Research Article

Black Seed (*Nigella sativa)* Oil Restores Smoke or Nicotine-Induced Vascular Impairment via Improvement in Endothelium-Dependent Relaxation: Role of Nitric Oxide Synthase and Voltage-Sensitive Potassium Channels

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ABSTRACT

Background: Despite the overwhelming evidence linking smoking and nicotine intake with vascular function impairments, the mechanisms involved and the possible ameliorative effect of black seed (*Nigella sativa* (NS)) oil administration are not clearly understood. This study sought to determine the involvement of nitric oxide synthase and voltage-sensitive potassium channels in the modulation of vascular reactivity in cigarette or nicotine-exposed rats treated with NS oil. Methods: Thirty male Sprague-Dawley rats were divided into 6 groups comprising vehicle control (Control), NS oil only (NS), Smoke only (SMK), Smoke + NS oil (SMKNS), Nicotine only (NCT) and Nicotine + NS oil (NCTNS). Animals were either passively exposed to cigarette smoke or nicotine vapour for 12 weeks, however, NS oil treatment commenced from 9th-12th week orally. At the end of the 12-week experimental period, vascular reactivity to norepinephrine (NE), acetylcholine (ACh) and sodium nitroprusside (SNP) were assessed with or without the presence of L-nitro-arginine (LNA) or 4-Amino-pyridine (4AP). Results: Percent contractile response to NE was higher (p < 0.01) while relaxation response to ACh was lower in the SMK and NCT (p < 0.05) groups. LNA-induced inhibition to ACh was significantly reduced in both SMK and NCT groups. 4AP-induced inhibition to ACh was significantly increased only in the NCT group. 4AP-induced inhibition to SNP was increased in SMK group. NS oil reduced only contractile response to NE in NCT group. It also significantly improved relaxation response to ACh as well as restored LNA-induced inhibition to ACh in the SMK and NCT groups. Interestingly, while NS oil reduced 4AP-induced inhibition in the NCT group, it reduced 4AP-induced inhibition to SNP in the SMK group. Conclusion: NS oil ameliorates vascular dysfunction by reducing contractile response in rats exposed to nicotine vapour while increasing endothelium-dependent relaxation as well as restoring differentially both LNA- and 4AP-induced inhibition in rats exposed to cigarette smoke and nicotine vapour.

Introduction

Smoking remains the most important preventable risk factor for the development of many common cardiovascular diseases (Jamal *et al*., 2018). It has been estimated to kill about 7 million direct users and more than a million non-users via second-hand smoke (SHS) inhalation annually (Rao *et al*., 2020). Several mechanisms have been reported to be responsible for the high mortality in smokers (Daiber *et al*., 2017), the most important of which is endothelial dysfunction via altered vascular reactivity (Daiber *et al*., 2017). The endothelium plays a central role in the control of vascular tone via regulated release of vasoconstrictors and vasodilators (Gori *et al*., 2012).

The desire to inhale nicotine, the main active constituent of cigarette smoke, is the main reason why humans smoke tobacco and their alternatives (Benowitz and Gourlay, 1997). Nicotine however does not only cause addiction but also enters the blood stream along with several other toxic chemicals to cause many of the widely reported negative health effects (Moghe *et al*., 2015), including high blood pressure and stroke risks (Escobedo and Zack, 1996). Nicotine is widely known to also cause enhanced release of catecholamines which in turn results in many adverse cardiovascular effects (Benowitz, 1988) including arrhythmias (Escobedo and Zack, 1996). As a matter of fact, while electronic cigarettes were invented to ameliorate some of the negative health effects of the traditional cigarette smoke as they were assumed to be safer (Kaisar *et al*., 2016), their use has been reported by several authors to not only worsen addiction but also cause alteration in vascular reactivity thereby leading to several cardiovascular diseases and death especially among vapers (Waziry *et al*., 2017; Qasim *et al*., 2019).

