

# Nutrigenomic effects of edible bird's nest on insulin signaling in ovariectomized rats

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**Abstract:** Estrogen deficiency alters quality of life during menopause. Hormone replacement therapy has been used to improve quality of life and prevent complications, but side effects limit its use. In this study, we evaluated the use of edible bird's nest (EBN) for prevention of cardiometabolic problems in rats with ovariectomy-induced menopause. Ovariectomized female rats were fed for 12 weeks with normal rat chow, EBN, or estrogen and compared with normal non-ovariectomized rats. Metabolic indices (insulin, estrogen, superoxide dismutase, malondialdehyde, oral glucose tolerance test, and lipid profile) were measured at the end of the experiment from serum and liver tissue homogenate, and transcriptional levels of hepatic insulin signaling genes were measured. The results showed that ovariectomy worsened metabolic indices and disrupted the normal transcriptional pattern of hepatic insulin signaling genes. EBN improved the metabolic indices and also produced transcriptional changes in hepatic insulin signaling genes that tended toward enhanced insulin sensitivity, and glucose and lipid homeostasis, even better than estrogen. The data suggest that EBN could meliorate estrogen deficiency-associated increase in risk of cardiometabolic disease in rats, and may in fact be useful as a functional food for the prevention of such a problem in humans. The clinical validity of these findings is worth studying further.

**Keywords:** ovariectomy, lipid metabolism, insulin resistance, antioxidant, aging

## Introduction

Menopause is characterized by depletion of the ovarian follicles and decreasing levels of estrogen and related hormones. As menopause sets in, the ovaries progressively fail to produce estrogen, resulting in symptoms that may be severe and disabling and have an impact on quality of life.<sup>1</sup> Estrogen has modulatory effects on metabolic processes including the maintenance of optimal glucose and lipid homeostasis, which it does through regulation of key hormones and genes involved in cardiometabolic health.<sup>2</sup> During menopause, fluctuations and/or relative or absolute deficiency of estrogen and other sex hormones will result in loss of the balance maintained by these hormones, thereby leading to a disturbed internal milieu and heightened risk of diseases including insulin resistance and associated problems.<sup>3,4</sup>

The symptoms and diseases that are a consequence of menopause have received considerable attention, due to their distressing nature and impact on quality of life. It is increasingly becoming a concern for women because of increasing life expectancy. Hormone replacement therapy has been used to restore premenopausal hormonal levels in order to reverse menopausal problems, but associated side effects including cancers and cardiovascular disease have necessitated the search for alternatives.<sup>5,6</sup> Phytoestrogens from plants have also been associated with side effects similar to those of hormone replacement therapy.<sup>7</sup> Alternatives like Remifemin<sup>®</sup> have not offered significant improvements in

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long-term sequelae of menopause like cancer and cardiometabolic disease risk, and side effects further limit their use.<sup>8</sup>

The burden of cardiometabolic disease has grown considerably over the years, and in women the risk of these diseases increases significantly after menopause.<sup>3,4</sup> Edible bird's nest (EBN) has been used as a traditional supplement mostly by Asians to improve wellbeing.<sup>9</sup> Recent evidence indicates that EBN may have anti-inflammatory and antioxidative properties,<sup>10–12</sup> which can be beneficial in cardiometabolic disease.<sup>13,14</sup> In this study, we determined the effects of EBN on cardiometabolic indices in ovariectomized rats, and a possible mechanistic basis for such effects. We hypothesized that the outcome of the study could provide the evidence needed for use of EBN as a functional food in preventing cardiometabolic disease in menopause, where the risk of such is increased due to loss of protection from estrogen and related hormones.

## Materials and methods

### Materials

A rat insulin enzyme-linked immunosorbent assay kit was purchased from Millipore (Billerica, MA, USA), while kits for superoxide dismutase (SOD) and estrogen were from Cell Biolabs (Cell Biolabs Inc, San Diego, CA, USA) and Cusabio Biotech Co Ltd (Wuhan, People's Republic of China), respectively. The GenomeLab™ GeXP start kit was from Beckman Coulter Inc (Miami, FL, USA) and the RNA extraction kit was from RBC Bioscience Corp (Taipei, Taiwan). MgCl<sub>2</sub> and DNA Taq polymerase were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA), while RCL2 solution was purchased from Alphelys (Toulouse, France). Glucometer strips were from Roche Diagnostics (Indianapolis, IN, USA) and lipid profile kits (low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, total cholesterol, and triglycerides) were purchased from Randox Laboratories Ltd (Crumlin, County Antrim, UK). Rat chow was obtained from Specialty Feeds (Glen Forrest, WA, Australia). Ketamine/xylazine was from Sigma Chemical Co (St Louis, MO, USA) and other solvents of analytical grade were purchased from Merck (Darmstadt, Germany).

### EBN samples

Ready-to-use EBN was supplied by Niah Bird's Nest Trading Company (Sarawak, Malaysia), and was incorporated into

standard rat chow for animal feeding. For determination of nutritional composition, EBN was dried at 50°C for 3 days, ground into powder, and used. Nutritional composition (protein, carbohydrate, fat, ash, and moisture; Table 1) was determined as reported previously.<sup>15</sup>

### Animal handling and feeding

Approval for use of animals was granted by the Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (approval UPM/IACUC/AUP-R012/2014), and the animals were handled as stipulated by the guidelines for the use of animals. Thirty-six Sprague-Dawley rats (3 months old, female, 160–180 g) were housed under controlled conditions (12-hour light/12-hour dark cycle, 20°C–22°C, 40%–50% humidity) with free access to water and food for 2 weeks prior to the experiments. After acclimatization, rats were ovariectomized under anesthesia using 10 mg/60 mg/kg xylazine/ketamine (intraperitoneally), except for the normal controls. The rats were then observed for 4 weeks and randomly assigned to one of five groups (n=6): a sham group, maintained on standard rat chow; an ovariectomy (OVX) control group maintained on standard rat chow; an OVX + estrogen group maintained on standard rat chow and daily estrogen (0.2 mg/kg body weight); an OVX + 3% EBN group maintained on standard rat chow containing 3% EBN; and an OVX + 1.5% EBN group maintained on standard rat chow containing 1.5% EBN. The normal group was not ovariectomized and was maintained on standard rat chow. Treatments lasted for 12 weeks, and food intake in each group was adjusted to the average intake each day according to observation of the OVX group the day before. Weights were measured weekly, and the total amount of feed (g) given was reviewed weekly based on the weekly weights of the rats. At the end of the experiment, all animals were exsanguinated after anesthesia (10 mg/60 mg/kg xylazine/ketamine, intraperitoneally). The livers were collected into RCL2 solution for gene expression studies.

