Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2021.118

RESEARCH ARTICLE

S-Allylcysteine Supplementation Effects on Vascular and Bone Health in Ovariectomized Wistar Rats

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ARTICLE HISTORY (21-118)

Received: March 14, 2021
Revised: May 05, 2021
Accepted: May 10, 2021
Published online: June 05, 2021

Key words:
Blood pressure
Bone health
Ovariectomy
Postmenopausal hypertension
S-Allylcysteine

ABSTRACT

Estrogen level deteriorates in postmenopausal women that is associated with increased risks of osteoporosis and cardiovascular diseases. This study was aimed to investigate SAC supplementation effects on vascular and bone health in ovariectomized rats. Twenty-four female Wistar rats were randomly divided into 3 groups: sham, ovariectomy (OVX) and SAC-supplemented OVX (OVX+SAC). Sixteen rats underwent OVX surgery (bilateral ovaries excision) and 8 rats were sham-operated. After a 3-week recovery, sham and OVX groups were given distilled water whereas OVX+SAC group was treated with 100 mg/kg SAC orally for 7 days. SAC supplementation showed a marked SBP reduction ($P<0.05$) and a potential increase in NO production compared to OVX group. However, SAC caused no changes in body weight increment, aortic and bone oxidative stress markers, as well as in all bone parameters in OVX rats compared to sham. SAC also sustained the increase ($P<0.05$) in intima-media thickness (IMT); but ameliorated circumferential wall tension (CWT) in OVX rats. These findings suggest that SAC supplementation may benefit the vascular health in the postmenopausal animal model by reducing the SBP-raising effect, possibly by modulating NO production.

To Cite This Article: Halim RM, Kamisah Y, Aziz NF, Sudirman UN, Ahmad NAN, Chin KY and Zainalabidin S, 2021. S-allylcysteine supplementation effects on vascular and bone health in ovariectomized Wistar rats. Pak Vet J, 41(4): 507-512. http://dx.doi.org/10.29261/pakvetj/2021.118

INTRODUCTION

Estrogen is an important sex hormone with various physiological roles, including its significance in cardiovascular health and bone density regulation (Patel *et al*., 2018). Menopause results after ovarian follicles depletion which causes low estrogen that is eventually accompanied by high luteinizing and follicle-stimulating hormones. The prevalence of disease risks, namely cardiovascular diseases (CVD) and osteoporosis are markedly increased after menopause than premenopausal women and associated with estrogen depletion (Pollow *et al*., 2019). A non-hormonal approach was recently suggested alongside hormonal therapy to prevent diseases related to the postmenopausal condition (Zaw *et al*., 2018).

Hypertension is a risk factor for CVD in postmenopausal women, from increased body mass to oxidative stress and disturbed endothelial function (Sabbatini and Kararigas, 2020). Nevertheless, the commonly prescribed antihypertensive medications were less satisfying in achieving desirable blood pressure in women than men (Ljungman *et al*., 2014). Apart from hypertension, oxidative stress augments osteoclast and reduces osteoblast activities, resulting in osteoporosis with increased bone fragility, making it easier to break (Domazetovic *et al*., 2017).

Plant-based diet is believed to improve human health and practiced globally amongst cultures (Bayan *et al*., 2014). SAC is an organosulfur compound from garlic that is widely studied for antioxidative properties by its potential to produce bioactive thiol and has been associated with various health benefits (Colín-González *et al*., 2012). Even so, the implications of SAC in a postmenopausal condition have yet to be investigated. Hence, its effect against vascular and bone health in a rat model that mimics postmenopausal state is sought after.

MATERIALS AND METHODS

Experimental design: Twenty four female Wistar rats (194 ~ 214 g) were obtained from the Laboratory Animal Resource Unit of Universiti Kebangsaan Malaysia (UKM), Kuala Lumpur under the approval of UKM Animal Ethics Committee (FSK/2018/SATIRAH/28-NOV./974-DEC.-2018-FEB.-2021). Rats were acclimatized for one week (25±2ºC temperature, 12 hours day and night cycle, rat chow diet and tap water supplied *ad libitum*) before randomly divided into 3 groups: sham-operated (Sham), ovariectomy (OVX) and SAC treated ovariectomy (OVX+SAC). Sixteen rats underwent bilateral ovariectomy while 8 rats were sham-operated under the anesthesia of cocktail mixture of ketamine, xylazine and zolazepam (1 ml/kg, i.v.).