While Zhang *et al*. (2006) hinted at the modulatory effect of cigarette smoke exposure on L-arginine transport and by extension the impairment of nitric oxide pathway in human arteries and veins (Tanriverdi *et al*., 2006), disruption of the nitric oxide pathway was recently confirmed by a marked reduction in endothelial nitric oxide synthase gene expression (Ardiana *et al*., 2021). Moreover, earlier studies had indicated the ability of nicotine from cigarette smoke to alter action potential characteristics in many tissue types (Tanriverdi *et al*., 2006) as well as block the voltage sensitive potassium channels especially in vascular smooth muscle cells following exposure to cigarette smoke (Wang *et al*., 1999). The voltage-sensitive potassium channels act as physiologic brakes and are the main regulators of vasoconstriction in vascular smooth muscle cells (Halliday *et al*., 1995). Nicotine from cigarette smoke has also been reported not to only block potassium channels (Wang *et al*., 1999) but to also cause dual effects on the potassium channels present in the rat vascular smooth muscle cells (Tang *et al*., 1999). It is therefore conceivable that it could have a role to play in the alteration of key enzymes and ion channels involved in the control of vascular reactivity especially in smokers or vapers. While endothelial nitric oxide synthase helps to convert L-arginine to nitric oxide, a potent vasodilator, the voltage-sensitive potassium channels (A-type K^+ current) are involved in the control of membrane repolarization and vascular function (Wang *et al*., 1998).

Nigella sativa (NS) oil is from a plant of the Ranunculaceae family (Ahmad *et al*., 2013). The oil consists of linoleic, palmitic, oleic and other fatty acids with the main active ingredient being thymoquinone (Leong *et al*., 2013). The oil has been reported by several studies to have vasorelaxant (Niazmand *et al*., 2014), antihypertensive (Leong *et al*., 2013), hypotensive (Fallah-Huseini *et al*., 2013), antihyperlipidemic (Ahmad and Beg, 2013), and ameliorative effects on endothelial dysfunction (El-Saleh *et al*., 2004). Considering the important role of the nitric oxide pathway in vascular function and the fact that the voltage-sensitive potassium channel type is involved in many cardiovascular diseases and also a special target for many drugs that modulate vascular functions (Wang *et al*., 1999), this study sought to determine the likely modulatory effects of exposure separately to either cigarette smoke or its active component, nicotine, on vascular reactivity with special focus on the role of the nitric oxide synthase enzyme and voltage-sensitive potassium channels. We also sought to determine the possible ameliorative effect of NS oil on vascular reactivity under this condition.

Methods
Experimental animals
Thirty (30) male Sprague-Dawley rats of weight range 80 to 100g and about 4-5 weeks old were obtained from the animal facility of the Faculty of Basic Medical Sciences, College of Medicine of the University of Lagos. The rats were fed normal rat chow *ad libitum*. The rats had free access to water. They were housed five per cage under a 12-hour dark-12-hour light cycle in room temperature (30 ± 2ºC) and humidity (55 ± 5%) controlled animal room. Before the commencement of the study, the rats were acclimatized in the new room for a week. Ethical approval was obtained from the College of Medicine of the University of Lagos Animal Care and Use Research Ethics Committee (CMUL-ACUREC) with registration number: CMUL/HREC/08/19/568. All animal experiments complied with and were carried out following the National Institutes of Health guide for the care and use of Laboratory animals (NIH, 1996).

Study design
Using computer generated numbers, Sprague-Dawley rats were randomly assigned into 6 groups as illustrated in Table 1 below:

during the last 4 weeks (9th to 12th week) of the experimental period.
Experimental procedures
Exposure Chamber
The exposure chamber consisted of two Plexiglas chambers (l = 40 cm × b = 25cm × w = 30 cm) each with a door (10 cm × 10 cm) in dimensions. The two chambers are linked by a central connecting chamber (10 cm × 40 cm × 10 cm) which also has a (10 cm × 10 cm) door through which the animals were introduced into either side of the chamber. The first side was designated to be the non-preferred side and consisted of white-colored walls with a rough, black floor. On the wall of the first side is an extractor, with power rating of 1200 rpm to prevent excess accumulation of the vapour in the chamber. Also present in the chamber is a Multigas MSA detector (ALTAIR5X) containing a gas analyzer sensor which is used to ensure carbon monoxide level in the chamber is kept between acceptable 150 to 250 ppm during the exposures. The Multigas MSA detector also helped ensure the other gases like sulphur IV oxide and methane were maintained at zero while oxygen was maintained at 20.8%. The other side of the chamber was the dark side designed and designated as the preferred side.

