

Edible Bird's Nest (EBN) Supplementation Ameliorates the Progression of Hepatic Changes and Atherosclerosis in Hypercholesterolaemic-Induced Rats

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Abstract

Persistence consumption of high-fat diet (HFD) is mainly attributed with the development of hypercholesterolaemia, which served as the major factor for the development of atherosclerosis in the blood vessels. As the hypercholesterolaemia progress, hepatic manifestation will occur including non-alcoholic fatty liver diseases (NAFLD). Therefore, the vascular and hepatic system is the most affected organs when chronic hypercholesterolaemia developed. In the present study, we have adopted consistence consumption of HFD and synergic xenobiotic administration (Triton X-100- TX100) to induce hypercholesterolaemia. This study involved 30 (N) male Sprague Dawley rats aged 10 weeks that randomly assigned into five different groups (n=6), including baseline control (normal diet rat- BC) and hypercholesterolaemic groups (HG) that consist of negative control (HFD and TX100- NC), positive control (HFD, TX100 and Simvastatin- PC), EBN soup-treated group (HFD, TX100 and EBN soup- EBNS) and EBN extract-treated group (HFD, TX100 and EBN extract- EBNE). After 12 weeks, the rats were euthanized and liver and cranial thoracic aortic were collected and processed accordingly for histological evaluation (H&E) and scanning electron microscope (SEM). Based on our observation, hepatic parenchyma of EBNS group had the least hepatic changes compared to PC and EBNS groups (which both groups have the same magnitude of hepatic changes); and moderately improved than NC group. Meanwhile, EBNS and PC showed great anti-sclerotic effect, while the EBNE had no effect in preventing atherosclerosis formation (equivalent with NC group). These findings suggested that EBNS have a good effect in slowing down hepatic changes and atherogenesis.

Keywords: Edible bird's nest (EBN), hypercholesterolaemia, liver, atherosclerosis, microscopic evaluation.

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Introduction

Emerging of metabolic syndrome (MetS) has been gaining tremendous worldwide attention as this syndrome is contributing to the top mortality causes in the human population. Metabolic syndrome can be collectively defined as the occurrence of central obesity, insulin resistance, atherogenic dyslipidaemia and hypertension. Epidemiologically, surging of this

syndrome is highly associated with prolonging consumption of high-calories diet, sedentary lifestyle and prevalence of gene polymorphism [1]. Nonetheless, most of the researchers have been claimed that high-calories intake that primarily composed of fat content, was the main factor to contribute in the emerging of this syndrome [2]. As human started to consume high-fat diet in extended period of time, they initiate development of atherogenic dyslipidaemia [3]. Atherogenic dyslipidaemia is characterized by the elevation of plasma triglycerides (TAG), apolipoprotein-B100-containing lipoprotein (low-density lipoprotein- LDL), and the reduction of high-density-lipoprotein (HDL) [1]. Based on the Ministry of Health (MoH) in 2016, 9.2% of Malaysian population was diagnosed with atherogenic dyslipidaemia or hypercholesterolaemia. Based on the epidemiologist prediction, the risk of cardiovascular diseases (CVD) is increased in the patient that was diagnosed with hypercholesterolaemia, and the major concern in the CVD is the development of atherosclerosis [4]. Atherosclerosis can be defined as the formation of solidified lipid-laden tissues in the highly-pressurized arterial lumen that consequently compromised the hemodynamics of blood circulation [1]. As hypercholesterolaemia progress, the hepatic manifestation of metabolic syndrome will start to develop. In human, this condition commonly known as non-alcoholic fatty liver diseases (NAFLD) and the hallmark of this disease is the abnormal accumulation of lipid content in the hepatic parenchyma that mainly stored in the lipid droplets (LDs) [2]. As the major gland and organ that primarily functioned in metabolizing cholesterol have been affected, advancement and severity of the metabolic syndrome will be accelerated. Therefore, in order to minimize the occurrence of CVD that can progress to the NAFLD in human, empirical targeting treatment of the initial problem, which hypercholesterolaemia was primarily addressed [4]. Prescription of statin drug or anti-cholesterol drugs has a beneficial impact to the hypercholesterolaemic patients was showing significant reduction of atherogenic dyslipidaemia. Although the efficacy of statin has been proven, the association of these drugs with hepatotoxicity was described and reported [5].