### OGTT and lipid profile

At the end of the intervention, an oral glucose tolerance test (OGTT) was performed on each animal after 12 hours of an overnight fast, and measurements were taken with a glucometer. Serum samples from blood collected on the day of euthanasia were used to analyze the rat lipid profiles (total

**Table 1** Nutritional values for edible bird's nest

Nutritional value*	Crude protein	Carbohydrate	Fat	Ash	Moisture
Edible bird's nest	67.72%±1.9%	31.07%±1.5%	1%±0.002%	0.06%±0.002%	0.15%±0.004%

**Notes:** \*Nutritional values are expressed as percent dry weight. All the values are shown as the mean ± standard deviation (n=3).

cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides) using a Dimension Xpand Plus Integrated chemistry system (Siemens, Germany) with commercially available kits.

## Determination of serum estrogen and insulin

Serum samples were used for measurements of estrogen and insulin using the respective enzyme-linked immunosorbent assay kits according to the manufacturers' instructions. Absorbance was read on a microplate reader (BioTek Synergy H1 Hybrid Reader, BioTek Instruments Inc, Winooski, VT, USA) and results were calculated from the respective standard curves: estrogen ( $y = -30.12x + 113.73$ ,  $R^2=1$ ), insulin ( $y = 0.762x - 0.143$ ,  $R^2=0.966$ ). Additionally, homeostatic model assessment of insulin resistance (HOMA-IR), a measure of insulin sensitivity, was computed from the fasting plasma glucose and insulin levels as reported previously.<sup>16</sup>

## Hepatic antioxidative markers

### Superoxide dismutase enzyme

Liver homogenates were used for SOD quantification using the enzyme-linked immunosorbent assay kit according to the manufacturer's instructions. Absorbance were read on a microplate reader (BioTek Synergy H1 Hybrid Reader) and results were calculated from the standard curve ( $y = 0.143x - 0.017$ ,  $R^2=1$ ).

### Thiobarbituric acid reactive substances assay

Thiobarbituric acid reactive substances were determined using a modified method based on the protocol of Chan et al.<sup>17</sup> Briefly, homogenized liver tissues (50 mg/100  $\mu$ L phosphate-buffered saline) were added to 0.25 N HCl, 15% trichloroacetic acid, and 0.375% thiobarbituric acid separately, then incubated at 100°C for 10 minutes, and centrifuged at 3,000 rpm for 15 minutes. Finally, absorbance of the supernatant were read at 540 nm using the Synergy H1 Hybrid Multi-Mode microplate reader. Malondialdehyde (MDA) was used as the standard ( $y = 0.1982x - 0.1898$ ,  $R^2=0.9947$ ).

## RNA extraction, reverse transcription, and multiplex polymerase chain reaction analyses

RNA was extracted from rat livers using the Total RNA isolation kit according to the manufacturer's instructions. Primer sequences were designed on the National Center for

Biotechnology Information website, except for the internal control (KanR), which was supplied by Beckman Coulter. The primers (Table 2) were supplied by Integrated DNA Technologies (Singapore), and reconstituted in 1 $\times$  TE buffer according to the protocol outlined in the GenomeLab GeXP kit. Reverse transcription and polymerase chain reaction were performed according to the GenomeLab GeXP kit protocol in an XP Thermal Cycler (Bioer Technology, Germany). The polymerase chain reaction products were finally analyzed with a GeXP genetic analysis system, and the results were normalized using eXpress Profiler software based on the manufacturer's instructions.

## Statistical analysis

Statistical analyses were done using one-way analysis of variance with Tukey's honest significant difference test. The data are expressed as the mean  $\pm$  standard error of the mean.  $P < 0.05$  indicates statistical significance. All values were statistically evaluated using Statistical Package for the Social Sciences version 20.0 (SPSS Inc, Chicago, IL, USA).

## Results and discussion

### Food intake and body weight

Menopause is characterized by a drop in estrogen levels, which results in lipid and glucose metabolic perturbation.<sup>1,3,4</sup> In this study, serum estrogen levels in the OVX group were significantly lower than in the other groups (Table 3), suggesting that removal of the ovaries produced a condition similar to menopause in the rats. The higher levels in the estrogen group could be attributed to exogenous estrogen administration, while the high levels of the hormone in the EBN group could have been due to increased extragonadal synthesis of estrogen<sup>18</sup> induced by EBN or possibly estrogen-like compounds absorbed from EBN. Body weights were similar for all groups at the start of the experiment (Table 3), and calorie intake remained similar throughout the intervention period. At the end, however, differences in weight gain were observed. The OVX group had the highest weight gain, indicating that loss of estrogen affected body fat deposition and distribution. Moreover, estrogen is believed to regulate weight in women, while menopause is associated with increased weight gain, which is linked to increased risk of cardiometabolic disease.<sup>2-4</sup> Estrogen treatment and EBN produced lower weight gains similar to the control group, suggesting that both treatments had some weight-modulating properties, possibly mediated through estrogen and/or estrogen-mimetic effects.

