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RESEARCH ARTICLE

Proteomic and Morphological Analysis of Bone and Articular Cartilage Changes in Osteoarthritic Rabbits Supplemented with Edible Bird's Nest (EBN)

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Abstract This study is aimed at assessing the effects of Edible Bird's Nest (EBN) on subchondral bone, articular cartilage and the expression of proteins in synovial fluid by micro-CT evaluation and histological analysis. 54 New Zealand white rabbits were induced by intra-articular injection of monosodium iodoacetate (8 mg) and divided into four groups: negative control (n=9): non-treated osteoarthritis; positive control (n=15): OA + diclofenac sodium 2 mg/kg daily orally; low dosage (n=15): OA + 75 mg/kg hydrolyzed EBN; and high dosage (n=15): OA + 150 mg/kg hydrolyzed EBN. The joints were harvested and subjected to micro-CT analysis and histological evaluation, and the synovial fluid was subjected to LCMS/MS analysis. Micro-CT analysis showed an increase in bone volume and a decrease in total porosity in the treatment group that showed bone integrity improvement. Histopathological results revealed comparable changes between the positive control group and the EBN treatment group. There was upregulation of proteins involved in the resolution of inflammation and downregulation of proteins associated with the bone resorption process. Morphology evaluation showed that EBN supplementation has a bone-improving effect by inhibiting osteoclastic activity. Protein expression showed chondroprotection and bone improvement through the action of several proteins via various signaling pathways. The morphological and molecular findings suggest the potential use of EBN as beneficial alternative for osteoarthritis treatment by improving bone quality and modulating inflammatory responses.

Keywords: Osteoarthritis (OA), subchondral bone, articular cartilage, micro-computed tomography (micro-CT), edible bird's nest (EBN).

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Introduction

Osteoarthritis (OA) is a joint disorder which is now widely accepted as a wide-ranging chronic condition affecting all components of the synovial joint, rather than just cartilage. It affects up to 15% of the global human population [1]. Many factors contribute to the onset of OA, including biomechanical, biochemical and genetics, and biomolecular and signaling feedback mechanisms [2]. Animal models are used to investigate the progression of disease and to assess the clinical potential of osteoarthritic treatments. Animal models of OA typically use rabbits as a result of their structural parallels to human knees (patellar ligament, two menisci, suprapatellar synovial recess). To measure the development of OA in animal models, histological and macroscopic evaluations are the gold standard. They allow for unambiguous visualization of articular cartilage and non-osseous components associated in OA pathology. Furthermore, modern non-invasive, multi-modality imaging techniques provide a unique and comprehensive approach to assessing bone microstructure in animal models of experimentally induced OA. Micro-computed tomography (micro-CT) has been shown to be a reliable method for determining improvements in bone thickness, density, and architecture [3, 4].

Current gold standard in OA diagnostics, only focuses on subchondral bone changes and not articular cartilage which is important in OA onset and progression. Articular cartilage is assessed indirectly using joint space width that reflects the spacing between subchondral bones ends in a joint. Overall, the widely used method does not allow early detection of structural deterioration. Moreover, OA diagnosis also occur with the appearance of pain, which is already at the advanced stage of the disease [5]. Therefore, proteomics is important in clinical applications of proteomics because they can help in early diagnosis of OA using potential biomarkers which is also useful in monitoring disease progression and in improving therapeutic outcomes. Biomarkers help clinicians in subclinical OA diagnosis and also determine the extent of disease progression. New discovery of biomarkers that indicate early response in the cartilage can be useful in detecting early, pre-radiographic OA. Also, proteomics technique can help to further understand OA pathophysiology and its underlying mechanisms. In recent years, the major focus of OA clinical research is to identify biomarkers that can help in early diagnosis and also to optimize custom individualized treatments [6].

Current OA medications are palliative in nature, with the aim of reducing pain, which is the disease's primary symptom. Intra-articular injections of hyaluronic acids (HA) and steroids, as well as administration of nonsteroidal anti-inflammatory drugs (NSAIDs) are a few treatment strategies used in addressing this disease [7]. However, these treatments come with several adverse effects such as renal toxicity, diarrhea, vomiting, gastrointestinal disturbances, nausea, or increased cardiovascular risks [8]. Surgical intervention by total joint replacement may be done at advanced stages of the disease; however, this is a major operation with significant morbidity and long-term complications such as joint injury and infection [9]. Natural products are gaining a lot of attention as an alternative remedy for a variety of health problems because of their high level of effectiveness [10]. Numerous traditional medicines including EBN have been studied extensively in order to create potential therapies for inflammatory disorders such as arthritis [11]. EBN is a natural product which is popular among Chinese populations all over the world. It is produced from saliva released by swiftlets specifically from *Aerodramus fuciphogus* and *Aerodramus maximus* species. The major composition of EBN is 62-63% of proteins and 25.62-27.26% of carbohydrate. Composition of EBN were previously characterized by [12].

Evidence suggests that EBN has nourishing and therapeutic qualities. It has been utilized for a variety of purposes, including anti-aging, anti-cancer, immunity enhancing, phlegm dissolvent, tuberculosis, voice improvement, stomach ulcers, hematemesis, asthenia, aphrodisiac, libido raising, renal dysfunction, asthma, cough, complexion enhancement, illness and surgical recovery, increasing energy and metabolism [12, 13]. A number of the studied properties of EBN includes cell proliferation induction, immunomodulatory effects, wound healing, neurodegenerative diseases amelioration, atherosclerosis, improvement of bone strength and skin sickness [14, 15, 16, 17]. However, EBN effectiveness and also the direct and indirect mechanism of action in osteoarthritis are not well understood. EBN exert chondroprotective effect based on an *in vitro* study by [18] using human chondrocytes. However, to date, there is no *in vivo* study on the effects of EBN treatment in OA. Therefore, this study is aimed to assess the effects of EBN treatment on subchondral bone and articular cartilage and the expression of proteins in synovial fluid of an experimental rabbit model of OA by micro-CT evaluation and histological analysis.