As the researchers are concerned about the hepatic toxicity of the statin drugs, flourishing of natural occurring anti-cholesterol product researches have been prominently observed. Most of the initial idea in searching for the beneficial natural product originally come from the ethnic beliefs. One of the most precious and legacy natural product in the past Chinese dynasties is edible bird's nest or commonly called EBN. This 'east caviar' is a polymerized c-shaped cup nest that derived from the salivary secretion of swiftlets sublingual glands [6]. Traditionally, EBN is consumed in a tonic soup that prepared by the double boiling method and normally consumed 1-3 nest per meal with 2-4 times intake per week [7]. EBN mainly composed of protein that made up 58-62% of their nutritional content and most of the protein formed is solubilized-glycoconjugates [8, 9]. Based on several Traditional Chinese Medicine (TCM) books, EBN has been claimed to have quite a number of medicinal effects, and one of it is to stimulate and booster metabolic rate including cholesterol metabolism [10]. Thus, it could be served as a potential anti-cholesterol natural-occurring product. Based on the claimed that has been made, the aim of our study is to observe the effect of EBN in the hepatic histological changes and development of atherosclerosis in hypercholesterolaemic-induced rats; as the liver and vascular system is the most affected organs in hypercholesterolaemia patients. Therefore, we hypothesized the EBN might be mitigating hepatic changes and atherogenesis in the hypercholesterolaemic-induced rats.

Methods and Materials

Animal study and experimental design

Our animal study was adopting 30 (N) male Sprague Dawley (SD) aged 10-weeks old as the animal model. The rats were outsourced from the Animal Research Unit (ARU), Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). Upon the rat arrival, they were acclimatized for 2-weeks in the open cage system and kept in a couple per cage. They were kept in the individual room without intervention from other research works. Internal room control was maintained at $22 \pm 2^{\circ}\text{C}$ with purified ventilation, and equal photoperiod 12 hours dark and 12 hours light. While the acclimatization was ongoing, they were fed *ad-libitum* with normal rat diet and constantly supplied of clean reverse osmosis (RO) water. Our experimental design was governed and obtained approval from UPM's animal ethic institution (UPM/IACUC/AUP-R084/2017).

Post-acclimatization, they were randomly assigned into two main different groups, including a baseline control group (BC) and hypercholesterolaemic-induced groups (HG). BC group (n=6) was fed continuously with normal rat diet (NRD). Meanwhile, the HG groups were induced into hypercholesterolaemic condition via synergic induction methods, which included constantly spontaneous feeding of high-fat diet (HFD- 55% fat and 1.25% cholesterol) from Envigo, United Kingdom (TD. 02028), and parenteral administration of xenobiotic agent, Triton X-100 (TRX100; 150 mg/kg, SQ q42d) from Sigma Aldrich, Germany [11]. The HG groups consisted of four different groups (n=6) including negative control group (NC; HFD and TRX100 only), positive control group (PC; HFD, TRX100 and Simvastatin via oral-gavage [10 mg/kg PO SID, Zocor 40 mg, Merck Sharp & Dohme (MSD), USA], EBN soup group (EBNS; HFD, TRX100 and EBN soup via oral-gavage [843.2 mg/kg PO SID]), and EBN extract (EBNE; HFD, TRX100 and EBN extract via oral-gavage [6.5 mg/kg PO SID]).

