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ORIGINAL RESEARCH

Edible bird's nest attenuates procoagulation effects of high-fat diet in rats

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Abstract: Edible bird's nest (EBN) is popular in Asia, and has long been used traditionally as a supplement. There are, however, limited evidence-based studies on its efficacy. EBN has been reported to improve dyslipidemia, which is closely linked to hypercoagulation states. In the present study, the effects of EBN on high-fat diet- (HFD-) induced coagulation in rats were evaluated. Rats were fed for 12 weeks with HFD alone or in combination with simvastatin or EBN. Food intake was estimated, and weight measurements were made during the experimental period. After sacrifice, serum oxidized low-density lipoprotein (oxLDL), adiponectin, leptin, von willibrand factor, prostacyclin, thromboxane and lipid profile, and whole blood coagulation indices (bleeding time, prothrombin time, activated partial thromboplastin time, red blood count count, and platelet count) were estimated. Furthermore, hepatic expression of coagulation-related genes was evaluated using multiplex polymerase chain reaction. The results indicated that EBN could attenuate HFD-induced hypercholesterolemia and coagulation similar to simvastatin, partly through transcriptional regulation of coagulation-related genes. The results suggested that EBN has the potential for lowering the risk of cardiovascular disease-related hypercoagulation due to hypercholesterolemia.

Keywords: edible bird's nest, coagulation, high-fat diet, hypercholesterolemia, nutrigenomics

Introduction

Cardiovascular diseases (CVD) cause huge morbidity and mortality burdens, and continue to grow despite advances in the management of the diseases.[1] Several variables are involved in the pathogenesis and progression of CVD including blood coagulation status. Effective control of CVD will therefore entail controlling multiple variables like hypercholesterolemia and coagulation.[2,3] There is heightened interest in the use of alternative therapies for CVD management due to concerns of side effects with synthetic drugs.[4] Edible bird's nest (EBN), popular among Asians, especially in the People's Republic of China, has a long history of traditional use. EBN has been a highly valuable food tonic among the Chinese people for thousands of years, and it was believed to treat diseases such as tuberculosis, cough, dyspnea, and stomach ulcers. It has also been used to improve general well-being, especially among the old and in postpartum women, as a result of its perceived abilities to enhance renal function, strengthen the immune system, increase energy and metabolism, and regulate circulation. Despite its traditional uses over the years, evidence-based studies on its functional effects are limited.[5,6] EBN is antioxidant-rich and can attenuate inflammation and oxidative stress,[7,8] which are disease states that have been closely linked with hypercoagulation states.[9] Accordingly, coagulation can be potentiated by inflammation and oxidative stress, which can be alleviated by antioxidants.[10] Based on the rich antioxidant nature

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of EBN and its ability to improve inflammation and lipid profiles, we hypothesized that it may have beneficial effects on CVD-related hypercoagulation.

Experience over the years with traditional use of EBN suggests that it may be free from any toxic effects since it has been used for generations without any documented deleterious effects. This means EBN may have potential use as a safe supplement or nutraceutical. In the present study, therefore, we aimed to study the effects of EBN on high-fat diet- (HFD-) induced hypercholesterolemia and hypercoagulation, including a possible mechanistic basis for observed effects.

Materials and methods

Materials

The Adiponectin ELISA (enzyme-linked immunosorbent assay) kit was purchased from Millipore (Billerica, MA, USA), while those of oxLDL (oxidized low-density lipoprotein), thromboxane, prostacyclin, leptin, and vWF (von Willebrand factor) were purchased from Elabscience Biotechnology Co., Ltd (Wuhan, Hubei, People's Republic of China). The RNA extraction kit was purchased from RBC Bioscience Corp. (Taipei, Taiwan), and the GenomeLab™ GeXP Start Kit was purchased from Beckman Coulter Inc (Miami, FL, USA). Simvastatin was purchased from Pfizer (New York, NY, USA), while Cholesterol and Cholic acid were purchased from Amresco (Solon, OH, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. RCL2 Solution was purchased from Alphelys (Toulouse, Plaisir, France), and analytical grade ethanol was purchased from Merck (Darmstadt, Hesse, Germany). Palm oil and Standard rat pellet were from Yee Lee Edible oils Sdn. Bhd. (Ipoh, Perak, Malaysia) and Specialty feeds (Glen Forrest, WA, Australia), respectively. Lipid profile kits were from Randox Laboratories Ltd (Crumlin, County Antrim, UK).

EBN

EBN was supplied by Blossom View Sdn. Bhd (Terrengganu, Malaysia). It was cleaned under tap water for 5 minutes, dried at room temperature and ground into powder manually using mortar and pestle. The ground EBN was stored at 4°C, and was used to prepare fresh pellets 1 day prior to being fed to rats. Only freshly prepared pellet was given to rats, while any leftover pellet on the rat cages from the previous day was discarded.