Group (Abbreviation)	Exposure (duration)	Treatment (duration)
Control (Ctrl)	No exposure	No treatment
Nigella sativa oil (NS)	No exposure	4 weeks treatment with oil (9th to 12th week)
Smoke exposed (SMK)	12 weeks smoke exposure (1st to 12th week)	No treatment
Smoke exposed + oil (SMKNS)	12 weeks smoke exposure (1st to 12th week)	4 weeks treatment with oil (9th to 12th week)
Nicotine-exposed (NCT)	12 weeks nicotine exposure (1st to 12th week)	No treatment
Nicotine-exposed + oil (NCTNS)	12 weeks nicotine exposure (1st to 12th week)	4 weeks treatment with oil (9th to 12th week)

Table 1: Grouping of animals

Rats administered with NS oil were given (10 mls/kgbw), orally once daily (Kanter *et al*., 2005)

The side has a smooth floor and a small vent through which the side is aerated.

For smoke exposure, the system consists of a pump (air pump) that has a direct connection to a syringe-system that blows air across the already lit cigarette. The air flow moves backward such that only filtered smoke gets into the chamber which directly mimics the kind of smoke inhaled by smokers in a passive whole-body exposure system.

For the nicotine vapour exposure, nicotine vapour was generated by bubbling air generated by an aerator (air pump) operating at a flow rate of 30 liters per minute through an Airistech vaporizer containing a solution of vaporized nicotine concentrations (80 mg/ml (FEELLiFE Vanilla Orange)) for 12 weeks (60 min/session/day) into the chamber. Nicotine vapour was produced by heating the nicotine solution to a temperature of 200ºC (392ºF) by the vaporizer. While there were other concentrations, it was observed that smokers preferred the 80 mg/ml concentration and that informed the choice of the concentration to mimic the likely effects observed in most smokers. Pure cotton wool was used to soak about 2mls of the pure nicotine and inserted into the vaporizer during each session that lasted for an hour per day for the two concerned groups of rats. Nicotine concentrations were selected putting the following factors into consideration: (1) the nicotine concentration (0-30 mg/mL) usually found in commercial e-cigarette liquids (Goniewicz *et al*. 2013), (2) companies' recommendation of e-cigarette liquids up to 60-100 mg/ml nicotine level; and (3) reported rapid metabolism of nicotine in rats compared to human smokers (Matta *et al*., 2007). Identical chambers with controlled untreated air were used as the Control group. These chambers were either used for nicotine vapour exposure with the door closed during exposure or with the doors free to open during conditioned place preference assessment.

Determination of Plasma Cotinine Concentration
Collection of blood samples for cotinine (a nicotine metabolite) measurement in all groups of rats was performed on the last day of exposure. Plasma (150 µL) was separated by centrifugation at 3000 rpm for 15 min and then stored at −80 °C until time for analysis. The concentration of cotinine was determined with Cotinine Direct ELISA (MBS580061), MyBioSource Inc., San Diego, CA 92195) according to the manufacturer's instructions.

Isolation and preparation of aortic rings
Rats were sacrificed by cervical dislocation to reduce pain to the barest minimum. The thoracic cage was cut open and heparin injected into one of the ventricles to prevent clotting of blood in the vessels. The abdominal aorta was excised (from the lower part of the diaphragm to the bifurcation of the iliac arteries), and quickly placed in a round Petri dish containing physiological salt solution (PSS). The PSS was maintained at 4°C and consisted of 119.0 mM/L NaCl, 4.7 mM/L KCl, 1.2 mM/L KH_2PO_4, 1–2 mM/L $MgSO_4$, 24.9 mM/L $NaHCO_3$, 1.6 mM/L $CaCl_2$, and 11.5 mM/L glucose. The abdominal aorta was cut into 2.5 mm ring segments. Care was taken to avoid rubbing the endothelial surface during the removal and mounting of the rings. Each ring was then mounted horizontally between two fine stainless-steel rods. The lower rod was connected to the base of the organ bath, while the upper part of the rod was attached to the clamp of the micropositioner, while the thread was attached to the isometric force transducer (top force transducer MLT 050/D; AD Instruments, Bella Vista, Australia). This was used in recording the force displacement by the tissue. The rings were perfused in a 20 ml double-jacketed organ bath with PSS at 37 °C and gassed with 95% O_2: 5% CO_2 mixture. The pH of the PSS was usually 7.4, and all baths used simultaneously had a parallel connection to the source of the PSS.