The amount of EBNS that was supplemented to the rats was derived from the human dosage based on the TCM books [7], and calculated according to the Animal Equivalent Dosage (AED) formula from the Food and Drug Administration (FDA) guidelines. Meanwhile, EBNE dosage was determined based on the effective dosage from our previous *in-vitro* study (unpublished data), and calculated via *In-Vivo* Conversion Dosage Formulary in the FDA guidelines. Our treatment was conducted for 12 weeks. Post-treatment completed, all rats were euthanized with the overdose of aerosol Ether inhalation, and liver and cranial thoracic aorta were collected for histological and electron microscopy evaluation, respectively.

EBN soup and extract preparation

EBN was purchased in raw and uncleaned form from a certified collector and supplier in Perak (U-Le Bird Nest Trading, Perak, Malaysia). EBNS (EBNS and EBNE) were prepared freshly on daily basis prior supplementation. Prior to the cleaning process, EBN was soaked in the distilled water for 5-10 minutes to allow softening of the EBN strands and eased the cleaning process. EBN was cleaned manually via manual feather and plumage plucking using sharp and blunt tip straight thumb forceps. Once cleaned, the EBN was dried up in the ventilated-incubator

for 24 hours at 100% fan speed and 25°C to prevent denaturing of the protein content. Dried up EBN was ground into small granules before processed into soup and extract.

For the EBN soup (EBNS), the required amount of EBN (843.2 mg/kg) was soaked in the PBS overnight prior to the stewing procedure. Based on our optimization and in order to get the proportionate soluble and insoluble part of EBNS, each 100mg of EBN granule must be soaked in 1.5 mL of PBS. Instead of double boiling method, we've modified sample preparation to optimized stewing method, and the stewing was conducted in the pre-warmed water bath incubator at 70°C for 5 hours. Meanwhile, to prepare the EBN extract (EBNE) similar procedures were implemented and continued with protein precipitation technique. In the post-stewing procedure, EBN was spinning down at 4,400 rpm for 30 minutes at 4°C. Two visible portions would be observed, which included soluble (supernatant water extract portion) and insoluble (bottom gelatinous portion) part. Soluble part or supernatant extract was aspirated and transferred into a sterile tube, and this part was subjected to cold acetone precipitation technique; to get the concentrated protein. Protein was measured by standard BCA protein quantification technique. The required amount of EBNE (6.5 mg/kg) was administered to the EBNS group. Based on our sequential calculation, dosage for EBNS and EBNE is comparatively equivalent in term of dosage concentration.

Hepatic histological examination

After rats were euthanized, part of hepatic lobes was collected and soaked in the 10% neutral buffered-formalin prior the histological processing. Standard histological slides preparation was done at the Histopathology Laboratory, Faculty of Veterinary Medicine, UPM. Post 24 hours fixation, liver samples were subjected for standard histological process including, serial ethanol dehydration, clearing with xylene, embedding within paraffin block and thin section; before stained with H&E staining. Histological slides were observed using an image analyzer (Olympus® BX51TF) and hepatic parenchyma was observed in between of portal triad and central vein (x20) to get a general idea of hepatic parenchyma morphology and lesions. The slides were blindly reviewed by two independent certified veterinarians and pathologist for slides validation. Liver scoring was done based on the specifically designed scoring standard for rodent and details are as following [12].

Table 1: Hepatic scoring method that specifically designed for rodent. (Modified from Liang et al, 2014)

Parameters	Grading
Steatosis (micro- and macrovesicular)	0 (<5%)
	1 (5-33%)
Hypertrophy	2 (33-66%)
	3 (>66%)
Inflammation	Normal (<0.5 foci)
	Mild (0.5-1.0 foci)
	Moderate (1.0-2.0 foci)
	Severe (>2.0 foci)

Aortic scanning electron microscopy (SEM)

The cranial thoracic aorta was collected for scanning electron microscopy (SEM) and qualitative observation of arterial plaque formation in the intraluminal of the aorta was done. After samples collection, aortas were subjected for standard SEM sample processing. Finally, samples were viewed under SEM at 50x magnification (JOEL IT-100). Atherosclerosis was observed in the aortic lumen to visualized aortic patency and this aortic patency was measured via false: true lumen ratio as described in previous study [13].