Animal study

The animal study was approved by the Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (Project approval number: UPM/IACUC/AUP-R011/2014). Sprague Dawley rats (10-week old, 230–280 g, n=30) were handled as stipulated by standard guidelines for handling animals. They were housed at 25°C±2°C, 12/12 hours light/dark cycle and allowed to acclimatize for 2 weeks with free access to normal pellet and water. Rats were then divided into five groups: the normal group were fed with normal pellet, the HFD group were fed with HFD containing 4.5% cholesterol and 0.5% cholic acid, the HFD + simvastatin (SIM) group were fed with HFD and SIM (10 mg/kg/day), and two EBN groups were fed with low- or high-dose EBN with HFD (Table 1). Intervention lasted 12 weeks, and body weights were measured weekly, while food intake was calculated every day by subtracting the leftovers from what was added the previous day. Rats were sacrificed at the end, and their blood and tissue samples collected for further analyses.

Activated partial thromboplatin time, prothrombin time, bleeding time, platelet count, and red blood cell (RBC) count

For prothrombin time (PT) and APTT (activated partial thromboplatin time) assays, blood was collected in the vacutainer tubes (buffered sodium citrate), and centrifuged to get the serum, which was analyzed using an automated coagulation analyzer (START-4, Diagnostica Stago, Parsippany, NJ, USA). Rat tail bleeding time (BT) was measured after anesthesia on a hotplate. The tail was heated at 37°C, and 2 mm of its tip was amputated with a blade. The blood was blotted onto a filter paper every 30 seconds until the paper

Table 1 Food composition of rat groups and intake

Animal group	Normal pellet (%)	Cholesterol/ cholic acid (%)	Palm oil (%)	Starch (%)	Others	Food intake (kcal/kg/d)	Initial weight (g)	Final weight (g)
Normal	100					215.5±33.5[a]	260.4±10.7[a]	384±22.9[a]
HFD	65	5	20	10		215.0±37.5[a]	262.6±17.7[a]	395.2±16.8[a]
HFD + SIM	65	5	20	10	Simvastatin (10 mg/kg)	215.7±36.6[a]	267.7±21[a]	375.7±53.4[a]
HFD + EBNH	45	5	20	10	20% EBN	216.1±36.8[a]	261.7±15.4[a]	380.7±25.6[a]
HFD + EBNL	62.5	5	20	10	2.5% EBN	216.5±35.8[a]	257±20.1[a]	368±29.3[a]

Note: Columns with a similar letter indicate no statistical difference ($P>0.05$).
Abbreviations: EBN, edible bird's nest; HFD, high-fat diet; EBNL, low dose EBN; EBNH, high dose EBN; SIM, simvastatin.

was no longer stained with blood. The period between amputation and the cessation of bleeding was considered the bleeding time (second). For hematologic cell counts, whole blood was collected in vacutainer tubes (K3EDTA) and analyzed using the MEDONIC CA530 hematology analyzer (Medonic, Sweden) within 2 hours after collection.

Ex vivo platelet aggregation

Ex vivo platelet aggregation was performed using baseline and final blood samples, with adenosine diphosphate (ADP) as agonist. Rat whole blood was collected in a vacutainer containing 3.2% sodium citrate (9:1, v/v), and thoroughly mixed by inverting the vacutainer several times. The blood was spun at 100× *g* for 20 minutes to get the platelet-rich plasma. Then, 50 ng/mL of prostacyclin was added in to prevent platelet activation. Platelet-rich plasma was again spun at 240× *g* for 10 minutes to obtain the platelet pellet. The platelet pellet was resuspended in Tyrode-HEPES buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 12 mM $NaHCO_3$, 20 mM HEPES, and 5 mM glucose, pH 7.3) to a cell density of 10^8 cells/mL. The solution (100 µL) was then mixed with 1 µL of ADP in a 96-well plate, and the optical density changes read at 405 nm for 20 minutes in a microplate reader. Readings were taken every minute, and the plate was shaken in double orbital mode.

Biochemical analyses and serum ELISA analysis

Serum samples were analyzed for lipid profile using Randox analytical kits on a Selectra XL instrument (Vita Scientific, Dieren, Rheden, the Netherlands). Serum ELISA analyses were performed using oxLDL, prostacyclin, thromboxane, Adiponectin, Leptin, vWF, and PAI-1 ELISA kits according to manufacturers' recommendations. Absorbances were read on a BioTeK Synergy H1 Hybrid Reader (BioTek Instruments Inc., Winooski, VT, USA) at the appropriate wavelengths, and results were expressed as fold changes in relation to the values for the normal control group.

Gene expression

Primer design

The primers were designed by GenomeLab eXpress Profiler software using input sequences from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/nucleotide/). Primers were tagged with an 18-nucleotide universal forward and 19-nucleotide universal reverse sequence, respectively (Table 1), and synthesized by Integrated DNA Technologies (Singapore).

RNA extraction, reverse transcription, and polymerase chain reaction

RNA was extracted using a Total RNA isolation kit (RBC Biotech Corp., Taipei, Taiwan), and diluted to 20 ng/mL. Reverse transcription and polymerase chain reaction (PCR) program were performed according to the GenomeLab™ GeXP Start Kit protocol (Beckman Coulter, Inc, Miami, FL, USA), as shown in Table 2.