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

Gene name	Accession number	Left sequence	Right sequence
IRS2	NM_001168633	AGGTGACACTATAGAATAAGGCACTGGAGCCTTAC	GTACGACTCACTATAGGGAGCAGCACTTACTCTTTTCAC
KCNJ11	NM_031358	AGGTGACACTATAGAATACTACTTCCAGGCAAACTCTG	GTACGACTCACTATAGGGAGAACTTCCAAATATTTCTTTT
Insr	NM_017071	AGGTGACACTATAGAATAAGCTGGAGGAGTCTTCAT	GTACGACTCACTATAGGGAAAGGGATCTTCGCCTT
GCK	NM_001270849	AGGTGACACTATAGAATAATCTTTTGCACACTCAGC	GTACGACTCACTATAGGGATTTGGTGCCCAAGA
PKLR	NM_012624	AGGTGACACTATAGAATAATCGGAGGTGAAATG	GTACGACTCACTATAGGGACTCTGGCCCGATTTT
B2M*	NM_012512	AGGTGACACTATAGAATAATGCTTGCAGAGTTAAACA	GTACGACTCACTATAGGGATGCATAAATATTTAAGGTAAGA
HPRT1**	NM_012583	AGGTGACACTATAGAATAATCTCATGGACTGATTATG	GTACGACTCACTATAGGGACTGGTCACTACAGTAGCTCTT
MAPK1	NM_053842	AGGTGACACTATAGAATAATTTTTGAAGAGACTGCTC	GTACGACTCACTATAGGGAAACTCTCTGGACTGAAGAAT
IKBKB	NM_053355	AGGTGACACTATAGAATACTTGAACCTTAAAGCTGGTTC	GTACGACTCACTATAGGGAAACAATTTTACTGTGTGTCAAAGAG
Kan (r)**			
GLUT4	NM_012751	AGGTGACACTATAGAATACTCTGAAGATGGGGAAC	GTACGACTCACTATAGGGAGGCTCTGTTCAACTCAGCTTTC
ACTB*	NM_031144	AGGTGACACTATAGAATAAACAACACTACATTCCAATCCATCA	GTACGACTCACTATAGGGATAAAAACGCCAGCTCAGTAAC
Pik3ca	NM_133399	AGGTGACACTATAGAATAACAAGGATCTGACTTATTTCC	GTACGACTCACTATAGGGACTAAACCATGCTGTTACCAA

**Notes:** \*Housekeeping gene. \*\*Normalization gene. Underlined sequences are left and right universal left and right sequences (tags). \*\*\*Internal control supplied by Beckman Coulter Inc, as part of the GeXP kit. Reverse transcription 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. Polymerase chain reaction 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.

### Serum lipid profile

Table 4 shows the lipid profiles for the different groups. Total cholesterol for the OVX group was highest although no significant differences were observed between the groups. Low-density lipoprotein cholesterol was also highest in the OVX group, while the others were significantly lower, except for the 1.5% EBN group. Triglyceride levels were significantly lower in the EBN groups in comparison with the OVX group, while high-density lipoprotein cholesterol levels were not significantly different between the groups. As expected, estrogen improved the lipid profiles. Estrogen modulates cholesterol metabolism, and in estrogen-deficient states similar to OVX, there is dysregulation of cholesterol metabolism in favor of higher cholesterol levels, with an increased risk of cardiometabolic disease.<sup>4</sup> EBN on the other hand was able to ameliorate lipid profile and cholesterol ratios (Table 4) similar to estrogen. This effect of EBN on the lipid profile may have been secondary to the enhanced estrogen concentration induced by EBN, but other EBN constituents may also have played a role since EBN is known to be bioactive-rich.<sup>9</sup> The improved lipid profiles observed in this study suggest that EBN could lower the risk of cardiometabolic disease without the associated risks of estrogen administration.

### OGTT, serum insulin, and HOMA-IR

The OGTT results are shown in Figure 1. The glycemic response in the OVX group was impaired in comparison with the other groups, which showed similar and significantly ( $P < 0.05$ ) lower responses (Figure 1A). Moreover, the area under the curve for glucose over 120 minutes (Figure 1B) was significantly lower ( $P < 0.05$ ) for the EBN and estrogen treatments in comparison with the OVX group. Additionally, the insulin level was highest in the OVX group, which could have been a counter-response to the loss of estrogen-regulated insulin action in the rats (Figure 2). The estrogen group, on the other hand, showed significantly lower insulin levels ( $P < 0.05$ ), while the EBN groups were not significantly different from the OVX group. HOMA-IR results showed that the OVX group had the highest tendency for insulin resistance, while estrogen therapy significantly improved insulin sensitivity ( $P < 0.05$ ). These effects of OVX and estrogen therapy mirror what has been reported previously;<sup>4</sup> OVX tends to increase the risk of insulin resistance, which can be prevented by estrogen therapy. EBN treatments showed dose-dependent effects on insulin sensitivity, but only the 3% EBN treatment showed significantly better results than those seen in the OVX group. Although estrogen may prevent the

**Table 3** Body weight, food intake, and serum estrogen levels in ovariectomized rats after 12 weeks of intervention

Rat groups	Body weight before intervention (g)	Body weight at end of intervention (g)	Total food intake (kcal/kg/day)	Serum estrogen (pg/mL)
Normal control	221.5±33.5 <sup>a</sup>	227.5±6.0 <sup>a</sup>	134±4.8 <sup>a</sup>	151.1±8 <sup>a</sup>
Sham control	217.8±44.0 <sup>a</sup>	224.6±6.8 <sup>a</sup>	134±4.8 <sup>a</sup>	156.7±13 <sup>a</sup>
OVX control	230±29.7 <sup>a</sup>	283.1±23.1 <sup>b</sup>	134±4.8 <sup>a</sup>	35.6±0.9 <sup>b</sup>
Estrogen-treated	236±33.3 <sup>a</sup>	239±3.0 <sup>a</sup>	134±4.8 <sup>a</sup>	169.8±11.4 <sup>a</sup>
3% EBN	228.1±53.9 <sup>a</sup>	242.8±14.6 <sup>a</sup>	134.97±4.99 <sup>a</sup>	147.8±8.7 <sup>a</sup>
1.5% EBN	216±31.5 <sup>a</sup>	242.6±26.7 <sup>ab</sup>	134.81±4.45 <sup>a</sup>	143.3±13.4 <sup>a</sup>

**Notes:** Values are shown as the mean ± standard deviation, n=6. All rat groups received standard rat chow for 12 weeks, and in addition, the estrogen-treated groups received 0.2 mg/kg/day of estrogen, while EBN groups received 3% or 1.5% EBN in their rat chow. Columns with different letters signify statistical difference ( $P < 0.05$ ).