All rats were subjected to a 3-week recovery, and estradiol level was determined via ELISA kit (Elabscience Biotech Co Ltd, China). Sham and OVX groups were given vehicle distilled water, whereas OVX+SAC was supplemented with 100 mg/kg SAC (Tokyo Chemical Industry, Tokyo, Japan) orally for 7 days (Zainalabidin *et al*., 2020). Blood pressure was measured on day 0 and day 7 of treatment by non-invasive tail cuff (Kent Scientific, USA). Body weight, food and water intake were measured weekly. After 7 days, blood was collected for serum nitrite determination using Griess reagent, and rats were sacrificed. The aortas, femurs and tibias were harvested for further studies.

Homogenate preparation and biochemical evaluation: Aortic and tibial homogenates were prepared based on Upston *et al*. (2001). Tissue was weighed, grounded and homogenized in pH 7.4 phosphate-buffered solution. The homogenates were centrifuged and supernatant was stored at -40℃. Malondialdehyde (MDA) was performed according to method by Khusniyati *et al*. (2014) and reduced glutathione (GSH) were quantified spectrophotometrically using Ellman's reagent.

Bone mineral content (BMC), bone mineral density (BMD) and mechanical strength evaluation: BMC and BMD were performed on the left femoral bone using a dual-energy x-ray absorptiometry (DXA) machine (Hologic Inc., USA). Mechanical strength was measured according to Shapiro and Heaney (2003) by Shimadzu Autograph AG 25 TA (Shimadzu Corporation, Japan). Pressure was applied to the femur bone until it was broken, and the bone strength was recorded.

Aortic histology: The formalin-fixed thoracic aorta was dissected at 5 $x10^{-3}$ m length, dehydrated with ascending series of alcohol and embedded in paraffin. Tissue was sectioned at 4 mm thickness for histological studies via hematoxylin and eosin (H&E), Verhoeff Van Gieson (VVG) and picrosirius red. The mean aortic elastic lamellae number were calculated; and collagen deposition (Ali *et al*., 2019) were quantified.

Aortic morphometry measurement: The images of aortic cross-sections from H&E were captured with a light microscope via "analySIS FIVE" software (Olympus Corporation). Morphometric measurement was carried out according to Fernandes-Santos *et al*. (2009) via ImageJ software with four sides per image were measured and averaged.

Statistical analysis: Analyses were conducted using IBM SPSS Statistics 23 and GraphPad Prism 8. Results were expressed as mean ± SEM. Analysis of variance (ANOVA) was applied, followed by post-hoc tests to compare significant variables. Welch t-test was performed for estrogen quantification.

RESULTS

Systemic characteristics: Significant ($P<0.05$) body weight increment in OVX and OVX+SAC groups achieved before respective treatment began at week 3 with a trend of estradiol reduction ($p=0.0527$) compared to sham. SAC supplementation neither affected the body weight increment ($P<0.05$) nor food consumption in OVX+SAC than the sham group after 7 days. SAC also did not improve the uterus weight reduction ($P<0.05$) in OVX rats against sham group. No difference in food intake, heart and kidney weights over with tibial length were shown across the groups (Table 1).

Blood Pressure and Serum Nitrite Level: OVX caused a significant increase ($P<0.05$) in SBP compared to sham group (Fig. 1A). A marked SBP reduction ($P<0.05$) on day 7 of SAC supplementation with no changes in diastolic blood pressure (DBP) (Fig. 1B) and mean arterial pressure (MAP) (Fig. 1C) against OVX rats. OVX+SAC group showed a pattern in potentially lowering all the blood pressure parameters before and after treatment (Fig. 1A, 1B and 1C). Serum nitrite is an indirect indicator of NO production in an organism. OVX rats showed a significant reduction ($P<0.05$) in serum nitrite level against sham and OVX+SAC group showed a potential of serum nitrite increment by 52% against OVX after 7 days of treatment (Fig. 1D).