GeXP genetic analysis system and multiplex data analysis

Sample loading solution (38.5 µL) and DNA size standard 400 (0.5 µL) (GenomeLab GeXP Start Kit; Beckman Coulter, Inc) were mixed with 1 µL PCR products, and the mixture was loaded onto a 96-well sample plate for analysis on the GeXP genomelab genetic analysis system (Beckman Coulter, Inc). Gene expression results were analyzed with the Fragment Analysis module of the GeXP system software, and the data exported and normalized on the eXpress Profiler software.

Table 2 Names, accession number, and primer sequences used in the study

Name	Left sequence	Right sequence
vWF	AGGTGACACTATAGAATAAGTACATTTGGGAGAAGAGG	GTACGACTCACTATAGGGACTCTTCCGACTTACAATCTC
B2m[a]	AGGTGACACTATAGAATAATGCTTGCAGAGTTAAACA	GTACGACTCACTATAGGGATGCATAAAATATTTAAGGTAAGA
Hprt1[a,b]	AGGTGACACTATAGAATATCCTCATGGACTGATTATG	GTACGACTCACTATAGGGACTGGTCATTACAGTAGCTCTT
Rpl13a[a]	AGGTGACACTATAGAATAATGGGATCCCTCCAC	GTACGACTCACTATAGGGAATTTTCTTCTCCACATTCTT
Kan(r)[c]		
Adipoq	AGGTGACACTATAGAATACCAAAAGTTCCAGGACTC	GTACGACTCACTATAGGGAGGTCACCCTTAGGACCA
Leptin	AGGTGACACTATAGAATACAAGTATCCCTGGTAGATGT	GTACGACTCACTATAGGGAAAGTTTTCTTGCATTGATCT
NOS3	AGGTGACACTATAGAATATTTTAAGGAAGTAGCCAATG	GTACGACTCACTATAGGGAGATTTTGTAACTCTTGTGCT
PAI-1	AGGTGACACTATAGAATAACAAGTCTTTCCGACCA	GTACGACTCACTATAGGGAAGAGGATTGTCTCTGTTGG

Notes: [a]Housekeeping genes. [b]Normalization gene. Underlined sequences are universal left and right sequences (tags). [c]Internal control supplied by Beckman Coulter Inc (Miami, FL, USA) as part of the GeXP kit. RT conditions were: 48°C for 1 minute; 37°C for 5 minutes; 42°C for 60 minutes; 95°C for 5 minutes, then hold at 4°C. PCR conditions were initial denaturation at 95°C for 10 minutes, followed by two-step cycles of 94°C for 30 seconds and 55°C for 30 seconds, ending in a single extension cycle of 68°C for 1 minute.

Data analysis

Data are presented as mean ± standard deviation, and comparisons of means was done using one-way analysis of variance on SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The significance of the difference between comparisons was determined by Tukey's range test. $P<0.05$ was considered significantly different.

Results and discussions

Body weight changes

Table 2 shows the food intakes (calories) and body weights for different groups during the intervention period. No significant differences were observed in terms of food intake and body weights between the groups after 12 weeks of intervention, although the percentage body weight changes were different. HFD, HFD + SIM, lose dose EBN with HFD, and high dose EBN with HFD groups had 50%, 40%, 45%, and 43% changes in body weights, respectively.

Lipid profile

Table 3 shows the lipid profiles of the different groups. The normal group had significantly lower cholesterol levels compared with the other groups. The cholesterol levels of the EBN groups were lower than that of the HFD group, although it was only significantly so for the HFD + EBNH group when compared with the HFD group. Typically, simvastatin is expected to reduce the cholesterol level, as seen with the present data.[11,12] Based on the results of the EBN group, it can be suggested that EBN can prevent worsening of lipid profiles, which may contribute toward attenuating the risk of coagulation since hypercholesterolemia is associated with hypercoagulatory states.[13]

APPT, PT, BT, platelet count, platelet aggregation, and RBC count

APTT and PT are important indices of the endogenous and exogenous coagulation systems, respectively.[14] Figure 1A shows that APTT was significantly shorter in the HFD group compared with the other groups ($P<0.05$). A similar pattern was observed for PT which was also shorter in the HFD group compared with the other groups (Figure 1B), although only the HFD + EBNH and normal groups were significantly different from the HFD group ($P<0.05$). These results indicated that HFD promoted hypercoagulation, while EBN attenuated this pathological change. Additionally, BT showed a similar trend with APTT; the HFD group had shorter bleeding time compared with the other groups, while EBN attenuated the HFD-induced changes (Figure 1C). With regard to RBC cell count, no differences were observed between all the groups (Figure 2A). However, the platelet count and platelet aggregation were significantly higher in the HFD group in comparison with the other groups except the HFD + SIM group (Figure 2B and C, respectively). Moreover, increased platelet count and higher platelet aggregation have been linked to increased risk of hypercoagulation.[15] Overall, these results indicated that HFD could promote hypercoagulation partly as a result of the hypercholesterolemia it induces,[16] and EBN may have prevented HFD-induced hypercoagulation partly through regulating cholesterol levels.

