HIBISCUS EXTRACT MITIGATES SALT INDUCED CAROTID ADVENTITIAL CHANGES IN RATS

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ABSTRACT
The tunica adventitia is an active vascular compartment that actively participates in modulation of vascular structure, function and pathophysiology. Adventitial thickness has recently been accepted as a surrogate marker of atherosclerosis. The effects of salt and chemicals that ameliorate those effects are important in understanding vascular structure, function and pathology. There are few studies on hibiscus and high salt induced vascular pathology. This study, therefore, investigated the effects of hibiscus on salt induced vascular changes on rat carotid artery. The experimental animals were divided into 3 groups of 8 animals each – (i) controls; (ii) high salt diet alone and (iii) high salt + hibiscus extract for a period of eight weeks. At ages 2, 5 and 8 weeks 2 – 3 animals were sacrificed for study. They were anaesthetized with ether and perfused with formal saline. Specimens were then obtained from the middle of common carotid artery, fixed in 5% formaldehyde solution, processed routinely for paraffin embedding and 5-micron thick sections stained with Hematoxylin / Eosin and also with Mason’s Trichome/ Aniline blue. Adventitial thickness and volumetric densities of collagen were measured using morphometric techniques. High salt consumption induced statistically significant increase in adventitial thickness from 297.45µm at week 2 to 659.4µm in week 8. In hibiscus fed rats, this increase progressively reduced to 482.55µm in week 8. Volumetric density of collagen was 57% in high salt fed rats but reduced to 45.66% in hibiscus fed rats (p<0.001). The increase in tunica adventitial thickness and collagen density which is induced by high salt can be mitigated by hibiscus extract. This implies that hibiscus has potential to restore salt induced vascular injury. Further studies are recommended to refine the extract.

Keywords: adventitial thickness, high salt, hibiscus, collagen, density

INTRODUCTION
Tunica adventitia is an active vascular compartment which participates in vascular homeostasis and pathophysiology (Ogeng’o et al., 2017). Adventitial thickness is now an accepted marker of vascular pathology (Skilton, 2009; 2011; 2012). Identification of substances that ameliorate injury induced increase in adventitial thickness is important in understanding, vascular structure, function, pathophysiology and mitigating vascular disease. Studies reveal that in rats fed on high salt diets, there is arterial wall stiffening attributable to increased collagen deposition (Safar et al., 2000). Further, a high salt diet has been reported to result in adventitial inflammation as a result of activation of pro-inflammatory monocytes and macrophages which produce cytokines that inhibit collagen degradation and promote collagen deposition in the tunica adventitia (Sullivan, 2009; Kanbay et al., 2011). Reduced collagen turnover in turn results in adventitial thickening which has been correlated with increased risk of developing atherosclerosis and other cardiovascular diseases [CVDs] [Skilton et al., 2009]. Hibiscus sabdariffa extract (HSE) is commonly used as a traditional remedy that provides an effectively manageable diet-imposed treatment that is low on cost and easily available (Seujange et al., 2013; Rawat et al., 2016). It is known to confer cardioprotective effects, partly through its...
antihypertensive actions (Onyenekwe et al., 1999; Sireeratawong et al., 2013; Serban et al., 2015). Consequently, it is important to understand further, the mechanisms of this cardioprotection. The common carotid artery (CCA) is one of the most commonly afflicted arteries. This study therefore examined the effects of HSE on salt induced adventitial thickness in CCA.

MATERIALS AND METHODS

This was experimental study involving administration of high salt and hibiscus to examine their effects on the rat common carotid artery. High salt diet was prepared by adding 77 grams of sodium chloride (NaCl) to 723 grams of standard rat chow to make 8% NaCl diet (Seujange et al., 2013). Hibiscus Sabdariffa Extract (HSE) was prepared from dry dark red calyces of the plant. 30 grammes of the calyces were immersed in boiling water 30 minutes. The mixture was filtered, and the filtrate allowed to evaporate leaving a dark red powder (Yield: 55%) which was then dissolved in distilled water at room temperature. It was administered by gavage. 24 two-month-old experimental animals, that is albino rats (Rattus norvegicus) were obtained from the Department of Biochemistry University of Nairobi. Ethical approval for the study was obtained from the BioSafety, Animal Use and Ethics Committee, Faculty of Veterinary Medicine, University of Nairobi, approval number: FVB/BAUE/2017/130. The rats were randomly selected by simple random sampling and marked 1-24. The numbers were then fed into a random number generating software. From this, 8 random numbers were obtained for each of the 3 groups (A – C). Group A was fed on a diet containing 8% sodium chloride, Group B was fed on 8% sodium chloride and HSE by gavage and Group C (the control group) was fed on standard pellets. Feeding was evaluated by a feed efficiency ratio to determine how much food each rat consumes. Water was provided ad libitum.