After mounting the rings, a passive tension of 2g was applied by adjusting the knobs on the micropositioner. The ring was then allowed to equilibrate in the PSS for 60 minutes during which each ring was subjected to a sub-maximal dose (10^{-6} mol/L) of noradrenalin at 30 min intervals (three times). Each of the stabilizing stimulations lasted for 5 minutes, after which the ring was rinsed with PSS. At the end of the period, the relaxation response study to acetylcholine (ACh) was assessed in endothelial intact aortic rings. Aortic rings were pre-contracted with 10^{-5}M noradrenalin, and after the contraction had reached a plateau, cumulative doses of ACh (10^{-9}–10^{-4} mol/l) were added to the organ bath while the recordings were taken continuously. Involvement of endogenous nitric oxide (NO) production and voltage-sensitive potassium channels in the relaxation response of abdominal aorta to ACh in all the groups were studied by using the endothelial nitric oxide

synthase (eNOS) inhibitor L-nitroarginine methyl ester (L-NAME) and 4-amino-pyridine (4AP). In separate experiments, the rings were incubated with L-NAME (10^{-4} M) and 4AP (10^{-5} M) for 20 minutes. After the 20 minutes incubation period, the rings were pre-contracted with 10^{-5} mol/l noradrenalin, after which cumulative doses of ACh (10^{-9}–10^{-4} mol/L) were added to the organ bath. Endothelium independent relaxation was assessed in endothelium-denuded rings with or without the above-mentioned inhibitors using sodium nitroprusside (SNP). The percentage relaxation and the negative logarithm to base 10 of the EC_{50} (50% of Emax (EC_{50}) were determined from the concentration response curve.

Chemicals and drugs
Noradrenalin, acetylcholine (ACh), sodium nitroprusside (SNP), methylene blue (MB) and L-NAME were dissolved and diluted in distilled water. Noradrenalin, ACh, and SNP were obtained from Sigma–Aldrich (St. Louis, Mo., USA).

Statistical analyses
The collected data were expressed as means ± SEM. The data were analyzed using one-way ANOVA. The Students–Newman– Keuls (SNK) post-hoc test was used to identify differences between individual means. The confidence interval was set at 95%, so that in all cases results with a value of $p < 0.05$ were considered significant. In the vascular reactivity studies, percent maximum relaxation responses to each agonist in each of the groups were compared using programmed statistical software (GraphPad Prism 6, GraphPad Software, Inc., La Jolla, Calif., USA).

Results
Effect of treatments on cotinine level
Figure 1 illustrates the plasma cotinine level at the end of the experiment. Both cigarette smoke and nicotine vapour exposure caused a statistically significant increase ($p < 0.01$) in the level of cotinine in the SMK and NCT groups when compared with the control group. NS oil administration in the cigarette smoke and nicotine vapour-exposed groups caused a statistically significant ($p<0.01$) reduction in cotinine level in the SMK+NS and NCV+NS groups compared to the SMK and NCV groups respectively.
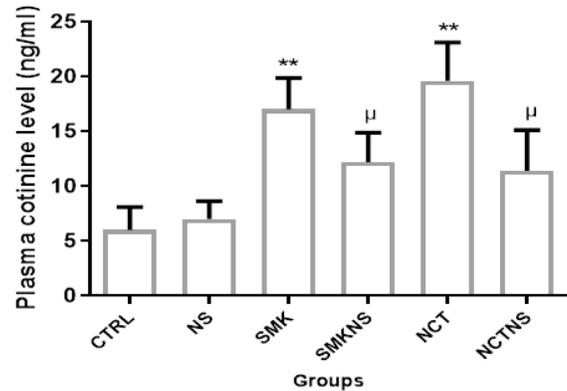

Figure 1: Cotinine level across the group at the end of the experiment. Values are expressed as mean ± SEM ($n = 5$ for each group). ** $p < 0.001$ versus Control group, $^\mu$ $p < 0.01$ versus SMK and NCT groups.
CTRL = Control, NS = *Nigella sativa*, SMK = Smoke-exposed, SMKNS = Smoke-exposed then administered *Nigella sativa*, NCT = Nicotine-exposed, NCTNS = Nicotine-exposed then administered *Nigella sativa*.