Oxidative stress marker and antioxidant level in aorta and bone homogenate: No significant changes in MDA and GSH levels in both aortic and tibial bone homogenate, with or without SAC supplementation in OVX rats against sham group. However, OVX group showed a trend of increasing MDA levels in aortic and tibial bone homogenate (Table 2).

Bone mineral content, bone mineral density and mechanical strength: All femoral bone parameters (BMC, BMD and mechanical strength) had no significant changes in OVX even with SAC supplementation compared to sham group (Table 3).

Aortic histological analysis: OVX and OVX+SAC groups showed normal histological aortic cross-sectional morphology (Fig. 2A) and regular contour in all tunica layers by H&E stain compared to sham. There was no difference in elastic lamellae units (Fig. 2B) by VVG stain with the continuation of arrangement and no visible fragmentation in the tunica media in all rat groups. No changes in collagen deposition by picrosirius red stain in OVX and OVX+SAC groups, although a potential increment by 13% in collagen deposition was demonstrated in OVX against sham (Fig. 2C).

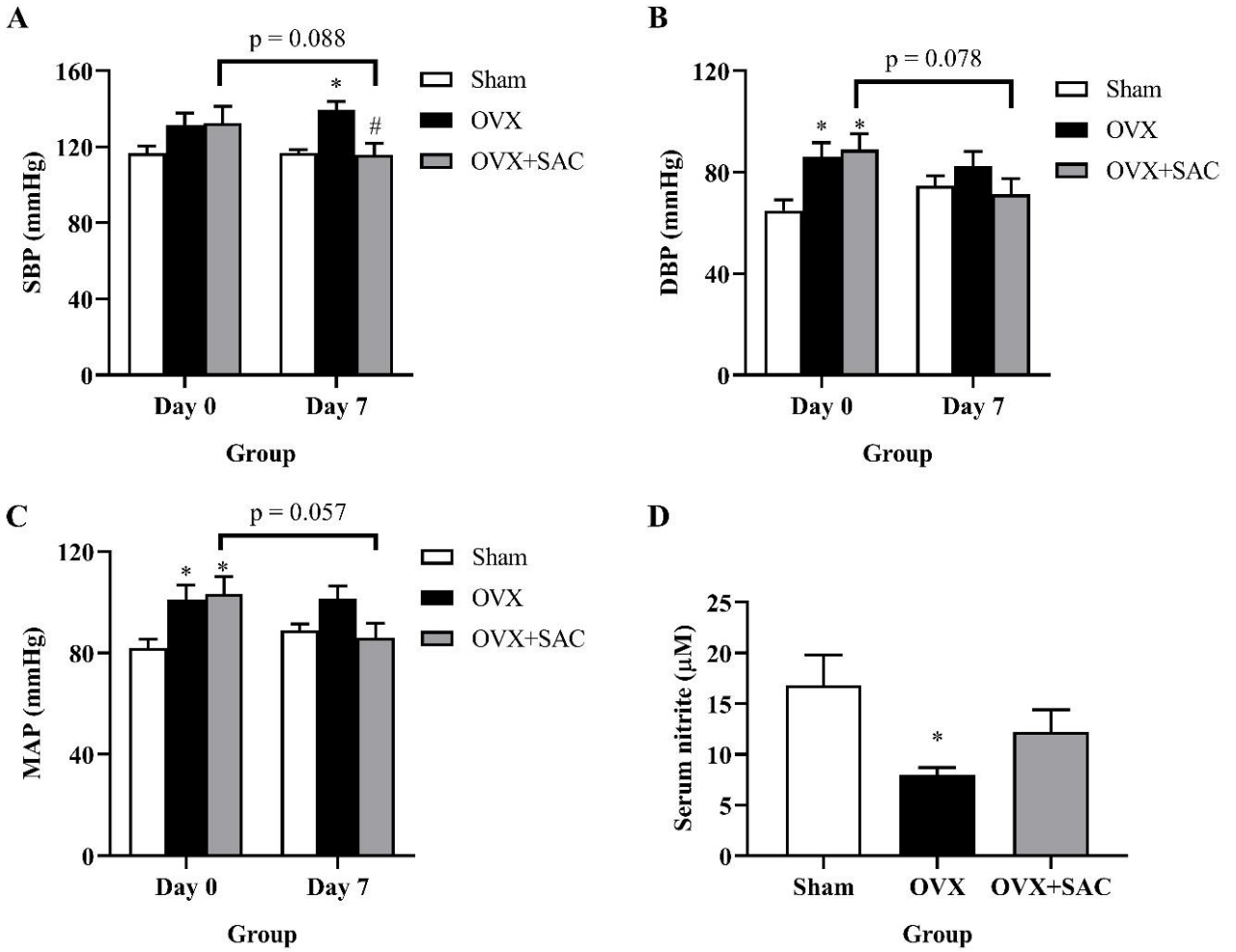

Fig. 1: Effects of OVX and SAC supplementation in SBP (A), DBP (B), MAP (C) and serum nitrite (D) (n= 6-8/group). *: significant difference (P<0.05) against sham group. #: significant difference (p<0.05) against OVX group. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

Table 1: Systemic characteristics

Parameters		Groups		
		Sham	OVX	OVX+SAC
Body weight (g)	Week 0	198.63±3.11	198.75±3.83	207.88±5.31