Serum oxLDL, adiponectin, leptin, thromboxane, vWF, and prostaglandin F1α

oxLDL is an important predictor of CVD risk, which is a product of LDL oxidation. In fact, higher levels have been associated with a higher risk of coagulation.[16] Figure 3A shows that EBN and simvastatin reduced oxLDL significantly in comparison with the HFD group. Moreover, simvastatin has been reported to lower oxLDL.[17] Additionally, adiponectin is an important regulator of lipid metabolism and coagulation.[18,19] As shown in Figure 3B, the HFD + EBNH group, but not the HFD + EBNL group, had a significantly higher adiponectin level in comparison with the HFD and HFD + SIM groups. Simvastatin, however, did not show improvement in adiponectin levels similar to

Table 3 Biochemical parameters

Rat groups	Chol (mmol/L)	Trig (mmol/L)	LDL (mmol/L)	HDL (mmol/L)	LDL/HDL	TG/HDL
Normal	1.55±0.43[a]	0.62±0.15[a]	0.28±0.11[a]	1.18±0.35[a]	0.24±0.04[a]	0.55±0.15[a]
HFD	7.47±1.13[b]	1.21±0.38[b]	4.98±1.03[b]	1.05±0.13[a]	4.77±0.98[b]	1.16±0.33[b]
HFD + SIM	4.99±1.11[c,d]	0.63±0.18[a]	3.6±1.1[b,c]	1.04±0.17[a]	3.46±0.94[b,c]	0.62±0.22[a,b]
HFD + EBNL	6.04±0.75[b,c]	0.54±0.1[a]	4.52±0.71[b,c]	1.17±0.18[a]	3.94±0.88[b,c]	0.46±0.08[a]
HFD + EBNH	4.17±1.06[d]	0.44±0.1[a]	2.98±0.83[c]	1.18±0.29[a]	2.63±0.87[c]	0.38±0.08[a]

Notes: Data are mean ± SD. Columns with different letters in each row indicate statistical difference ($P<0.05$).
Abbreviations: Chol, cholesterol; Trig, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HFD, high-fat diet; SD, standard deviation; EBNL, low dose EBN; EBNH, high dose EBN; SIM, simvastatin.

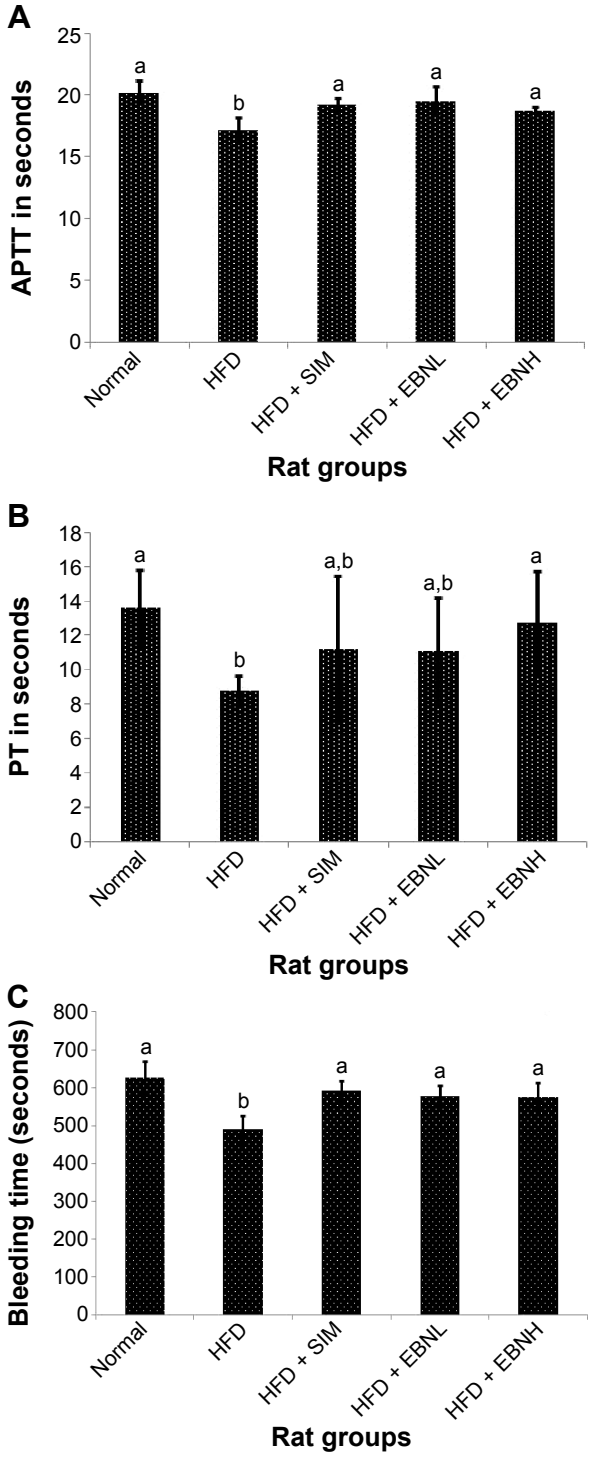

Figure 1 Effects of EBN on (**A**) APTT, (**B**) PT, (**C**) BT in HFD-fed rats.
Notes: Bars with different letters in each panel indicate statistical difference ($P<0.05$).
Abbreviations: EBN, edible bird's nest; APTT, activated partial thromboplatin time; PT, prothrombin time; BT, bleeding time; HFD, high-fat diet; EBNL, low dose EBN; EBNH, high dose EBN; SIM, simvastatin.