Materials and Methods

Animals

A total of fifty-four adult male New Zealand white rabbits (A-Sapphire Enterprise, Malaysia), aged 8-9 months and weighing 1.8-2.0 kg were housed in Animal Research Facility, Faculty of Veterinary Medicine, Universiti Putra Malaysia. Each rabbit was individually caged in stainless-steel cages and maintained with controlled temperature and humidity and regular light cycles, becoming acclimatized for a week. Rabbits had access to ad libitum feed and drink. The experiment was conducted in compliance with the current ethical regulations for animal care and use by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM/IACUC/AUP-R034).

Preparation of Animal Model of OA

To establish OA, rabbits were anaesthetized with Zoletil® (Virbac, Australia) at 2 mg/kg via intramuscular route and the stifle joint were injected with monosodium iodoacetate (MIA) (Sigma-Aldrich, USA) at 8 mg/joint via intra-articular route. MIA was dissolved in 0.9% sterile saline to a stock concentration of 25 mg/ml. The rabbits were randomized into four groups: negative control (no treatment, n=9), positive control (diclofenac, 2 mg/kg/day/oral, n=15), low dosage (EBN, 75 mg/kg/day/oral, n=15), and high dosage (EBN, 150 mg/kg/day/oral, n=15). Subsequently, three rabbits in negative control group and five rabbit from each treatment groups were sacrificed in week 4, 8 and 12 after model establishment.

Preparation of EBN Hydrolysate

EBN hydrolysate was purchased from Innovative Centre of Confectionery Technology (MANIS), UKM. Raw EBN was soaked in water with 1:100 ratio (w/v) and subsequently incubated at 4°C for 16 h. Sample was boiled at 100°C for 30 min and adjusted to suitable pH for enzymatic hydrolysis. Alcalase enzyme was added with 1% enzyme to substrate ratio and hydrolysis was conducted for 4 h. The hydrolysate was heated in boiling water for 5 min for enzyme inactivation and centrifuged at 4°C and 4000 rpm for 10 min. Supernatant was discarded and the filtrate was freeze-dried (EBN powder) [19]. For treatment, the EBN powder was dissolved in 15 ml distilled water and vortexed.

Sample Collection

Clinical symptoms (e.g., weight loss, immobility, etc.,) in rabbits were observed daily. At the end of week 4, 8 and 12, the rabbits from each group were euthanized with pentobarbital sodium (Veto quinol, France) at 120 mg/kg via intravenous route. Synovial fluids were collected via arthrocentesis in a 1.5 ml microcentrifuge tube and stored immediately at -80°C until subsequent analysis. Knee joints samples were harvested and immediately fixed in 10% buffered formalin (Sigma-Aldrich, USA). After 24 h, the muscle and tissue surrounding the femur were removed carefully to avoid damaging the cartilage surface. The proximal femur was fixed in 10% buffered formalin. Medial and lateral femoral condyle of stifle joints were photographed by P7000 digital camera (Nikon, Japan) at macro setting. The articular cartilage surface was examined for gross morphological changes, including erosions, fibrillations and fibro cartilaginous formation.

Micro-CT Evaluation

The distal femur of each rabbit was scanned and analysed using micro-CT (SkyScan1076, Belgium) with the following specifications: pixel size 18 µm, current 110 µA, voltage 70 kV, beam filtration filter 0.5 mm aluminum, exposure time 500ms. Projections were acquired with an angular range of 360° (angular step of 0.9°). Using the reconstruction software Skyscan NRecon (Skyscan, Belgium), two dimensional (2D) images were qualitatively evaluated by using DataViewer software (Skyscan, Belgium) in dorsal and sagittal planes for morphological changes. The lining of the joint and the presence or absence of osteophytes in the reconstructed dataset were identified and described. Micro-CT images were analysed quantitatively by using Skyscan CT-Analyser Software (Skyscan, Belgium). Measurement was taken from a stack of regions of interest (ROI) consisted within volume of interest (VOI) at distal femoral subchondral bone at the epiphyseal region with a semiautomatic contouring method. 3D micro-architectural parameters such as the ratio of bone volume over tissue volume (BV/TV; %), bone surface to volume ratio (BS/BV; mm^2/mm^3), trabecular thickness (Tb.Th; mm), trabecular spacing (Tb.Sp; mm), and total porosity (PO; %) were directly measured.

Histology

Femur samples were immediately fixed in 10% buffered formalin (Sigma-Aldrich, USA). The adjacent muscle tissue was cleaned and femur was decalcified in 10% formic acid (Nacalai Tesque, Japan). Formic acid was routinely changed every two days over 10 days period. The decalcified femur was cleaved in a dorsal plane, placed in cassette, and labelled. Tissue processing was done accordingly:

80% alcohol (2 h), 95% alcohol (2 h), 100% alcohol (3 h), chloroform (3 h), and paraffin (5 h 30 min) using Leica TP1020 Semi-enclosed Benchtop Tissue Processor (Leica Biosystem, Germany). Samples were embedded in paraffin wax using Leica EG1150H and EG11559 Modular Tissue Embedding Center (Leica Biosystems, Germany). The paraffin block was then sliced into 5 µm thin section using the Reichert-Jung 2045 Multicut Rotary Microtome (Leica Biosystems, Germany) and mounted on glass slide. The glass slides were dried on the slide warmer overnight, deparaffinized, and hydrated with distilled water. Samples were stained with Weigert's iron hematoxylin working solution and rinsed with running tap water, each for 10 min. Next, samples were stained with Fast green FCF solution for 5 min, rinsed rapidly with 1% acetic acid for 10-15 s, and counter-stained using 0.1% Safranin-O solution for 5 min. The slides were dehydrated with 95% ethyl alcohol and rinsed with absolute ethyl alcohol, alternately at 2 min each for two times. Lastly, the slides were mounted with resinous medium. The slides were then observed under microscope (Motic, China) at 10X and 40X magnification, and scored independently by two blinded observers and the severity of OA was evaluated based on OARSI scoring system [20]. For each sample, two slides were prepared and graded for five times at different sections along the articular cartilage. The two observers reached a consensus on each section.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software, version 7.0 for Windows (GraphPad Software, La Jolla, CA, USA). Micro-architecture parameters from week 4, 8 and 12 for all groups were compared using one-way ANOVA and Tukey's HSD multiple comparison post hoc test was used to identify significant differences between groups after checking for data normal distribution. Histological scoring was analyzed by a non-parametric test (Kruskal-Wallis followed by Dunn's post-hoc test) and *p*-values lower than 0.05 were considered significant.