The first 2 numbers generated for the control group were euthanized at day zero to determine the baseline histomorphometric variables. Two rats from the 3 groups were euthanized at week 2. Six experimental animals and 2 controls were then perfused at week 5 and week 8 from the start of the experiment (Table 1).

<table>
<thead>
<tr>
<th>Table 1: Timing for perfusion and harvesting</th>
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<td>Group</td>
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<td>High salt-fed</td>
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<td>High salt-fed + hse</td>
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<td>Controls</td>
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<td>Total</td>
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After perfusion, the CCAs were harvested by extending the midline body incision up to the neck region and reflecting the skin flaps laterally. The right CCA was identified at its junction with the brachiocephalic trunk and the right subclavian artery and the left was identified branching from the arch of the aorta. The whole length of the artery was harvested on both sides up to just before the bifurcation into internal and external carotid arteries. The rats’ whole common carotid arteries were fixed in 10% formal saline for at least 24hrs. 1mm long samples obtained from the middle of the specimen were dehydrated in increasing grades of alcohol, cleared in cedar wood oil and embedded in paraffin wax. Five-micron thick sections were deparaffinized in xylene,
rehydrated and stained with Hematoxylin/Eosin and Mason’s trichome. The slides were examined with a light microscope. Photomicrographs of the sections were taken using a high-resolution Canon digital camera (12 megapixels) mounted on a photomicroscope. These photographs were entered into Fiji Image J software (NIH image program) for morphometric and stereological analysis.

Five different 5 μm sections were sampled from each animal by simple random sampling. Photomicrographs were taken from each section and examined at a magnification of X400. The adventitial thickness (AT) was measured by tracing four random points on the wall of the CCA over the adventitial zone in a photomicrograph of each section [Figure 1]. The average measure was then calculated from the four points as shown below.

\[
AT = \frac{(AT_a + AT_b + AT_c + AT_d)}{4}
\]

Volumetric densities of collagen in the tunica adventitia were estimated based on the Cavalieri principle of point counting (Mandarim-de-Lacerda, 2003). Five different 5 μm sections were sampled from each animal by simple random sampling and 4 fields from each section examined at a magnification of X400. Images from these fields were captured and by the methods described by Gundersen et al. (1988) and Bancroft and Cook (1994), an 80-point grid was superimposed on the digital images in order to analyse the selected areas. Point probes from the grid system facilitated estimation of the volumetric densities for collagen distribution.
The volume densities for collagen were then calculated by the formula $V_v = P_p / P_t$, where $V_v$ is the volume density, $p$ is the adventitial collagen, $P_p$ is the number of test points associated with $p$, and $P_t$ is the total number of points of the test system that touched the tunica adventitial zone. This was done while unaware of the source of the tissues.

Morphometric data on carotid artery thickness was entered into the Statistical Package for Social Sciences (SPSS) software (version 21.0, Chicago, Illinois) for coding, tabulation and statistical analysis. Measurements are expressed in micrometers. The data are grouped into three; Group A, B and C. Normality of data was determined using histograms and box plots. Kruskal-Wallis H test was done to compare adventitial wall thickness and collagen fiber density among the groups. Dunn’s test was then applied for pair wise comparison. A p-value $\leq 0.05$ was considered statistically significant at a 95% confidence level.

**RESULTS**

The AT was seen to increase in all the groups across the total duration of the study [Figure 3]. There was a statistically significant increase in AT from week 2 [Figure 4A] to week 8 [Figure 4C] in the control group. The AT in the control group was 238.19μm at week 2 and 416.82μm at week 8. A similar statistically significant increase was seen in the experimental groups. In the high salt group, the AT increased from 297.45μm at week 2 to 659.4μm at week 8 [Figure 4B] whereas in the high salt with HSE group it was reduced to 482.55μm by the 8th week. Between the 5th and 8th week the increase in AT was slowed in the high salt with HSE group and control group. Concurrent administration of HSE with high salt loading led to a less pronounced increase in AT [Figure 4D] which was comparable to the normal control. At the 8th week the differences among the three groups were statistically significant.

All the experimental groups showed apparent increase in collagen density throughout the study duration [Figure 5]. Changes in the control group were not statistically significant between the 5th and 8th week [figure 6A and C]. The High salt group however had the highest collagen density by week 8 of 57% [Figure 6B] which was a statistically significant increase from the adventitial collagen density in week 2 of the same group (p<0.001). The HSE + High salt group had lower collagen density (45.76%). Although there was marked increase in the adventitial collagen density [Figure 6D], this was significantly different from the High salt group (p<0.001). Volumetric density of collagen in the tunica adventitia is represented below [Figure 5].
Figure 3: Line graph showing trends in adventitial thickness in control and experimental groups.