Contractile response to norepinephrine in the abdominal aorta of cigarette smoke-exposed rats

Figures 2a and b illustrate the contractile responses of abdominal aortic rings obtained from each group of animals. At lower concentrations of norepinephrine, abdominal aortic rings of rats in the SMK group showed significantly higher percentages of contraction compared to the Control group (62.71 ± 8.81 vs 32.92 ± 6.38, $p < 0.01$ at [10^{-8}]), 71.85 ± 8.88 vs 48.27 ± 4.31, $p < 0.01$ at [10^{-7}], 82.63 ± 8.98 vs 61.88 ± 3.30, $p < 0.01$ at [10^{-6}]). NS oil administration did not significantly ($p > 0.05$) change the final percentage contraction in the SMKNS group compared to the SMK group.

(p<0.01) in the SMKNS group compared to the SMK group (33.83 ± 2.16 vs 71.73 ± 2.60 at $[10^{-4}]$) (Figure 3a and b).
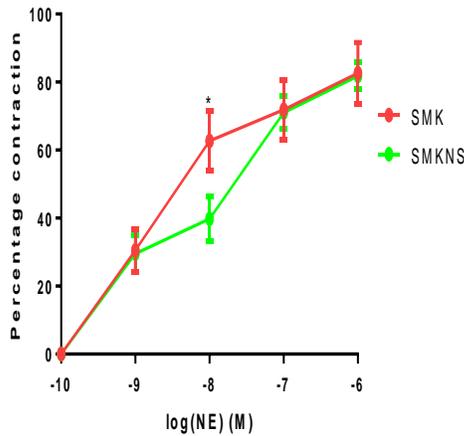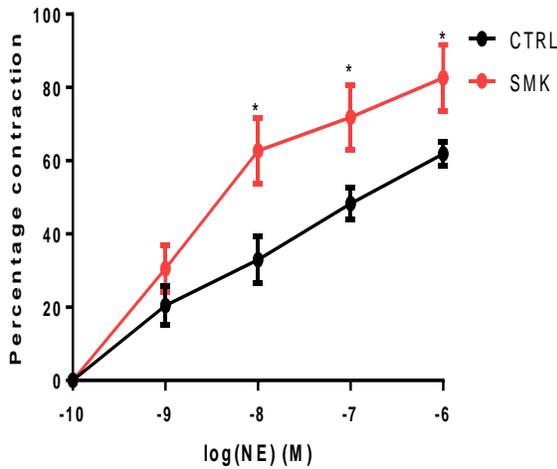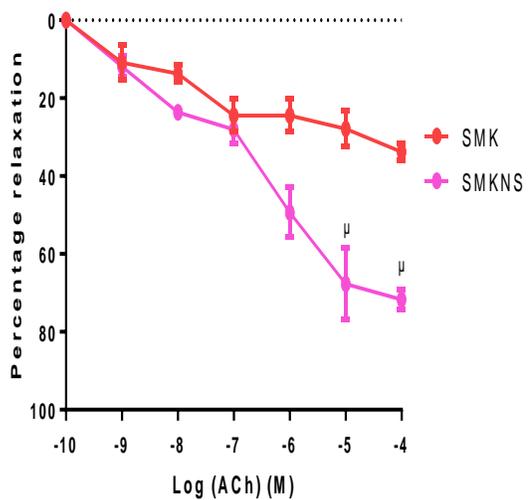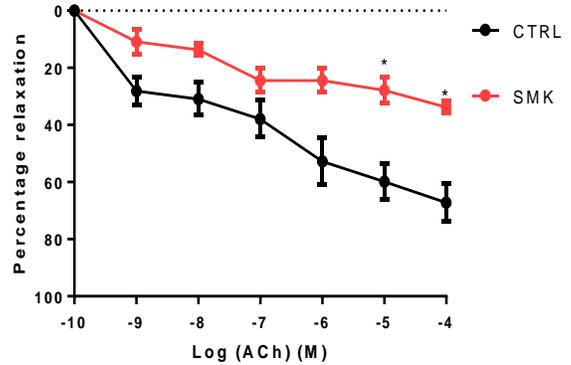