Proteomics Analysis of Synovial Fluid

A 100 µL aliquot from each pooled samples was treated with 10 µl of hyaluronidase (1 mg/ml) (Sigma-Aldrich, St. Louis, USA) at 37 °C for 1 h to reduce viscosity and then centrifuged for 5 min at 1000 × *g*. Protein concentration of each sample was measured using bicinchoninic acid (BCA) protein assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, USA) according to the manufacturer's instructions and the concentration was standardized to 50 µg/ml. Proteins from synovial fluid were precipitated using modified TCA/Acetone precipitation method [21, 3]. Subsequently, protein samples were prepared as mentioned by [22], prior to analysis.

Liquid Chromatography-Tandem Mass Spectrometry

The LCMS/MS analysis was performed using EASY-nano liquid chromatography (EASY-nLC) 1200 System (Thermo Scientific, MA, USA), coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, MA, USA). Each sample (2 µl) was injected into an Acclaim PepMap 100 C18 reversed phase column (3 µm, 0.075 x 150 mm) (Thermo Scientific, MA, USA) at the flow rate of 300 nl/min. Full scan spectra were collected by orbitrap MS (OTMS) with scan range within 310 – 1800 *m/z*, resolving power of 70000 and maximum injection time of 100 ms. Only precursors charged state of 2-8 were further analysed for MS2. The MS2 spectra were analysed by linear ion trap MS using rapid scan rate with resolving power of 17500 and a maximum injection time of 60 ms. Precursors were fragmented by collision induced dissociation and high energy collision dissociation at normalized collision energy of 28 %. Mass range scanned was from *m/z* 110–1800.

Proteomics Analysis of Synovial Fluid

Data were analysed using Thermo Scientific™ Proteome Discoverer™ Software Version 2.1 against *Oryctolagus cuniculus* database downloaded from NCBI. Mass tolerances for peptide and product ions were set to 20 ppm and 0.5 Da. Trypsin was designated as the protease with two missing cleavages allowed. Carbamidomethylating on cysteine and lysine was set as the fixed modification while oxidation of methionine and deamidation of asparagine and glutamine were searched as variable modifications. Proteins were accepted if they had at least one Rank 1 peptide. All database searches were also performed against the decoy database to determine the false discovery rate. All peptide spectral matches were validated using the Percolator® algorithm, based on q-value less than 1% false discovery rate (FDR).

Results and Discussion

In the present study, effects of EBN in ameliorating OA in bone and articular cartilage as well as differential expression of proteins at different time points were evaluated. MIA-induced OA progresses more slowly compared to anterior cruciate ligament transection-induced OA and therefore is a more

suitable choice for study to observe treatment efficacy [23]. MIA-induced OA has joint and cartilage degenerative features that are similar to human OA [24] and [25] demonstrated that diclofenac is effective to treat OA.

Gross Anatomy of The Femoral Condyle

Based on the macroscopic images (Figure 1), negative control group grossly showed presence of erosion along the surface of lateral and medial articular cartilage during week 4, whereas smooth, glistening and translucent appearance of articular cartilage were observed in positive control and high dosage EBN treatment group. In contrast, slight erosion at the lateral part of the articular cartilage was detected in low dosage EBN treatment group. At week 8, erosion was present in all groups as indicated by the red arrows. At week 12, these changes were observed accordingly: prominent erosion and presence of fibro-cartilaginous material covering lateral part of articular cartilage surface (negative control group), noticeable erosion at lateral part (positive control group), erosion of the medial part (low dosage EBN), and relatively intact articular cartilage surface with minimal erosion and roughness along the surface (high dosage EBN).

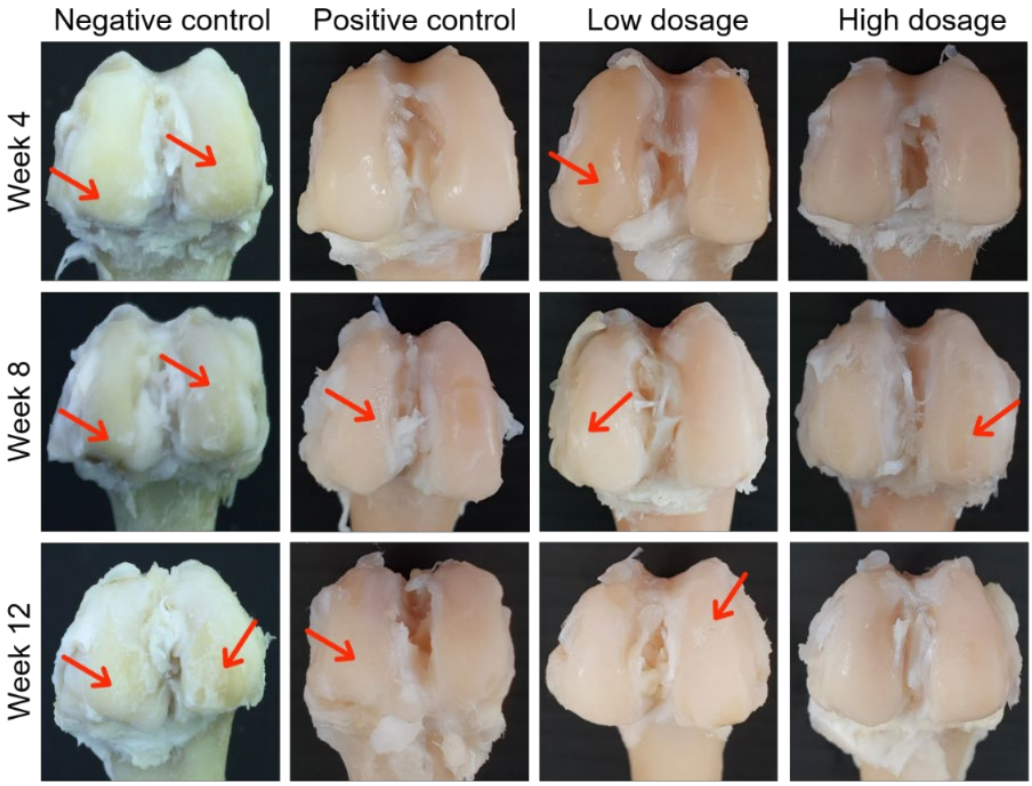

Figure 1. Gross morphology observation of the femoral condyle of negative control, positive control, low dosage EBN treatment and high dosage EBN treatment groups for week 4, week 8 and week 12. Osteoarthritic changes indicated by presence of erosion as shown by red arrows