Figure 4A- D: Photomicrographs showing changes in the Adventitial thickness of the common carotid artery with time in control and experimental groups. Hematoxylin/Eosin Stain x 400. AT = Adventitial Thickness; IMT = Intima Media Thickness. A: The control group at week 2 of the study. B: High salt group at week 8 of the study. Notice the prominent adventitial thickness (AT). This represents an increase in AT when compared to figure 4A. C: The control group at week 8 of the study. Notice the adventitial thickness (AT) almost comparable to that of week 2 and before intervention. D: Hibiscus sabdariffa extract (HSE) + high salt group at week 8 of the study. Notice the AT is reduced when compared to the high salt group at week 8 [figure 4B].
**Changes in Adventitial Collagen Density in Control and Experimental Animals**

![Figure 5](image)

**Figure 5:** Volumetric density of adventitial collagen in the control and experimental groups. (Mason’s Trichrome/aniline blue stain x400. A: The control group at week 2 of the study. Notice the collagen bundles (asterisks) in the tunica adventitia with numerous spaces in between. B: The high salt group at week 8 of the study. Notice the dense collagen bundles (asterisks) in the extensive tunica adventitia with few spaces in between. The extensive collagen represents a significant increase compared to figure A and C. C: The control group at week 8 of the study. Notice the collagen bundles (asterisks) in the tunica adventitia with numerous spaces in between. This is closely comparable to figure A. D: HSE with high salt group at week 8 of the study. Notice the collagen bundles (asterisks) in the tunica adventitia with numerous spaces in between. This represents a significant reduction when compared to figure B.)
DISCUSSION

Observations of the current study reveal that high salt intake increases adventitial thickness (AT) and collagen density. Both effects are attenuated by concomitant ingestion of HSE. Adventitial thickening commonly occurs with aging (Fleenor, 2012) and development of atherosclerosis (Ogeng'o et al., 2014b). The present study demonstrates a statistically significant increase in AT in the high salt fed rats. This is similar to the findings of Saka et al., (2016) on the aorta of adult Wistar rats. The AT attributable to invasion of macrophages and other inflammatory cells and release of pro-fibrotic cytokines, transforming growth factor [TGF - β] and Tumor Necrosis Factor α (TNF α) which stimulate adventitial fibroblasts to synthesize and secrete collagen fibres and ECM (Sullivan et al., 2009; Fleenor, 2012; Kanbay et al., 2011; Dai et al., 2016). Further, the TGF-β inhibits matrix metaloproteinases (MMPs) responsible for extracellular matrix degradation (Risinger et al., 2010) and collagen fiber degradation (Ferreira-sae et al., 2011)

Conceivably, if the pro-inflammatory process induces adventitial thickening in atherosclerosis, it may be associated with a high salt diet with onset of atherosclerosis through the same process (Dai et al., 2016).

The HSE reduced the high salt induced adventitial thickening, similar to the effect of garlic extract (Saka et al., 2016). The possible mechanisms may include the extract’s stimulation nitric oxide synthase of and its modulation of endothelial TGF-β, reducing oxidative stress and lowering TGF-β production which effectively checks pro-fibrotic activity of the induced cytokines in high salt diet (Sanders, 2009b).

Adventitial thickening together with IMT, provide a more complete picture in the assessment of atherosclerosis (Skilton et al., 2011; Ogeng'o et al., 2014) and has become a target for therapeutic intervention, (Skilton et al., 2012). Accordingly, the current study underpins the potential for use of HSE in mitigating CVD, in the background of high salt intake.

Concomitant ingestion of HSE significantly attenuated the high salt induced increase in TA collagen density. This is consistent with antifibrotic effects of HSE (Seujange et al., 2012) which are probably mediated through its antioxidant effects which upscale production of nitrous oxide (Sanders et al., 2009b) and the inhibition of fibrosis inducing effects of TNF – α and TGF – β (Chen et al., 2014; Lan et al., 2013; Meng et al., 2015). This attenuation of salt induced TA fibrosis may provide part of the explanation for the antihypertensive effects of HSE (Serban et al., 2015) and underpin its potential in the prevention of CVD including atherosclerosis.

In conclusion Hibiscus extracts ameliorates high salt induced adventitial thickening. This implies that it has therapeutic potential in management of atherosclerosis. Further studies are recommended.
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REFERENCES