**Abbreviations:** OVX, ovariectomy; EBN, edible bird's nest.

OVX-induced risk of insulin resistance as demonstrated in the present study (lower HOMA-IR in the estrogen group), EBN may be preferred since its consumption is not associated with side effects as with estrogen therapy, except when it is adulterated.<sup>9</sup>

### Hepatic antioxidant capacity of EBN

Oxidative stress is increased while antioxidants are decreased in estrogen-deficient states. This imbalance is thought to underlie some problems related to menopause.<sup>19</sup> Thus, in this study, MDA levels, indicative of oxidative stress, were potentiated in the OVX group while SOD, an antioxidant enzyme, was suppressed (Figure 3), in keeping with a report by Muthusami et al.<sup>20</sup> Further, estrogen treatment reduced MDA and improved SOD levels but not as well as EBN. Moreover, the side effects of estrogen therapy may be associated with the pro-oxidant effects of exogenous estrogen.<sup>21</sup> EBN significantly ameliorated oxidative stress status and improved antioxidant abilities, in agreement with reports on its antioxidant properties.<sup>11,12</sup> The results therefore suggest that improved antioxidant enzymes may partly form the basis for improved metabolic indices in ovariectomized rats fed EBN, since improved antioxidant status has been linked with better metabolic outcomes.<sup>22</sup>

### mRNA levels of hepatic insulin signaling genes

The results thus far indicate that EBN could lower the risk of estrogen deficiency-induced perturbations in glucose and lipid homeostasis. In an effort to understand some of the mechanistic bases for the observed effects, we evaluated mRNA levels for hepatic insulin signaling genes (Figures 4–6). Figure 4 shows the effects of the interventions on mRNA levels of the hepatic insulin receptor, insulin receptor substrate 2 (*IRS2*), and phosphoinositide-3-kinase (*PI3K*). Insulin exerts its effects after binding to its receptor

and activating a cascade of events mediated by *IRS2*,<sup>23</sup> in which *PI3K* plays a prominent role through enhanced insulin signaling in the cell.<sup>24</sup> Disruption of this pathway promotes insulin resistance. In this study, OVX downregulated *IRS2* and *PI3K*, suggesting that estrogen deficiency-induced interruption of cellular insulin signaling contributed to worsening of glucose and lipid homeostasis in the rats. Interestingly, EBN increased expression of the insulin receptor, *IRS2*, and *PI3K* even better than estrogen therapy, indicating that its ability to prevent estrogen deficiency-induced worsening of glucose and lipid homeostasis in ovariectomized rats was partly through transcriptional upregulation of insulin signaling.

Furthermore, OVX upregulated mitogen-activated protein kinase (*MAPK*) and inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (*IKK $\beta$* , Figure 5), which have both been implicated in impaired insulin signaling.<sup>25,26</sup> These genes have been reported to influence the activity of *IRS2*, and the OVX-induced low transcriptional levels of *IRS2* observed in this study may have been due to upregulation of *MAPK1* and *IKK $\beta$* .<sup>25,26</sup> The EBN groups had lower mRNA levels of these genes in comparison with other groups, further suggesting that the effects of EBN on transcriptional regulation of *IRS2* may have been through *MAPK1* and *IKK $\beta$* . Glucose transporter type 4 (*GLUT4*) transports glucose into cells for metabolism and is downregulated in insulin-resistant states.<sup>27</sup> Although the exact contribution of hepatic expression of *GLUT4* to glucose transport remains a matter of debate, there are indications that its dysregulation is linked to insulin resistance.<sup>28</sup> In this study, OVX downregulated *GLUT4*, while EBN treatments upregulated the gene in a dose-dependent manner. Moreover, lower levels of *MAPK*, with consequently higher *PI3K* levels, have been reported to enhance glucose uptake through increased *GLUT4* expression.<sup>27</sup> In keeping with findings of decreased *MAPK* and increased *PI3K* and *GLUT4* in this study.

Table 4 Serum lipid profiles after 12 weeks of intervention

Rat groups	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	LDL/HDL (ratio)	VLDL* (mg/dL)	TC/HDL (ratio)	TG (mg/dL)
Normal control	1.858±0.240 <sup>ab</sup>	0.194±0.035 <sup>a</sup>	1.268±0.127 <sup>ab</sup>	0.153±0.027 <sup>a</sup>	0.396±0.078 <sup>ad</sup>	1.465±0.189 <sup>ab</sup>	0.473±0.033 <sup>3c-c</sup>
Sham	1.690±0.234 <sup>a</sup>	0.198±0.035 <sup>a</sup>	1.092±0.120 <sup>a</sup>	0.181±0.032 <sup>a</sup>	0.400±0.079 <sup>ad</sup>	1.548±0.214 <sup>ab</sup>	0.587±0.090 <sup>0-b,d</sup>
OVX control	2.220±0.248 <sup>b</sup>	0.374±0.046 <sup>b</sup>	1.360±0.157 <sup>ab</sup>	0.275±0.034 <sup>b</sup>	0.486±0.045 <sup>a</sup>	1.632±0.183 <sup>a</sup>	0.657±0.067 <sup>b</sup>
Estrogen	1.885±0.105 <sup>ab</sup>	0.286±0.038 <sup>c</sup>	1.375±0.077 <sup>b</sup>	0.208±0.028 <sup>c</sup>	0.224±0.011 <sup>b</sup>	1.371±0.076 <sup>b</sup>	0.460±0.026 <sup>c</sup>
3% EBN	1.683±0.301 <sup>ab</sup>	0.298±0.021 <sup>c</sup>	1.316±0.135 <sup>ab</sup>	0.226±0.016 <sup>bc</sup>	0.068±0.014 <sup>c</sup>	1.278±0.229 <sup>ab</sup>	0.455±0.021 <sup>c</sup>
1.5% EBN	1.897±0.254 <sup>ab</sup>	0.320±0.020 <sup>bc</sup>	1.260±0.132 <sup>ab</sup>	0.254±0.016 <sup>bc</sup>	0.317±0.010 <sup>d</sup>	1.505±0.201 <sup>ab</sup>	0.527±0.006 <sup>d</sup>