Quantitative bone micro-architecture assessment

Trabecular bone micro-architecture is an important determinant of bone quality [26] and changes in micro-architectural properties have been reported previously in human OA [27, 28]. To investigate the effects of EBN treatment, micro-CT evaluation was performed and several parameters of the bone micro-architecture were measured as shown in Figure 2.

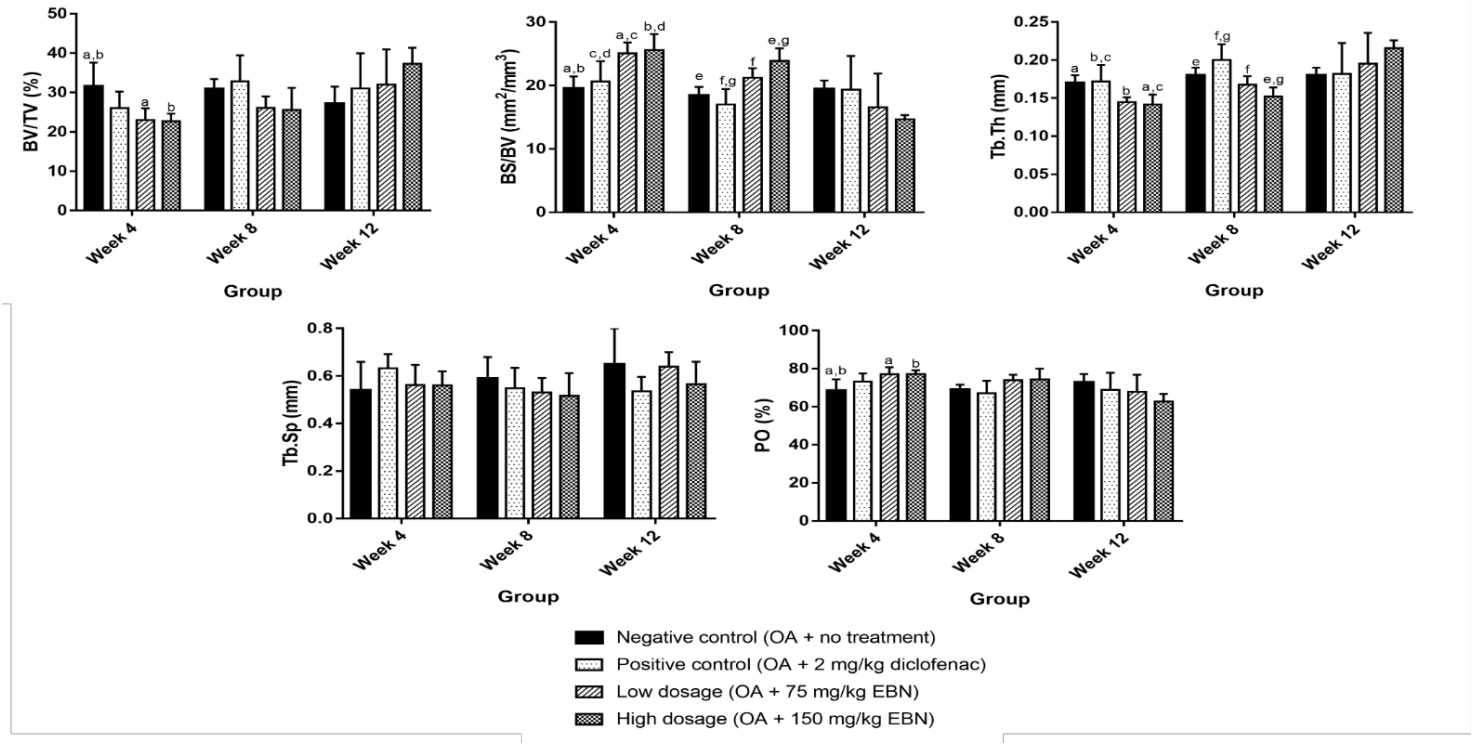

Figure 2. Progression of the subchondral bone micro-architecture of negative control, positive control, low dosage EBN treatment and high dosage EBN treatment group on week 4, week 8 and week 12. Results were presented as mean ± SD. Significant differences are in comparison with negative control group; *p<0.05 and indicated with the same letter within the groups between the columns

At week 4, the BV/TV value decreased in positive control, low dosage EBN, and high dosage EBN groups in comparison to the negative control group. At week 8, slight increased in BV/TV value was observed in positive control, both low dosage EBN and high dosage EBN groups, as compared to week 4. At week 12, increased BV/TV value was observed in positive control, low dosage EBN and high dosage EBN groups when compared to the negative control group. The decrease in BV/TV value in treatment group for week 4 and week 8 followed by an increase after 12 weeks treatment, with a 22% increase in high dosage group can indicate bone quality improvement. EBN treatment could slow down bone loss after long-term treatment (12 weeks). Components such as sialyl glycoconjugates are present in EBN, which may be effective for improvement in ovariectomized rats, as previously reported [29]. Also, phosphorous which is a bioactive component in EBN could help in improving bone strength as bone mineral consists of calcium phosphate, and phosphorus is as important as calcium in supporting bone augmentation and maintenance [30].

At week 4, increased BS/BV value was noticed in positive control, low dosage and high dosage EBN treatment groups as compared to the negative control group. At week 8, the BS/BV value decreased significantly in all groups as compared to the value obtained at week 4. A conflicting results of BS/BV value were recorded at week 12 whereby positive control group had increased value, while low and high dosage EBN group had decreased value when compared to the negative control group.