Results and Discussion

Liver scoring

Table 2 showed the liver scoring results to get a systematic morphological diagnosis. Liver of BC group was showing normal hepatocytes in the hepatic cord that arranged radially from the central vein. Focal macrovesicular steatosis was found in the hepatic parenchyma, which normally found in the normal liver as the liver served as major gland for nutritional storage and metabolism. On the other hand, liver of NC group that was fed with 12 weeks of HFD and bi-induction of TRX100 was showing the generalized formation of micro- and macrovesicular steatosis with moderately hepatic hypertrophy and severe infiltration of inflammatory cells. Meanwhile, hepatic parenchyma of PC group that fed with HFD, bi-induction of TRX100 and treated with Simvastatin (anti-cholesterol drug) was showing diffused micro- and macrovesicular steatosis with mild hepatic hypertrophy and mild inflammatory cell infiltration. Remarkably, liver of EBNS group that was induced with hypercholesterolaemia demonstrated generalized microvesicular steatosis with mild hepatic hypertrophy only without the presence of macrovesicular steatosis and inflammatory reaction. Based on our observation, hepatic parenchyma of EBNE was reflecting generalized microvesicular steatosis and diffuse macrovesicular steatosis with mild hepatic hypertrophy and infiltration of inflammatory cells. In contrast, hepatic parenchyma of EBNS group had the least hepatic changes compared to PC and EBNS groups (which both groups have the same magnitude of hepatic changes); and moderately improved than the NC group. (Fig. 1)

Table 2: Sub-parameters scoring for each groups. BC: Baseline control group; NC: Negative control group; PC: Positive control group; EBNS: EBN soup group; EBNE: EBN extract group

Parameters	Grading				
	BC	NC	PC	EBNS	EBNE
Microvesicular Steatosis	0	3	2	2	3
Macrovesicular Steatosis	0	3	1	0	1
Hepatic Hypertrophy	0	2	1	1	1
Inflammation	0	3	1	0	1

Histological evaluation of the liver

Based on our histological findings, BC group was showing normal morphological hepatic parenchyma and our finding was reflecting the whole batch of our animal model is initially healthy, when fed with NRD without causing a hepatic lesion. After synergic induction of hypercholesterolaemia (HFD and TRX100) in the NC group with the absence of other treatment, hepatic parenchyma was showing extensive hepatic changes with a mixture of micro- and macrovesicular steatosis with moderate to severe inflammatory infiltration. From this finding, it was reflecting that our method of hypercholesterolaemia induction was producing extensive hepatic changes. When a similar method of hypercholesterolaemia induction supplemented with EBNS (EBNS and EBNS), the severity of hepatic changes was delayed compared to the NC group. This might be explained by a previous study that had mentioned the EBN contains two novel and potent antioxidant that existing in pentapeptides sequences [14]. An antioxidant is one of a co-treatment regime that supplemented in NAFLD patients and it had been demonstrated to ameliorate hepatic changes [4]. In fact, in the cell culture setting EBN had demonstrated hepatic antioxidant properties by up-regulating natural occurring antioxidant in the hepatic cell line. Besides that, anti-inflammatory properties that had been discovered in the EBN reflecting less inflammatory cells infiltration in the hepatic parenchyma that treated with EBNS [15]; particularly in EBNS group. However, when we compared the hepatic changes of EBNS and EBNE group, the EBNS had a better effect in deferring hepatic changes. This finding is parallel with metabolomic and proteomic studies that have scientifically proven the bioactive ingredients of EBN is persistently preserved in the insoluble part of EBN [16-18]; which presence in the EBNS group in this study. We also postulated that the antioxidant molecules are existing in the EBNE, but when the molecules were exposed to constant enzymatic and harsh pH reaction along the gastrointestinal tract (GIT) during the digestion process, it might be modified and degraded part of the antioxidant molecules causing less or no effect. As we expected, hepatic changes in the PC group was greater than EBNS group, and this finding is consistent with studies that have shown statin drugs will induce hepatotoxicity via lipid accumulation that will lead to cholestasis [19]. Moreover, the pharmacodynamics of statin itself was modulating natural-occurring in the hepatocytes, namely selenoprotein [20]; which can maintain the integrity of liver when experiencing tremendous and prolong oxidative stress from the constant metabolizing activities.