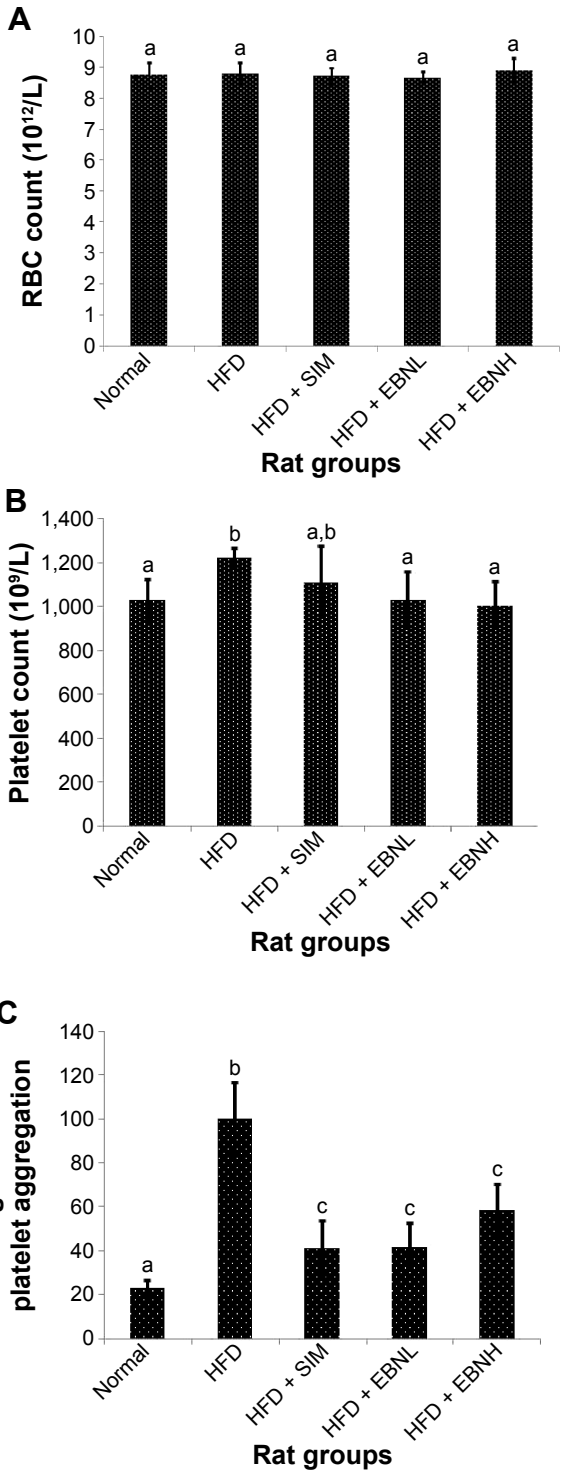

Figure 2 Effects of EBN on (**A**) platelet count, (**B**) platelet aggregation, (**C**) RBC count in HFD-fed rats.
Notes: Bars with different letters in each panel indicate statistical difference ($P<0.05$).
Abbreviations: EBN, edible bird's nest; RBC, red blood cell; HFD, high-fat diet; EBNL, low dose EBN; EBNH, high dose EBN; SIM, simvastatin.

previous reports.[20] Furthermore, leptin is a regulator of lipid metabolism and has implications for coagulation.[21] As shown in Figure 3C, the HFD + SIM and HFD + EBNH groups significantly lowered leptin levels, while the HFD + EBNL group did not produce as much change as with the former groups. Thromboxane levels reflect the state of thrombosis, and increased thromboxane levels are reported to denote a higher risk of CVD.[22] Figure 4A shows that the HFD group had significantly higher thromboxane levels than the