Figures 2a and b: Contractile responses to norepinephrine in the abdominal aorta of smoke-exposed rats. Contraction responses are expressed as percentages as elicited by NA (10^{-5}M). Each point on the graph represents Mean ± SEM. *=Significantly higher (p < 0.01) vs CTRL. CTRL = Control, NS = *Nigella sativa*, SMK = Smoke-exposed, SMKNS = Smoke-exposed then administered *Nigella sativa*.
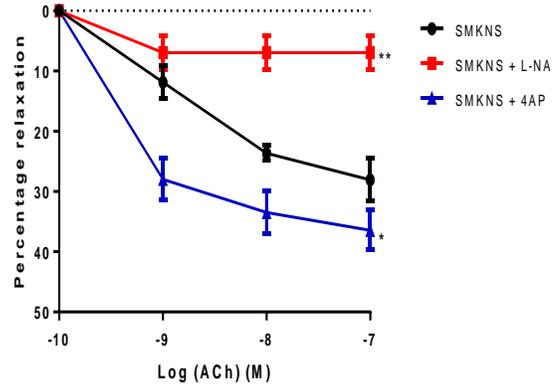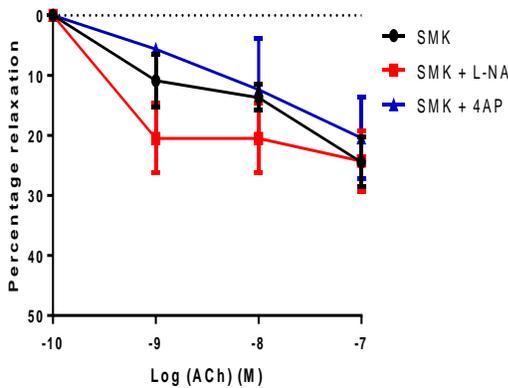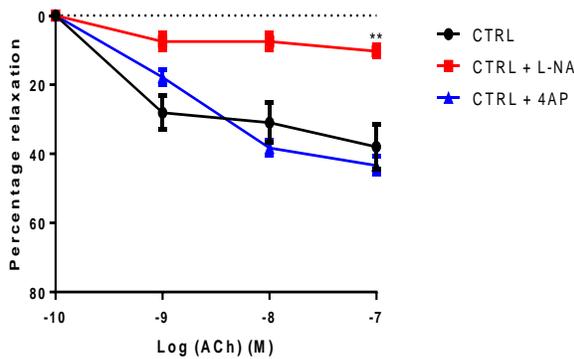

Figures 3a and b: Percentage relaxation response to acetylcholine in the smoke-exposed groups. Relaxation responses are expressed as percentage of decrease in sub-maximal contraction elicited by NA (10^{-5}M). Each point on the graph represents mean ± SEM. * = Significantly lower (p < 0.01) vs Control. μ = significantly higher (p < 0.01) vs SMK group.

Relaxation response to acetylcholine (ACh) in the abdominal aorta of cigarette smoke-exposed rats

Percentage relaxation responses were significantly lower (p < 0.01) in the SMK group especially at higher concentrations of ACh compared to the CTRL group (27.88 ± 4.55 vs 59.87 ± 6.45, p < 0.01 at $[10^{-5}]$, 33.83 ± 2.16 vs 67.17 ± 6.59, p < 0.01 at $[10^{-4}]$). Black seed oil administration significantly improved endothelium-dependent relaxation as relaxation response to ACh was significantly higher

Relaxation response to ACh in the presence of L-nitro-arginine (LNA) or 4-Amino-Pyridine (4AP) in cigarette smoke-exposed rats

In the Control rats, relaxation response to ACh was significantly lower (p < 0.01) in the presence of LNA (CTRL + LNA) (10.27 ± 1.69) but not significantly different (p > 0.05) in the presence of 4AP (CTRL + 4AP) (43.34 ± 2.50) when compared