Patency of aortic lumen

The patency of aortic lumen is the quantitative approach that was implemented in evaluating the plaque formation in the aorta lumen. There was absence of arterial plaque formation in the aortic lumen for BC group. In contrast, NC group showed visible formation of arterial plaque which contributing 69.30 ± 6.53 % aortic patency, and our finding is consistent with the hepatic changes. As we expected, there was very mild arterial plaque formation in the anti-cholesterol-treated group (PC group) with aortic patency of 99.63 ± 0.15 %, and a similar finding was observed in the EBNS (99.73 ± 0.07 %), which reflects the effect of EBNS is comparatively equivalent as an anti-cholesterol treated group. Although the EBNE was showing the similar hepatic changes as compared to the PC group, the effect of anti-sclerotic in EBNE (70.02 ± 5.96 %) was not as good as PC group; that was showing similar progressive and visible arterial plaque in the NC group. From these findings, EBNS and PC were showing great anti-sclerotic effect, while the EBNS had no effect in preventing atherosclerosis formation. (Fig. 2).

Based on the SEM micrographs, NC group demonstrated progressive and extensive atherogenesis in the aortic lumen and this finding proved the synergic induction purely produced visible arterial plaque. When the same induction was co-treated with Simvastatin[®] (PC group), a significant reduction of atherosclerosis formation was observed. Our finding is consistent with the epidemiological study that had claimed the statin drugs can reduce the formation of atherosclerosis between 20-30% and consequently improve hemodynamic of blood circulation [1]. Likewise, EBNS group also demonstrated an anti-sclerotic effect comparatively equivalent to the PC group, which not in the EBNS group. Our finding correspond with a recent study that stated the possibility of EBN serving as cardio protectant agent via delaying coagulation cascade [21], which can be one of the contributing factors in atherosclerosis development. More so, this finding is also supported with another finding that claimed EBN can reduce circulating total cholesterol in the blood, which served as the main factor in atherogenesis [9]. In further supporting that claim, another recent study also had shown anti-cholesterol metabolites that serve as prodrugs in statin pharmacodynamics, called paraoxonase/lactonase 3 [22, 23].