For Tb.Th value, a negative correlation was observed in respect to the BS/BV values wherein at week 4, the Tb.Th value of positive control was similar as negative control group. Also at week 4, decreased Tb.Th value was observed in low dosage and high dosage EBN groups in contrast to the negative control. At week 8, the Tb.Th value was higher in positive control, but decreased in low dosage and high dosage EBN groups against the negative control. At week 12, both negative and positive control groups had identical Tb.Th value, whereas the low and high dosage EBN groups had increased value in comparison to the control groups.

Increased value of Tb.Sp in all groups at week 4 and decreased at week 8 in comparison to the negative control was observed. Conversely, at week 12, increased Tb.Sp value was observed in positive control and high dosage EBN groups against the negative control group. the PO value indicated that there was

an increased in positive control and significant increased in low dosage and high dosage EBN treatment group at week 4. The value was decreased at week 8 as observed in positive control, however, increased PO value was notified in low and high dosage EBN groups. Surprisingly, the PO value of the treated group was attenuated in comparison to the negative control.

Tb.Th, which is correlated with BS/BV value, also showed a decreasing trend in week 4 and week 8 in treatment group, but increases during week 12 of treatment. The increase in Tb.Th was accompanied by increase in BV/TV and lower PO. This showed that treatment with EBN can mitigated trabecular bone thinning and prevent bone loss after 12 weeks. This may be related to osteoprogenterin, which can reduce osteoclastic activity thereby enhancing osteoblastic activity and bone mineralization [31]. Previous study reported high level of osteoprogenterin in EBN treatment group of ovariectomized rats which may have contribution towards enhancing bone density and preventing bone loss [32].

Histopathological Grading and Statistical Analysis

The effects of EBN on articular cartilage were evaluated by OARSI score (Table 1) and histochemical analysis (Figure 3). Negative control, positive control, low dosage and high dosage EBN groups showed comparable OARSI score, implying similar lesions observed. Cartilage degradation was mild during week 4, where positive control group showed a median value of 0 (range: 0-1) and negative control, low dosage and high dosage EBN treatment group showed a median value of 1, with range of 1-2, 1-2 and 0-1 respectively. Cartilage degeneration became more severe at week 8, evidenced by markedly increase OARSI scores median of 5 for negative control (range: 5) and high dosage EBN treatment group (range: 3-6). Positive control and low dosage EBN treatment group showed a median score of 4, with range of 4-5 for positive control group and 4-6 for low dosage treatment group.

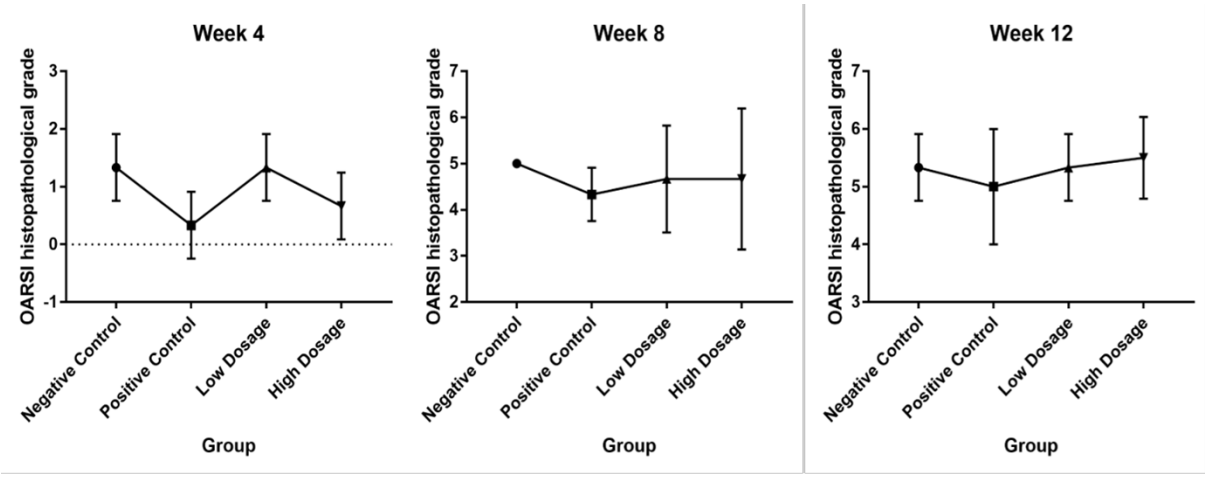

Figure 3. OARSI histological scores for negative control, positive control, low dosage EBN treatment and high dosage EBN treatment group for week 4, week 8 and week 12. Values are median with 95% confidence interval (n=5)

Week 4 showed a continuous, smooth, intact cartilage layer with no obvious cartilage destruction for all groups. However, during week 8, significant osteoarthritic cartilage degradation features can be observed, including articular surface fibrillation and erosion at superficial and mid-zone layer. The result is consistent for all groups. Similar result can be observed during week 12 where profound destruction of articular cartilage indicated by severe surface irregularity, chondrocytes apoptosis and disorganization and these results have not much differences among the groups. In summary, positive control and EBN treatment group did not showed any improvement in cartilage structure in comparison with negative control based on histology result. No significance difference was found in any of the compared group.