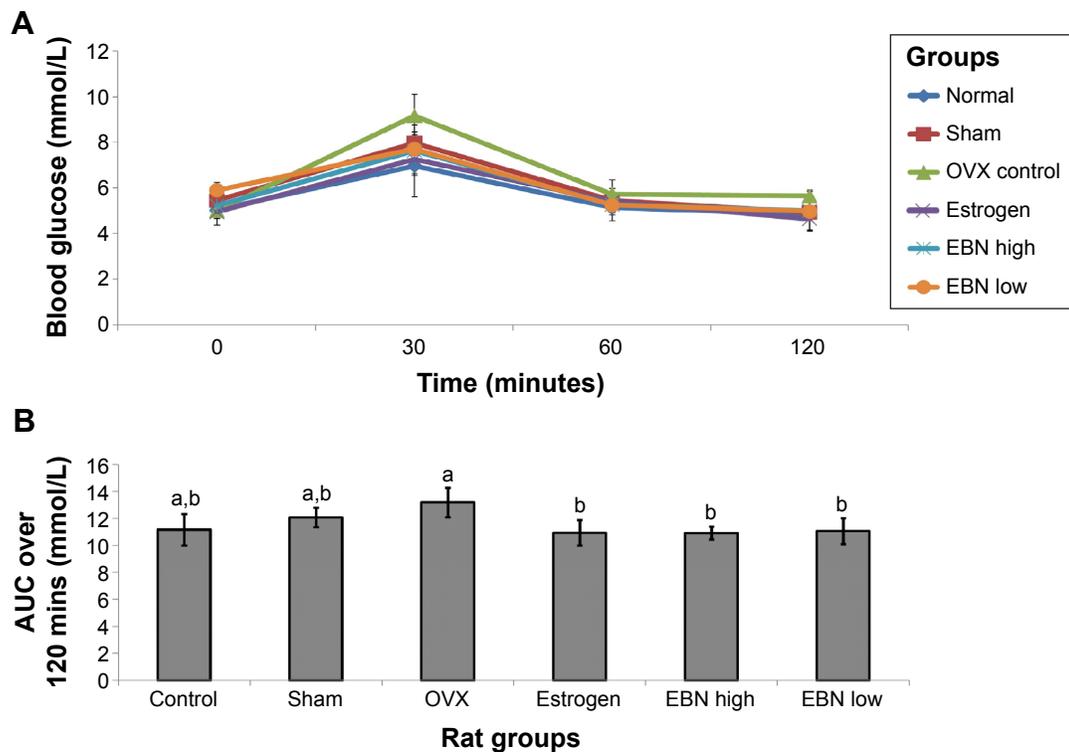
Notes: Values are shown as the mean ± standard deviation. Columns with different letters indicate statistical difference ( $P < 0.05$ ). \*Total cholesterol – (HDL + LDL). All rat groups received standard rat chow for 12 weeks, and in addition, the estrogen treated groups received 0.2 mg/kg/day of estrogen, while EBN groups received 3% or 1.5% EBN in their rat chow.

Abbreviations: TC, total cholesterol; TG, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very low-density lipoprotein; EBN, edible bird's nest; OVX, ovariectomy.

Transcriptional levels of other genes that contribute to enhanced cellular glucose sensing and homeostasis (glucokinase, pyruvate kinase-liver isoform, and potassium inwardly rectifying channel, subfamily J, member 11 [*KCNJ11*] genes) were downregulated in the OVX group (Figure 6). Conversely, the EBN group showed increased expression of these genes, except *KCNJ11*, even better than estrogen therapy. The implications of these effects could be increased hepatic glucose storage and utilization (glucokinase and pyruvate kinase-liver isoform),<sup>29</sup> which may then enhance cellular adenosine triphosphate levels and subsequent upregulation of *KCNJ11* with improved glucose homeostasis.<sup>30</sup>

Based on the effects of EBN observed in this study, we propose that EBN transcriptionally regulates multiple targets in the insulin signaling pathway (Figure 7) and that this is the basis for the improved insulin signaling and glucose and lipid homeostasis.