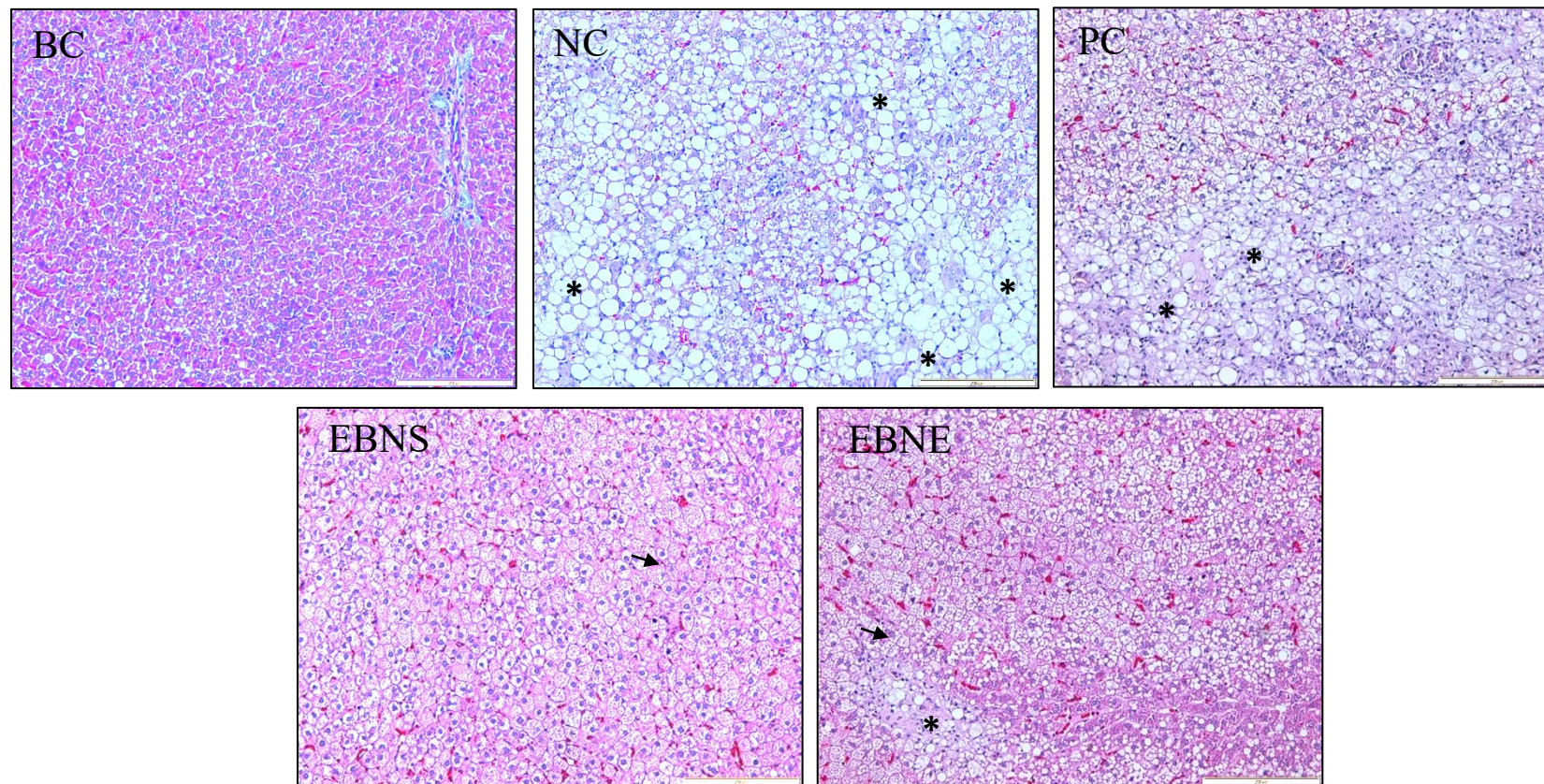


Figure 1: Photomicrographs of liver in various group.

BC: Baseline control group; NC: Negative control group; PC: Positive control group; EBNS: EBN soup group; EBNE: EBN extract group. Normal morphology of hepatocytes with focal hepatic steatosis in BC. NC demonstrated generalized and severe micro- and macrovesicular steatosis in the hepatic parenchyma (*). Meanwhile, PC group showed moderate to severe hepatic steatosis. EBNS group showed generalized intracytoplasmic microvesicular steatosis (marked by arrow) only, while in EBNE generalized microvesicular and diffuse macrovesicular steatosis. (H&E staining, x20 magnification).