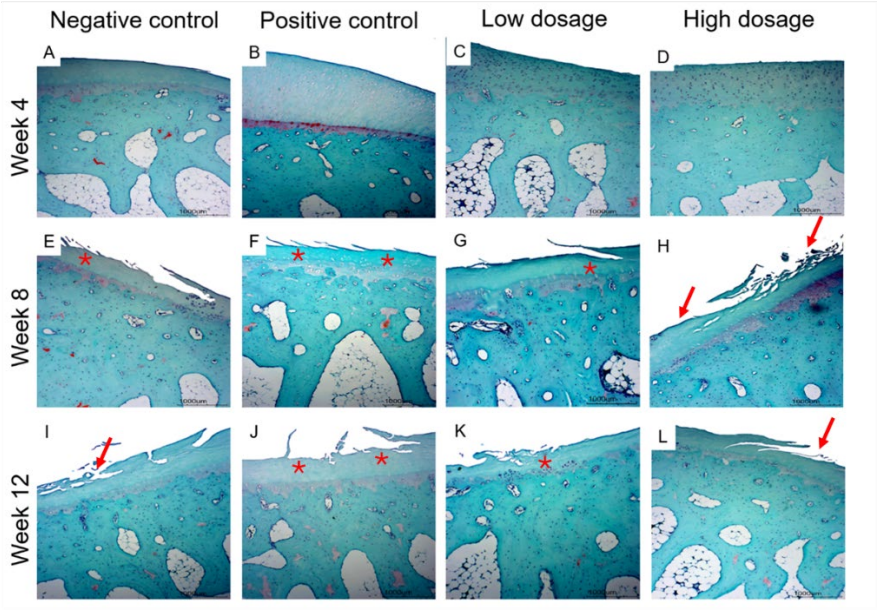

Figure 4. Representative histology images of femoral articular cartilage for negative control, positive control, low dosage EBN treatment and high dosage EBN treatment group for week 4, week 8 and week 12. Safranin O/fast green stained with 20x magnification. At week 8, articular cartilage fibrillation and erosion into the mid zone (asterisk) and surface denudation (red arrow) can be seen in all groups. Similar result is observed in week 12 group whereby erosion (asterisk) and surface denudation (red arrow) can be seen in all groups

Comparative Proteome Profiling

A total of 2782 proteins were identified across 12 pooled samples. From these initial proteins, 325 proteins were further enriched to assess biological process and pathways involved, and thus further elucidate the effects of EBN treatment. The GO enrichment analysis results were represented in Figure 4.

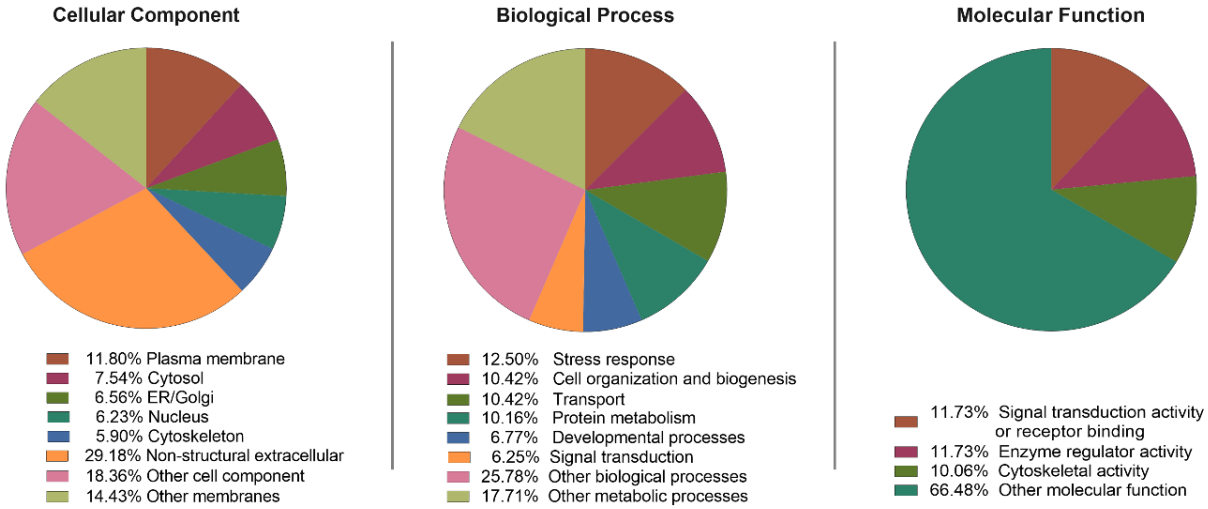

Figure 5. Gene Ontology (GO) domain overview of all identified proteins. All identified proteins were input into the three GO domains – Biological Process, Cellular Complex, and Molecular Function – and the resultant terms and percentage of proteins associated with these terms are visualized as pie charts. Term names and percentages are located next to their position on the chart. Percentages correspond to all 475 proteins identified across all groups

Majority of the identified proteins were from extracellular component. Stress response contributed most of the biological process of the proteins (48; 12.5%) and signal transduction activity/receptor binding contributed most of the molecular functions of proteins (21; 11.73%). Proteins involved in signal transduction biological process (in response of EBN towards OA) were listed in Table 1.

Table 1. List of upregulated and downregulates proteins identified from control and treated samples

Week 4			
Accession no.	**Proteins**	**Gene name**	**Fold change**
291400487	transferrin receptor protein 1	TFRC	6.64
28394750	myocilin	MYOC2	-2.75
162287061	caveolin-1	CAV1	-6.64
291391454	actin-related protein 3	Arp3	-1.68
Week 8			
Accession no.	**Proteins**	**Gene name**	**Fold change**
291391890	collagen alpha-1(III) chain	COL3A1	1.74
655607983	EGF-containing fibulin-like extracellular matrix protein 1 isoform X1	EFEMP1	1.88
655898269	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GNB1	2.74
291383947	ras-related protein Rab-39A	RAB39A	6.64
118582299	Carbonic anhydrase 2	CA2	2.24
28394750	myocilin	MYOC	1.80
49037475	Calmodulin	CALM	-6.64
162287061	caveolin-1	CAV1	-6.64
126723401	glucose-6-phosphate isomerase	GPI	-6.64
291402252	vimentin	VIM	-1.98
291389089	tubulin alpha-1A chain	TUBA1A	-1.67
291388361	14-3-3 protein zeta/delta	YWHAZ	-1.84
307548922	collagen alpha-2(I) chain precursor	COL1A2	-1.98
291400487	transferrin receptor protein 1 [Oryctolagus cuniculus]	TFRC	-6.64
291407758	collagen alpha-5(IV) chain isoform X1	COL4A5	-6.64
P60712	Actin, cytoplasmic 1	ACTB	-2.13
Week 12			
Accession no.	**Proteins**	**Gene name**	**Fold change**
118582299	Carbonic anhydrase 2	CA2	3.77
291407758	collagen alpha-5(IV) chain isoform X1	COL4A5	6.64
284004994	rab GDP dissociation inhibitor alpha	GDI1	6.64
655859660	angiotensinogen	AGT	-1.68
291400487	transferrin receptor protein 1	TFRC	-6.64
291391454	actin-related protein 3	Arp3	-1.63
162287061	caveolin-1	CAV1	-6.64
291402252	vimentin	VIM	-2.39
291383777	heat shock cognate 71 kDa protein	HSPA8	-1.84
291389089	tubulin alpha-1A chain	TUBA1A	-2.82
291388361	14-3-3 protein zeta/delta	YWHAZ	-1.90
655898269	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GNB1	-2.28
284004994	rab GDP dissociation inhibitor alpha	GDI1	-1.57
291391890	collagen alpha-1(III) chain	COL3A1	-1.72
307548922	collagen alpha-2(I) chain precursor	COL1A2	-3.30
1052873	annexin I	ANXA1	-2.20
49037475	Calmodulin	CALM	-6.64