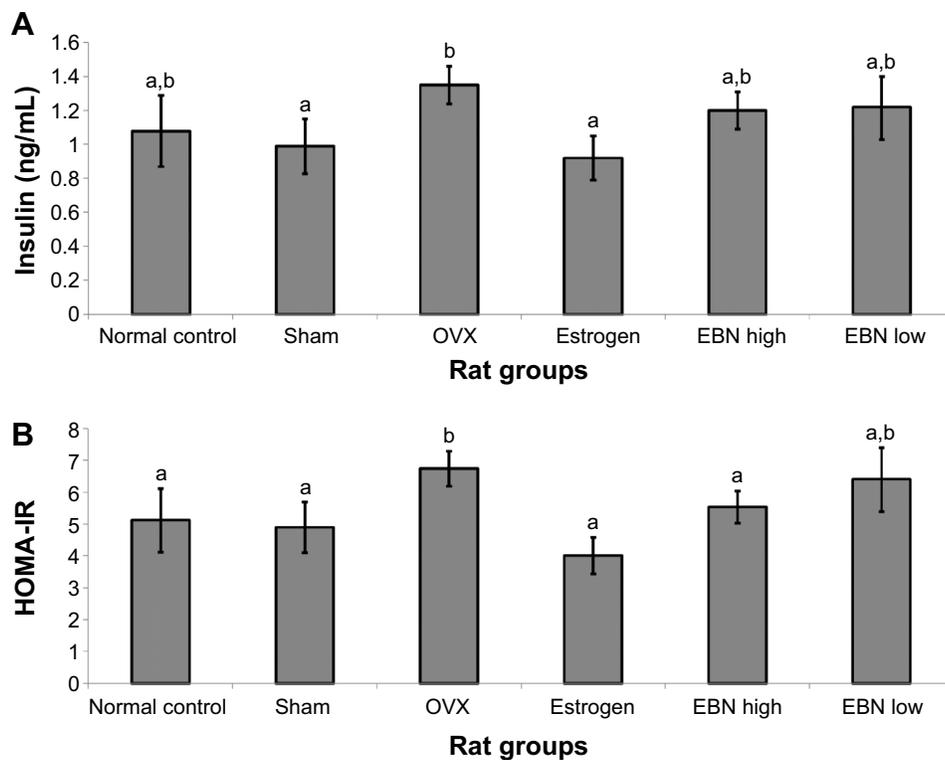
Overall, the data show that OVX worsened glucose and lipid homeostasis and oxidative stress (low insulin and SOD levels; high body weight, HOMA-IR, MDA, and lipid profile levels; and impaired insulin signaling) in rats. Estrogen therapy, on the other hand, was able to improve the lipid profile and OGTT, SOD, and MDA levels, and was even associated with some transcriptional changes in hepatic insulin signaling genes that tended toward better glucose and lipid homeostasis. However, EBN improved glucose and lipid homeostasis, lowered weight and oxidative stress, and enhanced estrogen deficiency-induced impaired signaling of insulin better than estrogen. Simple linear regression analyses indicate that estrogen levels significantly correlated with changes in HOMA-IR ( $r = -0.82$ ,  $P = 0.046$ ), SOD ( $r = 0.82$ ,  $P = 0.046$ ), MDA ( $r = -0.87$ ,  $P = 0.023$ ), and PI3K-mediated insulin signaling ( $r = 0.86$ ,  $P = 0.027$ ). Additionally, SOD levels ( $r = -0.86$ ,  $P = 0.028$ ) and PI3K-mediated insulin signaling ( $r = -0.85$ ,  $P = 0.033$ ), but not MDA levels ( $r = 0.53$ ,  $P = 0.28$ ) correlated with HOMA-IR in this study. Multiple linear regression analyses indicate further that changes in estrogen were overall strong determinants of changes in SOD, MDA, HOMA-IR, and PI3K expression changes ( $R = 1$ ,  $R^2 = 0.995$ ), and that estrogen, SOD, MDA, and PI3K expression was a significant predictor of HOMA-IR ( $R = 0.987$ ,  $R^2 = 0.873$ ). The combined effects of EBN on these markers of cardiometabolic disease, coupled with the known side effects of estrogen therapy, suggest that EBN may be better than hormone replacement therapy at preventing menopause-associated cardiometabolic disease, especially impaired glucose and lipid homeostasis.



**Figure 1** Effects of 12 weeks of supplementation with EBN on (A) oral glucose tolerance test and (B) AUC for glucose in ovariectomized rats.

**Notes:** EBN high, 3% EBN; EBN low, 1.5% EBN. Different letters for bars in (B) indicate a statistically significant difference ( $P < 0.05$ ). All rat groups received standard rat chow for 12 weeks, and in addition, the estrogen treated groups received 0.2 mg/kg/day of estrogen, while EBN groups received 3% or 1.5% EBN in their rat chow.

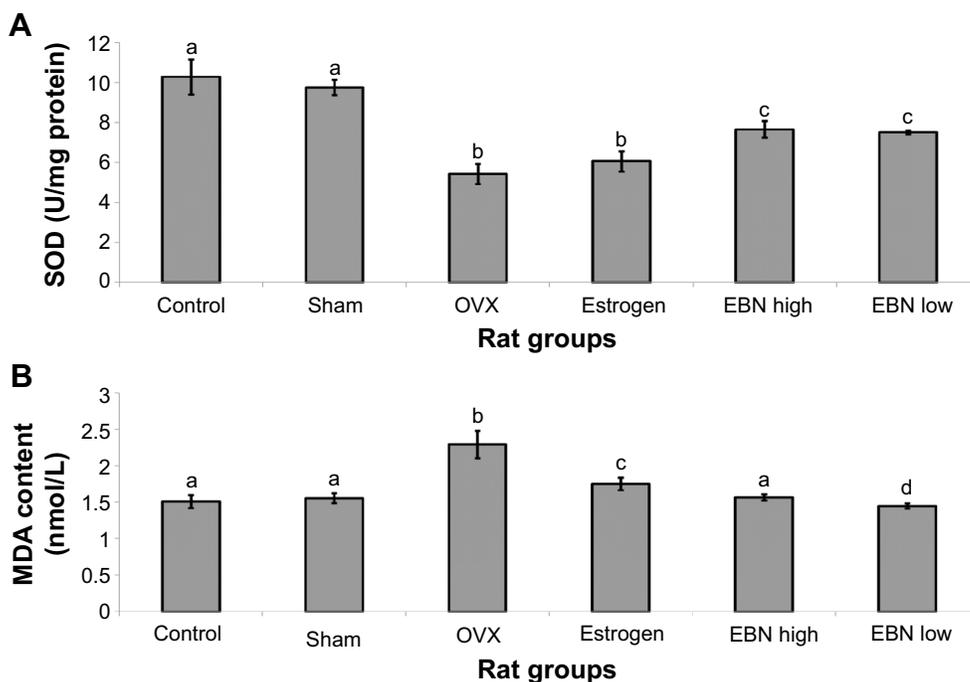
**Abbreviations:** AUC, area under the curve; EBN, edible bird's nest; OVX, ovariectomy.