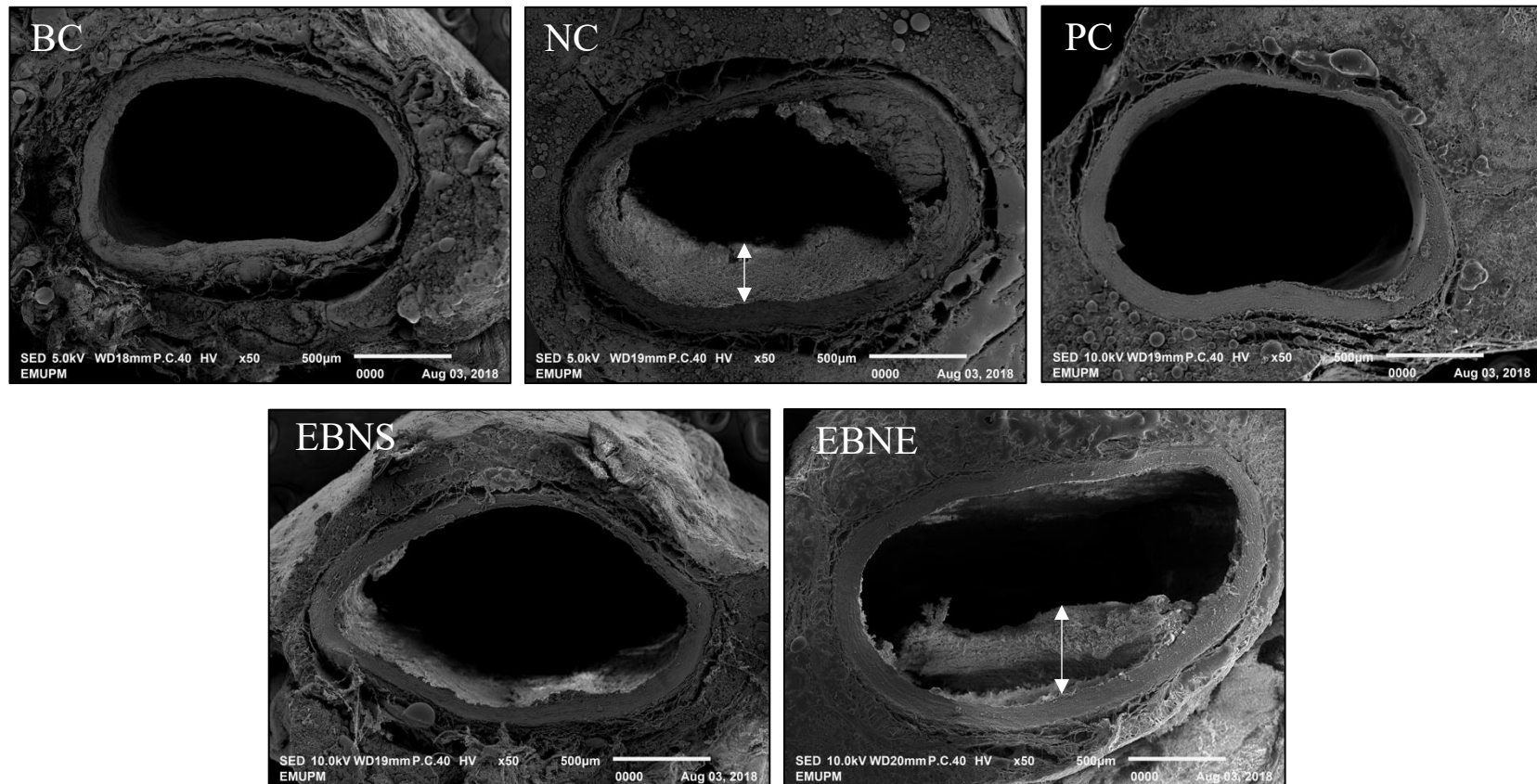


Figure 2: Photomicrographs of the aortic lumen under SEM. BC: Baseline control group; NC: Negative control group; PC: Positive control group; EBNS: EBNsoup group; EBNE: EBN extract group. Absence of arterial plaque formation in BC group. NC group showed a significant formation of atherosclerosis (marked by arrow). PC and EBNS groups demonstrated the insignificant formation of arterial plaque in the aortic lumen. EBNE group demonstrated no effect on preventing atherosclerosis development (marked by arrow). (X50 magnifications).

Conclusion

Based on our histological and ultramicroscopic studies, we have demonstrated that the EBNS can slow down the hepatic changes and atherogenesis in hypercholesterolaemic-induced rats. Nonetheless, EBNS have a better effect in differing those changes compared to PC and EBNE groups, even NC group. In future, in order to improve our decisive findings, it is recommended to include the biochemical evaluation for better understandings of the physiological and pathological changes in the rats.

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Author contributors

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest

The authors have no disclosures to declare.

Compliance with ethical standards

The work is compliant with ethical standards.

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