Among all the proteins identified, most of the proteins were involved in cartilage and were extracellular matrix (ECM) proteins which includes caveolin-1, collagen alpha-1 (III) chain, collagen alpha-5 (IV), EGF-containing fibulin-like extracellular matrix protein 1, angiotensinogen, 14-3-3 zeta/delta and heat shock cognate 71 kDa. Angiotensinogen and EGF-containing fibulin-like extracellular matrix protein 1 were proteins exclusively identified in the positive control group and the expression of these proteins may be related to the pharmacokinetics and pharmacodynamics of diclofenac in response to the onset of OA.

Caveolin-1 is a transmembrane protein that has been proposed to function as regulator for many cellular processes such as caveolae biogenesis, vesicular transport, cholesterol homeostasis and in stress induced senescence [33, 34]. In comparison with negative control, caveolin-1 expression is upregulated in high dosage EBN treatment group during week 4, positive control and low dosage EBN treatment group during week 8 and positive control and high dosage EBN treatment group during week 12. [35] demonstrated that caveolin-1 expression is upregulated in induced oxidative stress of chondrocyte senescence while other study reported lower caveolin-1 expression is observed in more advanced cartilage degeneration in OA. [36] reported that caveolin-1 significantly correlates with osteopontin (OPN), a protein which regulates hyaluronic acid (HA), type II collagen and proteoglycan expression at cartilage through β_1-integrin mediator signaling pathway. Therefore, upregulation of caveolin-1 could indicate the expression of cartilage protective factor in EBN treatment group.

At week 8, it was observed that collagen alpha-2 (I) chain precursor upregulation expression in low dosage EBN treatment group and in high dosage EBN treatment group during week 12. As for collagen alpha-1 (III) chain expression, upregulation of this protein can be observed in high EBN dosage group also during week 12. Collagen type I and III are usually related as they both belong to the large fibrillar collagens. The lining layer of synovium consists of collagen type III while the sub lining layer consists of type I collagen. Type I and type III collagen are usually elevated in osteoarthritic condition. Previous studies have shown that in disease process, the production of predominant type II collagen will switch to type I collagen [37] due to upregulation of TGF β1 [38]. Type III collagen was speculated to bind collagen network in attempt to repair ECM collagen network from further deterioration and damage as an early response of chondrocyte in osteoarthritis [39]. Similarly in this study, upregulation of collagen alpha-1 (III) may indicate early repair mechanism in response to damage.

14-3-3 zeta/delta protein was upregulated in low dosage EBN treatment group during week 8 and in low dosage and high dosage EBN treatment group during week 12. This protein consists of seven isoforms, and these proteins involved in many processes involving cell cycle progression, apoptosis, intracellular protein trafficking and signal transduction [40]. However, recent evidences have elaborated more on this protein function in inflammatory response in TLR signalling via suppression of TLR3 [41] and the upregulation of this protein in EBN treatment group may indicate anti-inflammatory response of EBN.

Heat shock cognate 71 kDa protein act as a molecular chaperone protein which assist in intracellular cell folding, transport and disassembly [42]. This protein also protects cells from apoptosis and necrosis thus inhibit oxidative damage to the cells [43]. Some studies reported overexpression of heat shock protein in OA and considered as protective phenomenon at the cartilage [44]. The anti-apoptotic effect of this protein is due to interference in downstream caspase activation and activity [43]. In this study, this protein was upregulated in low dosage and high dosage EBN treatment group during week 12 and it can be suggested that EBN treatment protect cells from oxidative damage.

Some of the proteins identified are cytoskeletal proteins including actin, vimentin, tubulin 1A, myocilin, calmodulin, guanine nucleotide binding protein and actin related protein 3. Vimentin is a fibroblast intermediate filament protein in smooth muscle cells/tissues that regulate actin cytoskeleton in smooth muscle [45]. In chondrocytes, vimentin is involved in modulating mechanotransduction [46], a process as a response to mechanical stimulus which converts to biochemical signals which produce cellular responses. Vimentin is also associated with fibroblast proliferation and involved in wound healing. The decrease of vimentin is associated with OA and cause by continuous mechanical stress in OA. In this study, vimentin was identified and upregulated in low dosage EBN treatment during week 8 and continue to be upregulated in low dosage and high dosage EBN treatment during week 12, compared with negative control group. The upregulation of this protein may indicate fibroblast proliferation, which in turn will activate collagen production and TGF-β secretion. EBN was previously reported to increase fibroblast differentiation and in this study, it may be by the action of vimentin.

Other cytoskeletal proteins that were upregulated in EBN treated group in comparison with negative control group were actin cytoplasmic 1, tubulin alpha-1A chain, calmodulin and actin related protein 3. The continuous upregulation of these cytoskeleton proteins may be associated with joint protection in OA. Previous study by [47] showed increase cytoskeletal proteins in OA bone marrow adherent cells that were associated with joint structure conservation in OA. Mesenchymal stromal cells (MSCs) may be differentiated in bone marrow to be recruited into the joint cavity to repair joint destruction. Similarly in this study, upregulation of cytoskeletal protein may be due to MSCs differentiation in bone marrow. MSCs differentiation may be induced by EBN supplementation as observed in previous study which showed increased proliferative capacity of mesenchymal cells [25, 48].

