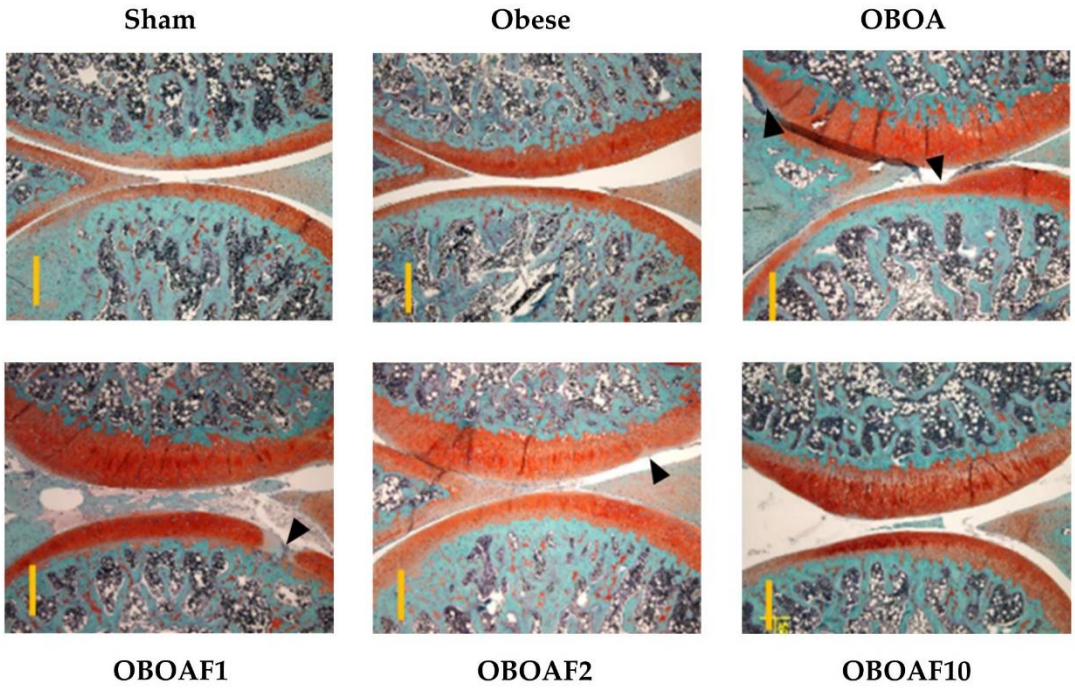

Figure 5. The histopathological difference between the knee joints in HFD-induced obese and ACLT+MMx surgery induced OA male rats. Representative cartilage sections from right medial condyle of femur and tibia were stained with Fast Green and Safranin-O. Specimens were observed with 40× magnification. Scale bar length is 500 μm.

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4. Discussion

Obesity and overweight act as one of the risk factor in OA progression [14,29]. The overload effect on joint cartilage may explain part of the increased risk of osteoarthritis, at least for osteoarthritis of the knee [16]. Reduction of body weight is a strategy for OA treatment due to it will reduction of joint loading or mechanical force on knees [30,31]. In animals with obesity, there is a huge increase in white fat (adipose tissue) deposits due to the hyperplasia and hypertrophy of their adipocytes [32]. Oral administration of fucoidan reduced the body weight in HFD-induced obese rat. In addition, fucoidan supplemented decreased the adipose tissue weight such as perirenal and epididymal fat tissues (**Table 1**). Furthermore, fucoidan administration reduced the triglycerides (TG), total cholesterol (TC), and LDL-Cholesterol (**Table 2**). Obesity condition associated with increase of plasma TG, TC, and LDL-Cholesterol level. In particular, triglyceride and cholesterol levels are closely related to cardiovascular disorders [33-35]. The previous study showed that fucoidan decreased the body weight of HFD-induced obese mice and reduced the epididymal fat tissue [27]. There was also decreased the plasma level of TG, TC, and LDL-Cholesterol in mice fed with fucoidan.

Oxidative stress is involved in pathological processes such as obesity, diabetes, cardiovascular disease, and atherogenic processes [36]. When obesity persists for a long time, antioxidant sources can be depleted, decreasing the activity of enzymes such as superoxide dismutase (SOD) and catalase (CAT) [37]. The activity of SOD and glutathione peroxidase (GPx) in individuals with obesity is significantly lower compared with that in healthy persons, having implications for the development of obesity-related health problems [38]. In addition, higher levels of malondialdehyde (MDA) in obese subjects as compared to normal-weight subjects [39,40]. The determination of MDA is used for monitoring lipid peroxidation in biological samples [41]. Supplementation with antioxidants would reduce the risk of complications related with obesity and oxidative stress [42]. The results of this study showed that fucoidan increased the SOD activity and reduced the malondialdehyde (MDA) level (**Figure 1**). The previous studies reported that fucoidan extracted from *Undaria pinnatifida* and *Sargassum bideri* showed potential antioxidant activity with high inhibition of free radicals [22,23].