	Week 1	196.25±6.97	217.25±4.27*	223.50±4.63*
	Week 2	218.13±5.74	236.25±5.89	245±4.66*
	Week 3	217.88±6.46	254.50±7.52*	255.75±7.48*
	Week 4	218.13±8.06	260.50±6.80*	260.75±6.79*
Heart weight (g/body weight)		$3.3 \times 10^{-3} \pm 1 \times 10^{-4}$	$3.5 \times 10^{-3} \pm 2 \times 10^{-4}$	$3.3 \times 10^{-3} \pm 2 \times 10^{-4}$
Left ventricle weight (g/body weight)		$2.6 \times 10^{-3} \pm 1 \times 10^{-4}$	$2.7 \times 10^{-3} \pm 2 \times 10^{-4}$	$2.4 \times 10^{-3} \pm 1 \times 10^{-4}$
Kidney weight (g/body weight)		$3.3 \times 10^{-3} \pm 2 \times 10^{-4}$	$3.4 \times 10^{-3} \pm 1 \times 10^{-4}$	$3.3 \times 10^{-3} \pm 1 \times 10^{-4}$
Tibia length (cm)		3.35±0.067	3.53±0.13	3.44±0.09
Uterus weight (g)		0.82±0.15	0.30±0.02	0.30±0.05
Estradiol (pg/ml)		1199±44.91	1069±38.36	
Food intake (g)	Week 1	79.25±6.35	86.38±8.30	89.75±7.83
	Week 2	113.56±5.14	124.38±6.86	127.50±6.28
	Week 3	119.06±5.73	136.00±9.93	133.16±8.49
	Week 4	118.88±6.41	109.94±4.74	111.75±4.41
Water intake (ml)	Week 1	165.94 ±17.38	196.75 ±17.88	179.75 ±14.43
	Week 2	210.31±13.54	181.00±7.28	194.63±10.45
	Week 3	189.31±10.70	203.56±11.25	176.63±11.71
	Week 4	159.56±7.81	186.13±9.46	183.50±10.71

*: significant difference (P<0.05) against sham group.

Table 2: Antioxidant and lipid peroxidation level in the aorta and tibial bones

Parameter	Organ	Groups		
		Sham	OVX	OVX+SAC
MDA	Aorta	35.26±4.70	38.90±6.02	33.57±9.19
(nmol/g protein)	Bone	13.01±2.15	14.48±1.23	11.96±2.13
GSH	Aorta	0.13±0.007	0.12±0.009	0.13±0.018
(mmol/mg protein)	Bone	0.15±0.014	0.13±0.010	0.13±0.007