A protein associated with bone functions was identified which is carbonic anhydrase II which play a role in osteoclast differentiation. Carbonic anhydrase II induced osteoclast formation and bone resorption by

mediating hormones involved in the biological process and effecting the intracellular pH and Ca^{2+} normal state [49]. In this study, the expression of this protein is downregulated in low dosage EBN treatment group during week 8 and week 12. Thus, it may be speculated that EBN possess bone protection effects by inhibiting protein associated with bone resorption that may cause imbalance in bone turnover that will result in OA progression. A study by [29] showed bone strength improvement and higher calcium concentration in oral administration of EBN extract.

One of the important proteins identified was annexin-1, which was upregulated in high dosage EBN treatment group during week 12. Annexin-1 is a glucocorticoid- (GC-) regulated protein which involved in resolution of inflammation via several mechanism [50]. It suppresses secretion of pro-inflammatory mediators and restricts neutrophil recruitments. Moreover, this protein also induces neutrophil apoptosis, mediate monocyte recruitment and also enhance apoptotic cells removal by macrophage [51]. The expression of this protein in high dosage EBN treatment group may indicate the anti-inflammatory properties of EBN in ameliorating OA.

STRING version 11.5 was utilized to further characterize and predict these differentially expressed proteins interactions (Figure 5). The result showed that these proteins were involved in inflammation, (e.g., annexin-1, collagen alpha-1(III) chain and collagen alpha-2(I) chain precursor), regulation response to external stimulus and positive regulation of cellular process. These results indicate the anti-inflammatory effect of EBN treatment in OA, for example annexin-1 protein which involves in resolution of inflammation.\

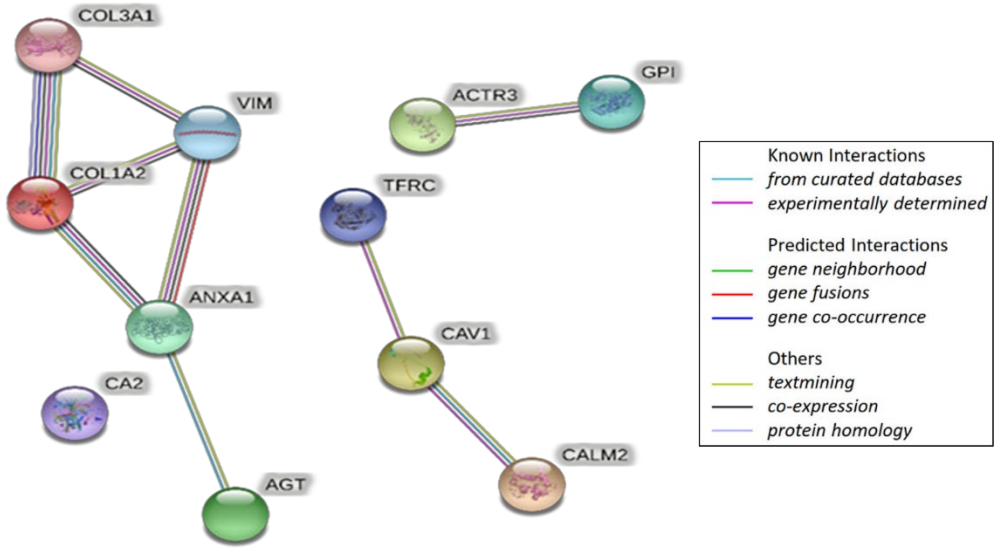

Figure 6. Protein-protein interaction network of treatment effects. The STRING database was searched for protein interaction analyses in order to elucidate the effect of EBN on synovial fluid proteins. As shown, most of the altered proteins interact with each other to constitute a large network. These proteins are involved in several processes, such as regulation response to external stimulus and positive regulation of cellular process

Damage associated molecular patterns (DAMP) will be released following stress and injury and will trigger inflammatory response through toll like receptor (TLR) pathway [52]. EBN will suppress TLR expression by increasing the expression of 14-3-3 zeta/delta protein which will inhibit TLR expression [41] and thus will reduce inflammation. Furthermore, EBN will also increase the expression of annexin-1, an anti-inflammatory protein which will reduce LPS-stimulated inducible nitric oxide synthase (iNOS) [53] and consequently reduce inflammation.

NF-κB signalling pathway will be activated during onset of OA and will upregulate hypoxia-inducible factor-2α (HIF-2α) expression. This in turn will increase hypertrophic cell markers such as matrix metalloproteinase-13 (MMP-13), matrix metalloproteinase-3 and consequently cause chondrocyte hypertrophy [54]. Hypertrophic chondrocytes situated at the growth plate will secrete RANKL and OPG and will promotes osteoclast formation and increase bone resorption [55]. EBN treatment downregulate

the expression of carbonic anhydrase II protein that involve in osteoclast differentiation and will reduce bone resorption activity. Fibroblasts maintain the condition of extracellular matrix (ECM) in cartilage and its action is mediated by vimentin protein. In this study, EBN chondroprotective effect is observed by upregulation expression of vimentin and thus will maintain the homeostasis of the cartilage. Previous study has shown the effect of EBN in increasing the proliferation of mesenchymal stem cells (MSCs) and this also can have chondroprotective effect by the upregulation of several cytoskeletal proteins such as actin cytoplasmic 1, tubulin alpha-1A chain, calmodulin and actin related protein 3.

Conclusions

Some limitations were identified in this study, which includes small sample size of animals. This is based on the recommendation by the Institutional Animal Care and Use Committee to minimize the number of animals due to the consideration for the welfare of the rabbits. Although this restricts the ability to draw more definitive conclusions, it does offer valuable preliminary data that could support the need for larger-scale studies in the future. Nevertheless, the findings from this study manage to highlight the use of EBN as potential osteoarthritis treatment. Further studies should be done to characterize nutritional composition and bioactive components in EBN hydrolysate and also elucidate the mechanisms of EBN bioactive components against bone resorption and inflammatory response. In conclusion, morphological evaluation showed that EBN supplementation have bone improvement effects by inhibiting osteoclastic activity but have no effects on cartilage damage. Protein expression showed chondroprotection and bone improvement by the action of several proteins via various signaling pathways.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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