The increase in obesity-associated oxidative stress is probably due to the presence of excessive adipose tissue itself, because adipocytes and preadipocytes have been identified as a source of pro-inflammatory cytokines, including TNF-α, IL-1, and IL-6 as well as adipokine such as leptin, adiponectin, resistin, and visfatin; thus, obesity is considered a state of chronic inflammation [16,37]. Inflammatory cytokine TNF-α and IL-1 may stimulate mitogen-activated protein kinase (MAPK) pathway and p38/c-Jun N-terminal kinase (JNK) pathway to synthesize matrix metalloproteinase-1 (MMP-1), MMP-3 and MMP-13 [43,44], also combined with leptin will stimulate Janus kinase 2 (JAK2) pathway and induce nitric oxide synthase (NOS) II and produce nitric oxide (NO). Nitric oxide produced in joint may cause cartilage degradation and chondrocyte apoptosis [45]. On the other hand, leptin regulates chondrocyte proliferation and differentiation [46]. Excessive leptin exposure might stimulate the differentiation of chondrocytes and formation of osteophytes [47,48].

Osteoarthritis in many cases causes joint swelling, pain, and disability [5,6]. Pain caused by the imbalanced of ipsilateral with contralateral limb (weight-bearing imbalance) and result would change their posture. In addition, in molecular inflammation, prostaglandin E_2 (PGE_2) is involved in all processes leading to the classic signs of inflammation such as redness, swelling, and pain. Pain results from the action of PGE_2 on peripheral sensory neurons and on central sites within the spinal cord and the brain [49,50]. In OA cartilage, IL-1β and TNF signalling mediated by the transcription factors NF-KB and AP-1 results in autocrine production of these cytokines, as well as expression of other inflammatory and chrondrolytic mediators including prostaglandin E_2 [45]. In the present study, the weight-bearing test show that rats induced by OA surgeries have higher differences in force applied by both hind limbs. On the other hand, oral administration of fucoidan protected the weight-bearing in ALCT+MMx-induced OA on HFD-induced rats. Lee *et al.* [6] reported that fucoidan showed the protected effects on monosodium iodoacetate (MIA) induced OA rat. Joint swelling is one clinical feature of OA attributed to inflammation and reflecting the presence of synovitis due to thickening of the synovium or to effusion [51]. Fucoidan treatment reduce the

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swelling of joint with lower knee joint width compared than non-treated OA rat model (**Figure 3**). Fucoidan has been studied for its bioactivities and show benefits for its anticoagulation [52,53], anti-inflammatory [54,55], hypolipidemic [27,56], and immunomodulatory properties [57,58]. The previous study investigated the anti-inflammatory effect of fucoidan on collagen-induced arthritis. In this study, the results suggested that lower molecular weight of fucoidan works better in lowering inflammation [28].

Under stained observation (**Figure 4** and **5**), rats supplemented by fucoidan showed the reduce of cartilage thickness and protected the matrix cartilage degeneration. Cartilage degeneration caused by overexpression of matrix metalloproteinases (MMPs). Overexpression MMP-1 stimulated the production by IL-1β and TNF-α [4,45,59]. In the present study, fucoidan-treated suppressed the expression of IL-1β and TNF-α, we hypothesized that fucoidan also suppress the expression of MMP-1 at the articular surface and inhibited cartilage degeneration. Overall, the administration of fucoidan prevents the progression of OA rat model.

In this study, we used ACLT+MMx with HFD to mimic the joint injury caused by overweight and obese with the results increased the mechanical force in joint, especially in knee joint. Recent studies suggest surgery-based OA model was more similar to natural occurring OA as a slow progressing disorder [60]. Others model such as iodoacetic acid induction method might able to mimic OA in a short time. These models, however, are more similar to chemical-induced chondrocyte death rather than OA model [61]. Due to the additional weight applied on both hind limbs, the effect of ACLT+MMx induced OA would be more significant. In the case of obesity, we also measure the inflammatory cytokines in their circulatory system

5. Conclusions

Fucoidan extracted from *Cladosiphon okamuranus* showed the anti-inflammatory effects on HFD induced inflammation, hypolipidemic properties against fat accumulation and protected the joint and cartilage on ACLT+MMx surgery induced OA in HFD fed obese rats. In addition, supplemented with fucoidan decreased leptin and IL-1β level. Our results suggest that oral administration of fucoidan may improve the meniscal/ligamentous injury and obesity-induced OA

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