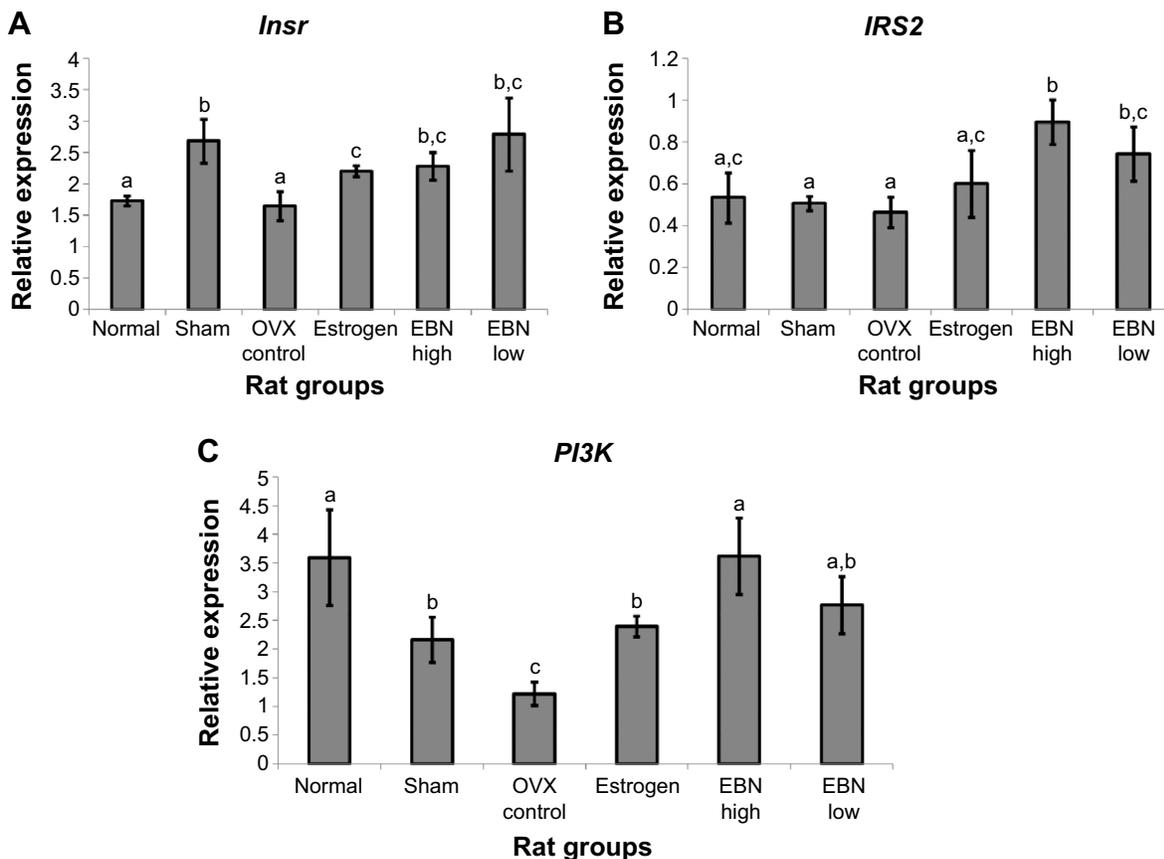
**Figure 2** Effects of 12 weeks of supplementation with EBN on (A) serum insulin and (B) HOMA-IR in ovariectomized rats.

**Notes:** EBN high, 3% EBN; EBN low, 1.5% EBN. Different letters for bars in each panel indicate a statistically significant difference ( $P < 0.05$ ). All rat groups received standard rat chow for 12 weeks, and in addition, the estrogen treated groups received 0.2 mg/kg/day of estrogen, while EBN groups received 3% or 1.5% EBN in their rat chow.

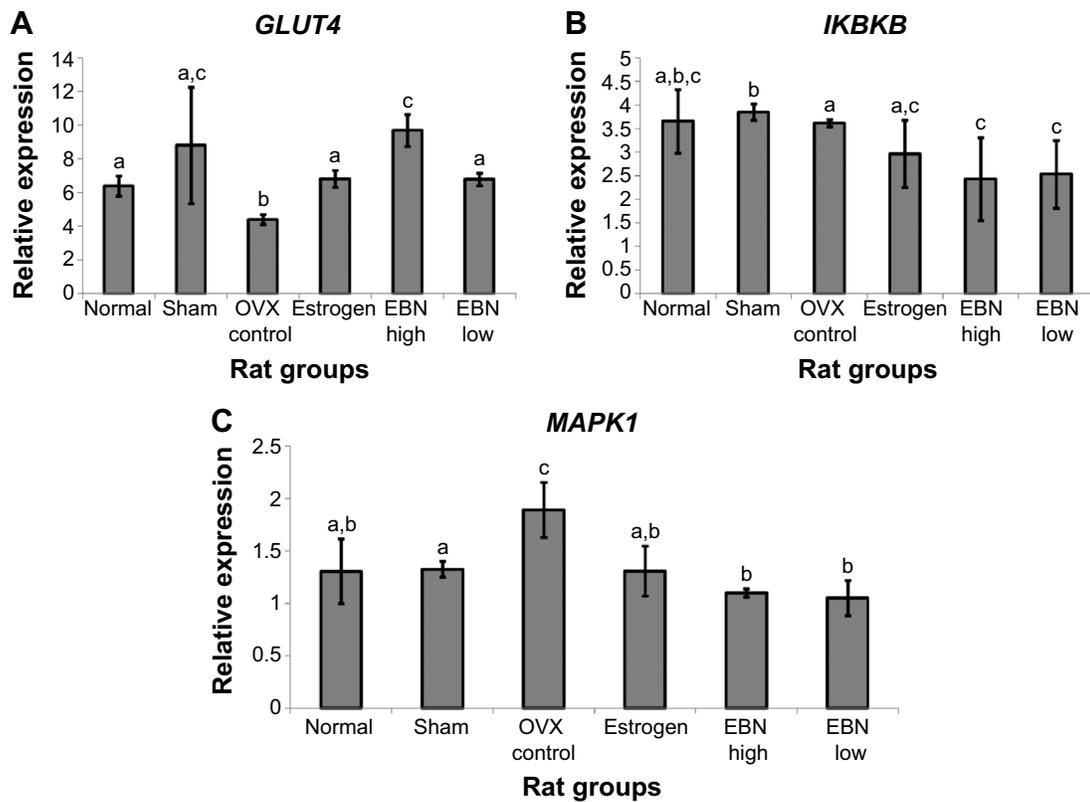
**Abbreviations:** EBN, edible bird's nest; OVX, ovariectomy; HOMA-IR, homeostatic model assessment of insulin resistance.