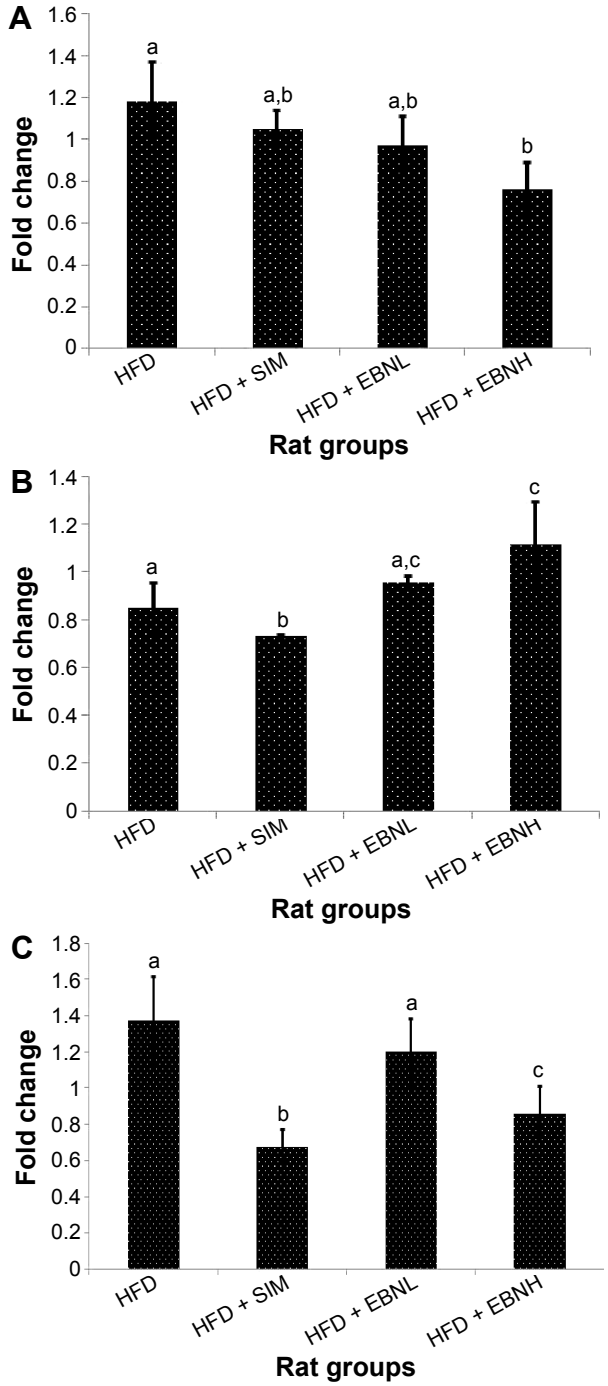

Figure 3 Effects of EBN on (**A**) serum oxLDL, (**B**) serum adiponectin, and (**C**) serum leptin in HFD-fed rats.

Notes: Bars with different letters in each panel indicate statistical difference ($P<0.05$).

Abbreviations: EBN, edible bird's nest; oxLDL, oxidized low-density lipoporotein; HFD, high-fat diet; EBNL, low dose EBN; EBNH, high dose EBN; SIM, simvastatin.

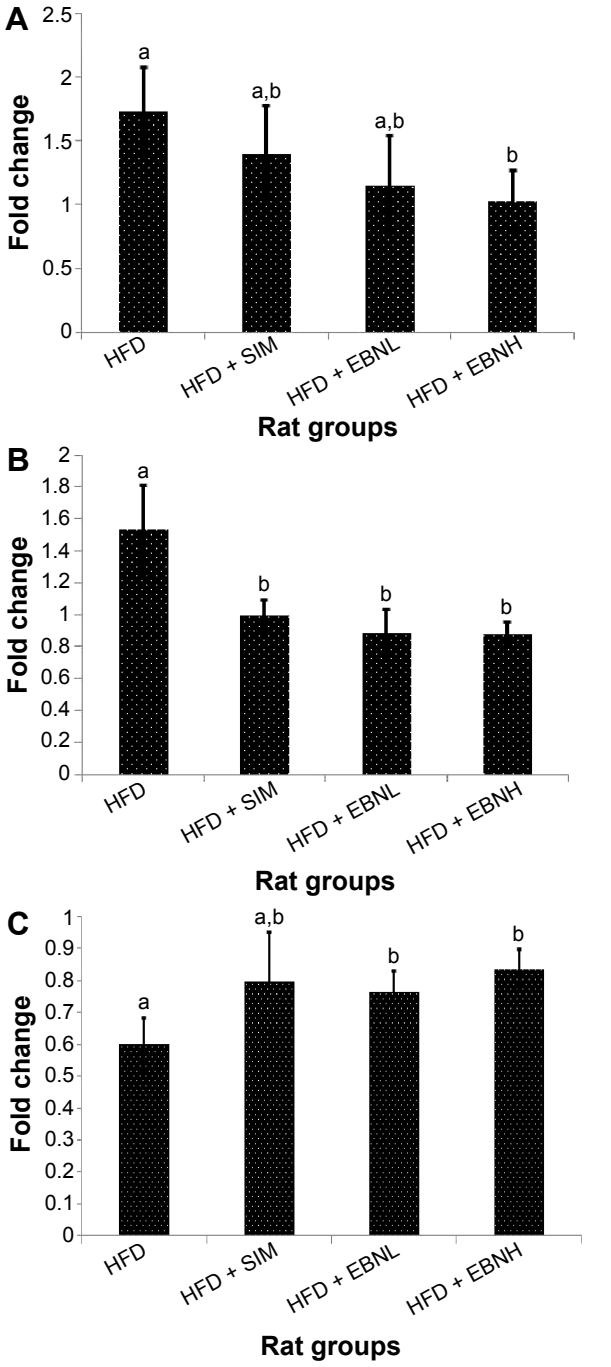

Figure 4 Effects of EBN on (**A**) serum thromboxane, (**B**) serum vWF, and (**C**) serum prostaglandin, in HFD-fed rats.

Notes: Bars with different letters in each panel indicate statistical difference ($P<0.05$).