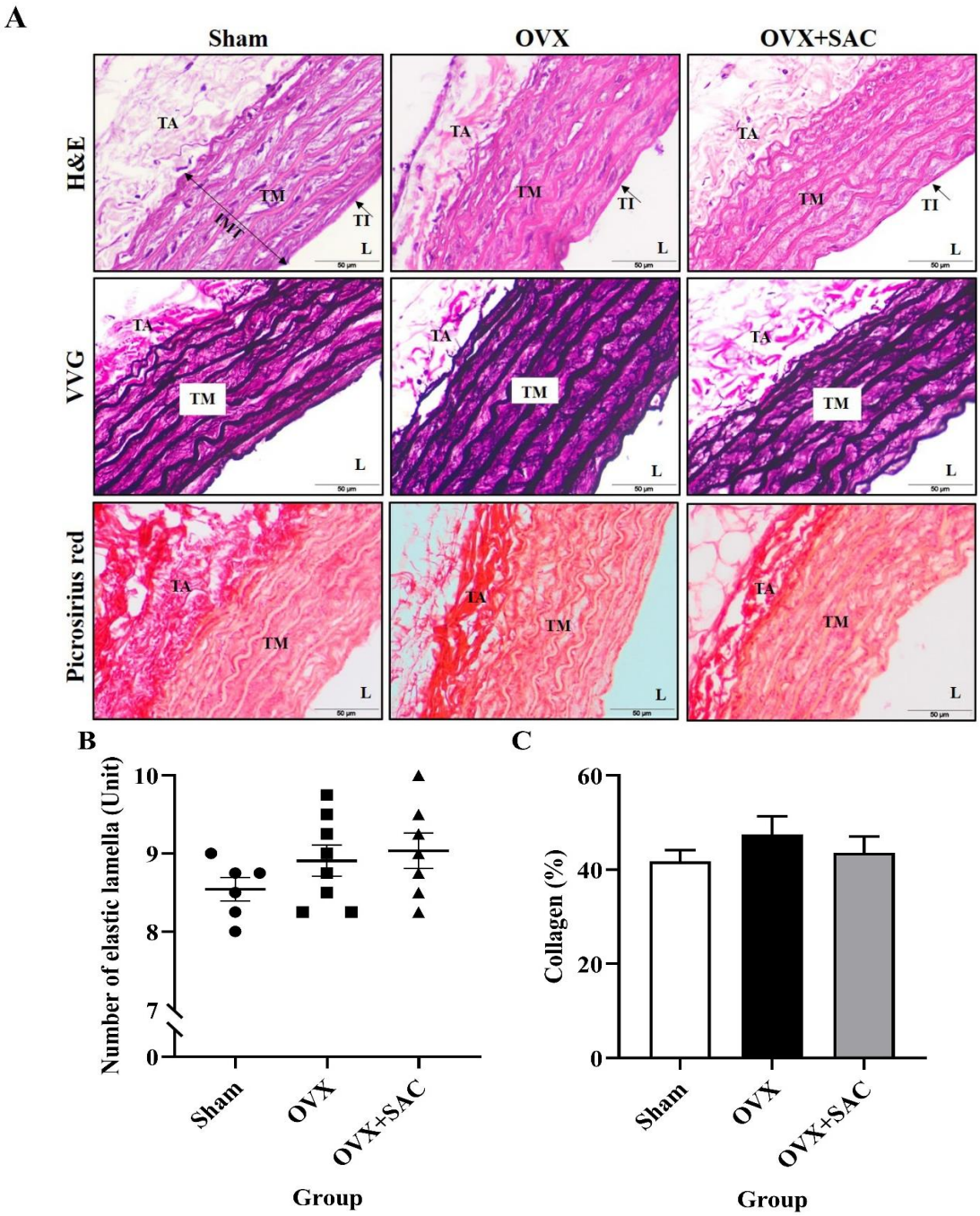

Fig. 2 Effects of OVX and SAC supplementation in aortic cross-sectional histology studies at 400x magnification (A), elastic lamellae numbers (B) and collagen deposition (C) (n= 6-8/group). L, lumen; TI, tunica intima; TM, tunica media, TA, tunica adventitia; IMT, intima-media thickness.

Table 3: Bone mineral levels and biomechanical strength in the femoral bones

Bone Parameter	Groups		
	Sham	OVX	OVX+SAC
Bone mineral content/body weight (g)	$1.4\times10^{-3}\pm9.63\times10^{-5}$	$1.3\times10^{-3}\pm9.35\times10^{-5}$	$1.3\times10^{-3}\pm4.60\times10^{-5}$
Bone mineral density/body weight (g/cm^2)	$1.3\times10^{-3}\pm5.95\times10^{-5}$	$1.1\times10^{-3}\pm3.59\times10^{-5}$	$1.1\times10^{-3}\pm3.04\times10^{-5}$
Biomechanical Strength			
Stress (N/mm^2)	2.81±0.18	2.40±0.17	2.45±0.15
Strain (%)	4.89±0.41	4.75±0.35	4.90±0.45
Displacement (mm)	0.026±0.0031	0.017±0.0010	0.018±0.0015
Maximum load (N)	0.40±0.023	0.36±0.02	0.36±0.023
Young's modulus (N/mm^2)	134.09±9.73	121.35±9.03	124.2743±21.94