**Figure 3** Effects of 12 weeks of supplementation with EBN on serum (A) SOD levels and (B) MDA levels in ovariectomized rats. **Notes:** Different letters for bars in each panel indicate a statistically significant difference ( $P < 0.05$ ). All rat groups received standard rat chow for 12 weeks, and in addition, the estrogen treated groups received 0.2 mg/kg/day of estrogen, while EBN groups received 3% or 1.5% EBN in their rat chow. **Abbreviations:** EBN, edible bird's nest; MDA, malondialdehyde; SOD, superoxide dismutase; OVX, ovariectomy.



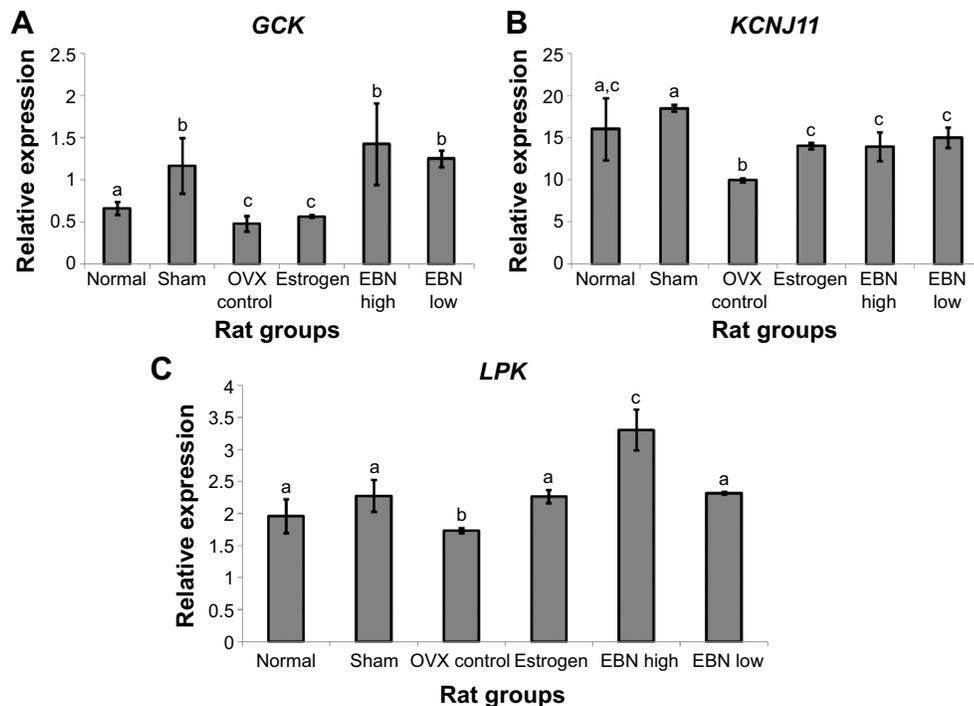
**Figure 4** Hepatic mRNA levels of the (A) *Insr*, (B) *IRS2*, and (C) *PI3K* in ovariectomized rats fed with EBN for 12 weeks. **Notes:** Different letters for bars in each panel indicate a statistically significant difference ( $P < 0.05$ ). All rat groups received standard rat chow for 12 weeks, and in addition, the estrogen treated groups received 0.2 mg/kg/day of estrogen, while EBN groups received 3% or 1.5% EBN in their rat chow. **Abbreviations:** EBN, edible bird's nest; *PI3K*, phosphoinositide-3-kinase; *IRS2*, insulin receptor substrate 2; OVX, ovariectomy; *Insr*, insulin receptor.



**Figure 5** Hepatic mRNA levels of (A) *GLUT4*, (B) *IKBKB*, and (C) *MAPK1* in ovariectomized rats fed with EBN for 12 weeks.

**Notes:** Different letters for bars in each panel indicate a statistically significant difference ( $P < 0.05$ ). All rat groups received standard rat chow for 12 weeks, and in addition, the estrogen treated groups received 0.2 mg/kg/day of estrogen, while EBN groups received 3% or 1.5% EBN in their rat chow.

**Abbreviations:** EBN, edible bird's nest; *GLUT4*, glucose transporter type 4; *IKBKB*, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta; *MAPK1*, mitogen-activated protein kinase 1; OVX, ovariectomy.



**Figure 6** Hepatic mRNA levels of (A) *GSK*, (B) *KCNJ11*, and (C) *LPK* in ovariectomized rats fed with EBN for 12 weeks.

**Notes:** Different letters for bars in each panel indicate a statistically significant difference ( $P < 0.05$ ). All rat groups received standard rat chow for 12 weeks, and in addition, the estrogen treated groups received 0.2 mg/kg/day of estrogen, while EBN groups received 3% or 1.5% EBN in their rat chow.

**Abbreviations:** EBN, edible bird's nest; *GSK*, glucokinase; *KCNJ11*, potassium inwardly rectifying channel, subfamily J, member 11; OVX, ovariectomy; *LPK*, pyruvate kinase-liver isoform.



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