Abbreviations: EBN, edible bird's nest; vWF, von Willebrand factor; HFD, high-fat diet; EBNL, low dose EBN; EBNH, high dose EBN; SIM, simvastatin.

other groups, suggesting that the group had a higher risk of thrombosis-related CVD, while EBN and simvastatin had lower risks. vWF, like thromboxane, is indicative of the state of thrombosis,[23] and just as with the thromboxane levels in the present study, vWF was significantly higher in the HFD group (Figure 4B). The results again suggest an increased risk of thrombosis-related events in this group, and lower risks of such events in the EBN and simvastatin groups. Prostacyclin is a potent vasodilator,[24] and the present study showed that the HFD group had lower levels (Figure 4C) in comparison with the EBN groups, suggesting that HFD promoted vasoconstriction, while EBN promoted vasodilatation.

In aggregate, the data demonstrated that HFD will promote hypercholesterolemia and hypercoagulation, while EBN attenuates the HFD-induced changes and may therefore lower the risk of CVD.

Hepatic tissue mRNA levels of coagulation genes

In order to provide insights into the mechanistic basis for the effects of EBN, mRNA levels of related hepatic genes were measured. Figure 5 shows the mRNA levels of hepatic leptin, adiponectin, and NOS genes. Similar to the serum markers, adiponectin and leptin mRNA levels were equally modulated by the interventions. The HFD group showed leptin and adiponectin mRNA changes that reflected the serum changes and were in keeping with worsening of metabolic control, while EBN prevented the HFD-induced changes. NOS is a potent mediator of vasodilatation,[25] and its increased expression by EBN may have underlined the changes in serum prostacyclin levels observed. Moreover, NOS has been reported to modulate prostacyclin levels as part of the basis for its vasodilatory effects.[26] Additionally, in Figure 6, vWF gene expression was reflective of the serum changes observed in this study, indicating that the transcriptional changes in vWF induced by EBN similar to those of adiponectin and leptin may have been the basis for the changes in serum markers. PAI-1, another marker of coagulation,[27] was also upregulated in the livers of the HFD-fed rats, while EBN and simvastatin attenuated such effects. Overall, the gene expression data are in keeping with the biochemical changes observed, and suggest that EBN is able to prevent the HFD-induced procoagulatory hypercholesterolemic condition partly through transcriptional regulation of genes related to coagulation and regulation of metabolism.