Table 4: Aorta morphometric measurement

Groups	IMT (mm)	Lumen diameter (mm)	IMA (mm^2)	CWT (x10^4 dyne/cm)	TS (x10^4 dyne/cm^2)
Sham	80.31±5.15	1.28±0.07	0.35±0.04	1.02±0.03	129.4±7.44
OVX	102.80±5.00*	1.31±0.05	0.46±0.04	1.24±0.07*	117.9±12.08
OVX+SAC	103.80±5.78*	1.33±0.05	0.47±0.04	1.16±0.05	114.3±4.99

*: significant difference (P<0.05) against sham group after 7 days of treatment. IMT, intima-media thickness; IMA, intima-media area; CWT, circumferential wall tension; TS, tensile stress

Aorta morphometry study: Thicker IMT ($P<0.05$) and a tendency of increased IMA was observed in OVX and OVX+SAC groups compared to sham. Besides, OVX showed an increase ($P<0.05$) in CWT while there was no difference in CWT in OVX+SAC against sham group. Also, no changes in aortic lumen diameter and TS were shown in all rat groups (Table 4).

DISCUSSION

Food from plants have been widely used for disease treatment and prevention, including garlic or *Allium sativum* (Bayan *et al*., 2014). SAC is a stable water-soluble compound from garlic with various health-promoting properties (Colín-González *et al*., 2012). To our knowledge, this is the first time SAC was tested in a postmenopausal animal model.

Animal OVX involves ovaries removal surgically to mimic human menopause, which is easy to perform and cost-effective with a low level of estrogen attained in a short duration of time (Medina-Contreras *et al*., 2020). Part of its success is determined by body weight increment after the surgery (Yousefzadeh *et al*., 2020), as shown in our findings. Estradiol is frequently referred to as estrogen as it is the most biologically active form of estrogens with physiological importance (Fuentes and Silveyra, 2019). Nevertheless, insignificant estradiol reduction after a 3-week post-OVX recovery might be from the adiposity increment that causes increased body weight by *ad libitum* food supply during the study, which may cause higher estradiol production from adipocyte (Marks *et al*., 2013). However, OVX showed a marked reduction in uterus weight that was also an essential parameter for surgery success (Yousefzadeh *et al*., 2020). Low uterus weight indicates the uterus atrophy after bilateral ovariectomy and SAC treatment did not result in the anti-atrophic effect of the uterus after OVX.

Clinically, estrogen-deficient women have approximately 0.8 kg annual weight gain with body fat accumulation (Lee and Kim, 2001) that can also be achieved in OVX rat model as estrogen deficiency is a factor that triggers obesity, although its exact mechanism in causing weight gain remains uncertain (Yoon *et al*., 2015). Our results showed that SAC supplementation did not affect the body weight increment in OVX rats with no changes in food consumption. As this is the first time SAC was tested in a postmenopausal condition, our results contradict a study by Asdaq (2015) where 32.76 mg/kg SAC supplementation for 5 days yielded a significantly lower body weight in a high-fat diet rat model from the hypolipidemic effect and oxidative stress attenuation by SAC. Therefore, it would be beneficial to consider the lipid profile and its metabolism status to evaluate SAC's impact in the future that might influence the body weight and adiposity in OVX model.

In the present study, OVX caused an increment in SBP which is highly related to endothelial dysfunction characterized by low serum NO bioavailability. Estrogen deficiency impaired the cardiovascular system's protection by dysregulation of renin-angiotensin pathway and endothelial-derived vasodilators (Delgado *et al*., 2017; Sabbatini and Kararigas, 2020). SAC supplementation in OVX rats lowered SBP, which may be associated with its potential augmentation in NO production. These findings are consistent with Brahmanaidu *et al*. (2017) that showed increased NO level in a diabetic rat model after 150 mg/kg SAC supplementation that was postulated from higher endothelial nitric oxide synthase (eNOS) expression to alleviate endothelial dysfunction.

OVX increases reactive oxygen species (ROS) production that hasten lipid peroxidation activity in the cell membrane component of polyunsaturated fatty acids, resulting in a higher MDA level (Lemini and Franco, 2005). Our study suggests an increasing pattern in aortic and bone MDA levels in OVX rats with the potential of SAC to reduce its levels as sham. In addition, the bone and aortic GSH levels were unaffected in this model with or without SAC supplementation after OVX. Surprisingly, our results are not in agreement with previous studies where SAC is known to boost the antioxidant system in animal models (Saravanan and Ponmurugan, 2013; Zainalabidin *et al*., 2020) related to GSH as SAC augments the enzymes involved in GSH production via Nrf2-ARE pathway activation. Also, intracellular GSH is closely associated with hydrogen sulfide (H_2S) production to enhance cysteine transport that contributes to cell's protection from oxidant stress (Rodrigues and Percival, 2019). Therefore, future assessment in H_2S level should be considered to support the insignificant GSH levels in OVX rats.

Four weeks of post-OVX did not cause postmenopausal osteopenic changes to the rats, as femoral bones needed a longer time for BMD reduction (Yousefzadeh *et al*., 2020). SAC supplementation in OVX rats also showed no significant differences against sham in bone mineral properties, all extrinsic (load) and intrinsic biomechanical parameters (stress, tension, elasticity, and Young's Modulus). The insignificant estrogen reduction may be partly responsible for the non-osteopenic postmenopausal changes. To date, there are minimal studies that evaluate the effect of SAC on bone health. Nevertheless, Uddandrao *et al*. (2019) showed that 150 mg/kg SAC for 45 days managed to improved bone mineral properties in a diabetic nephropathy rat model related to the upregulation in antioxidant gene mRNA and protein expressions. Hence, a longer duration in post-OVX and SAC treatment can be considered for postmenopausal osteopenic impact.

Endothelial dysfunction may alter aortic mechanical properties from disproportionate composition in extracellular matrix (ECM) components in the estrogen-deficient state (Lino *et al*., 2018). Passive aortic stiffness may occur in hypertension by ECM shift of aortic mechanical properties from elastin to stiffer collagen fibers (Wagenseil and Mecham, 2012). Our findings noted these changes as OVX showed a tendency of higher aortic collagen deposition, which may indicate early changes to aortic stiffness. However, SAC supplementation caused no significant aortic collagen deposition amongst OVX rats alongside with unaltered elastic lamellae unit. Although there is currently limited study that relates the effects of SAC against the cardiovascular system, Zainalabidin *et al*. (2020) showed SAC's potential in reducing post-myocardial infarction fibrosis activity in rats via attenuation in fibrotic gene expression and oxidative stress.

Furthermore, increased IMT and CWT from the morphometric study suggest a secondary aortic remodeling to hypertension in OVX rats. Hypertension results in the rise of intraluminal pressure, which may increase CWT that can further contribute to aortic thickness. Aortic wall thickness increased primarily because of medial thickening, presumably due to the compensatory process to prevent the increase in arterial diameter and TS (Siti *et al*., 2017). The SBP-lowering effect from SAC supplementation in OVX rats results in normal CWT that was probably from SAC's potential in augmenting NO production as a vasodilator. Therefore, SAC-treated postmenopausal subjects might have a protective effect against aortic remodeling from the rise in SBP.

Conclusions: Our study showed that OVX caused high SBP, at least in part from low NO production, resulting in secondary aortic remodeling in a postmenopausal animal model. SAC supplementation reduced SBP in OVX rats by its potential effect improving NO level. Therefore, further studies should be warranted to understand better SAC mechanisms towards blood pressure improvement in the postmenopausal animal model.

Acknowledgements: This work was supported by Fundamental Research Grant Scheme from the Ministry of Higher Education, Malaysia (FRGS/1/2020/STG03/ UKM/02/2).

Author's contribution: Conceptualization: SZ; Investigation and methodology: RMH, NFA, UNS, NANA; Supervision and methodology: SZ, YK, CKY; Formal analysis and writing (original draft): RMH, UNS, NANA; Writing (review & editing): RMH, NFA, UNS, NANA.

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