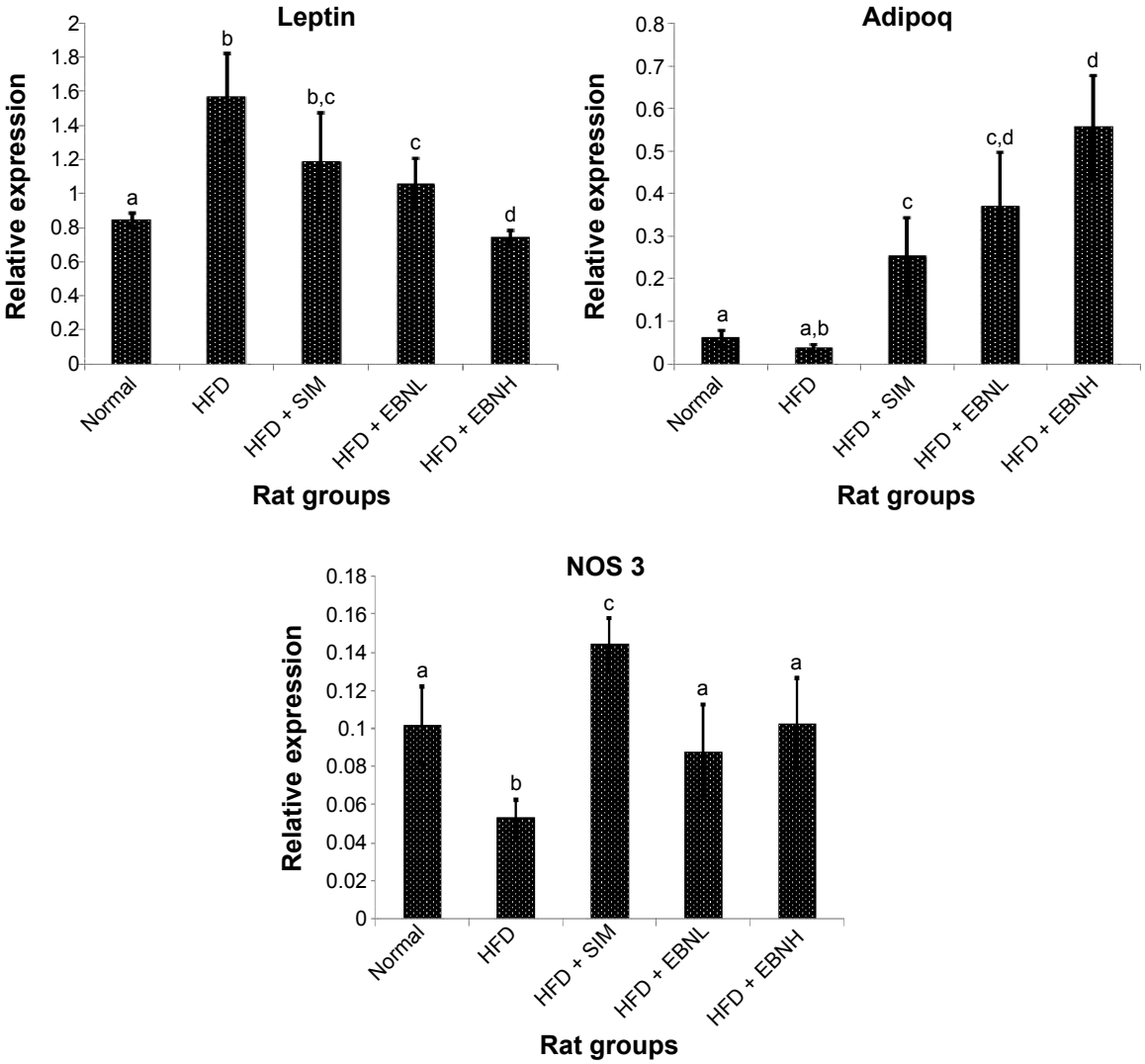

Figure 5 Effects of EBN on hepatic tissue mRNA levels of leptin, adipoq, and NOS 3 in HFD-fed rats.
Notes: Bars with different letters in each panel indicate statistical difference ($P<0.05$).
Abbreviations: EBN, edible bird's nest; Adipoq, adiponectin; NOS, nitric oxide synthase 3; HFD, high-fat diet; EBNL, low dose EBN; EBNH, high dose EBN; SIM, simvastatin.

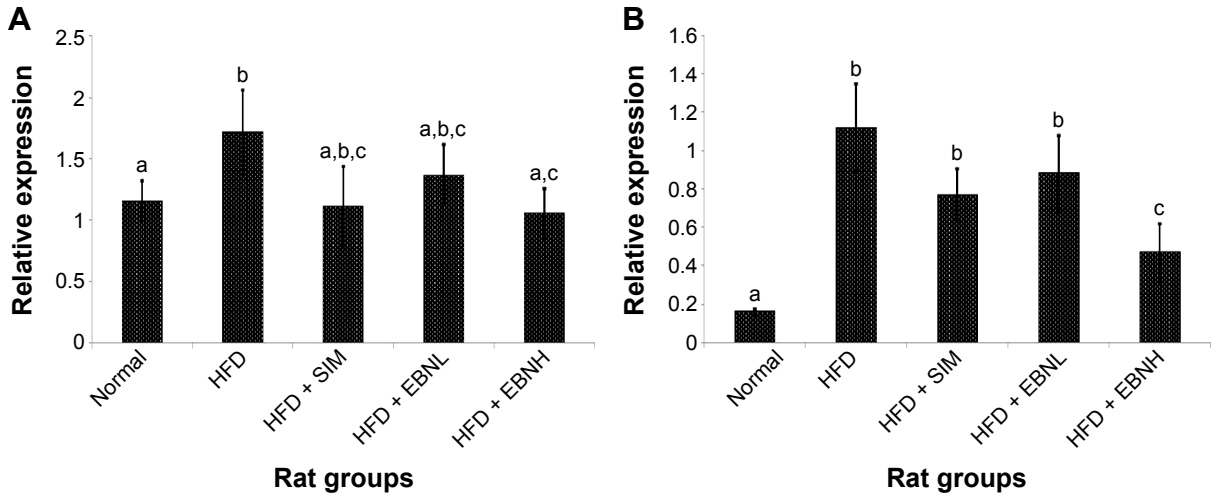

Figure 6 Effects of EBN on hepatic tissue mRNA levels of (**A**) vWF, and (**B**) PAI-1 in HFD-fed rats.
Notes: Bars with different letters in each panel indicate statistical difference ($P<0.05$).
Abbreviations: EBN, edible bird's nest; vWF, von Willebrand factor; PAI-1, plasmibnogen activator inhibitor-1; HFD, high-fat diet; EBNL, low dose EBN; EBNH, high dose EBN; SIM, simvastatin.

The effects of EBN may not be explained by the presence of a single bioactive in view of the wide variety of its components. Moreover, the concept of food synergy has been proposed to suggest that food is the functional unit of nutrition, since the effects of food are not normally explained by the presence of a single compound.[28] Accordingly, the multiple bioactive compounds in EBN may have produced their effects through synergism.

Conclusion

The results of this study showed that HFD induced hypercholesterolemia and hypercoagulation by worsening lipid profile and elevating procoagulation factors. Transcriptional modulation of related genes seems to be involved in EBN-associated attenuation of the HFD-induced changes. EBN showed effects similar to those of simvastatin, although the results suggested that EBN may be better because it did not show a tendency for worsening of metabolism. The present data demonstrate an important health benefit of EBN, the implications of which may mean more targeted patronage of EBN because of the increasing scientific evidence for its health benefits. The translational implications of these findings, however, need to be confirmed through clinical trials, which have the potential to establish EBN as a functional food for lowering the risk of CVD-related hypercoagulation due to hypercholesterolemia.

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Disclosure

The authors report no conflicts of interest in this work.

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