with the Control group (CTRL) (37.94 ± 6.47). This is illustrated in Figure 4a below. In the SMK group, the LNA-induced inhibition was blunted as there was no significant difference (p > 0.05) in the relaxation response to acetylcholine both in the presence of LNA (SMK + LNA) (24.28 ± 5.05) and 4AP (SMK + 4AP) (20.40 ± 6.78) compared to the absence of the inhibitors SMK (24.44 ± 4.18). This is illustrated in Figure 4b. In the rats treated with Black seed oil after exposure to cigarette smoke, relaxation response to ACh was significantly lower (p < 0.01) in the presence of LNA (SMKNS + LNA) (6.94 ± 2.80) but higher in the presence of 4AP (SMKNS + 4AP) (36.43 ± 3.27) when compared with the (SMKNS) (28.07 ± 3.56) group. This is illustrated in Figure 4c below.
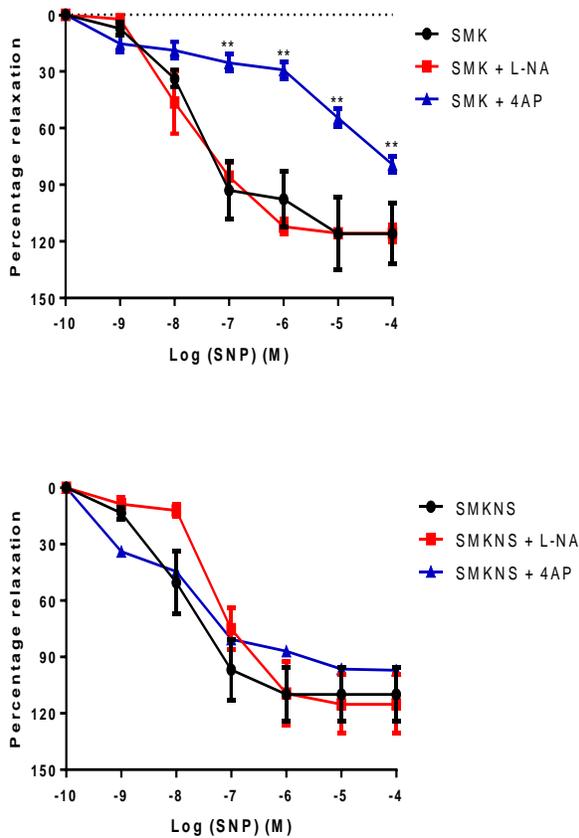

Figures 4a, b and c: Percentage relaxation response to acetylcholine in the control and smoke-exposed groups. Relaxation responses are expressed as percentage of decrease in sub-maximal contraction elicited by NA (10^{-5}M). Each point on the graph represents mean ± SEM. Significantly lower (*p < 0.05, **p < 0.01) vs Control. µ=significantly higher (p < 0.01) vs SMK group.

Relaxation response to sodium nitroprusside (SNP) in the presence of L-nitro-arginine (LNA) or 4-Amino-Pyridine (4AP) in cigarette smoke-exposed rats

In the control rats, relaxation response to sodium nitroprusside (SNP) was not significantly different in the presence of the inhibitors. Smoke exposure significantly increased (p < 0.01) 4AP-induced inhibition in the rats. This is illustrated in Figures 5a, b and c below.
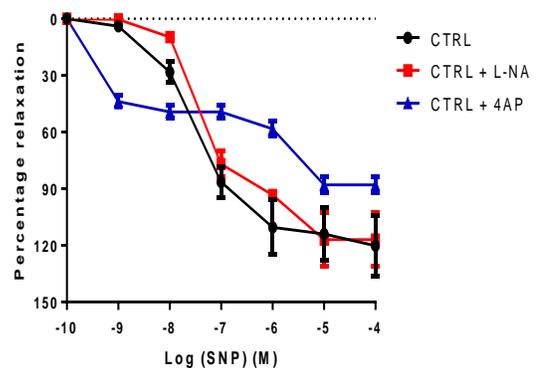
Figures 5a, b and c: Percentage relaxation response to sodium nitroprusside in the control and smoke-exposed groups. Relaxation responses are expressed as percentage of decrease in sub-maximal contraction elicited by NA (10^{-5}M). Each point on the graph represents Mean ± SEM. Significantly lower (*$p < 0.05$, **$p < 0.01$) vs Control.

Contractile response to norepinephrine in the abdominal aorta of nicotine-exposed rats

As illustrated in Figures 6a and b, contraction responses were significantly higher in the NCT group compared to the Control (CTRL) (52.71±4.26 vs 32.92±6.38, $p < 0.01$ at [10^{-8}] 69.87±5.97 vs 48.27±4.31, $p < 0.01$ at [10^{-7}] 83.51±6.51 vs 61.88±3.30, $p < 0.01$ at [10^{-6}]). In the group administered with the Oil (NCTNS), the contractile responses were observed to be significantly lower compared to the NCT group (40.04±4.65 at [10^{-8}] $p < 0.05$, 51.19±4.97 at [10^{-7}] $p < 0.05$).
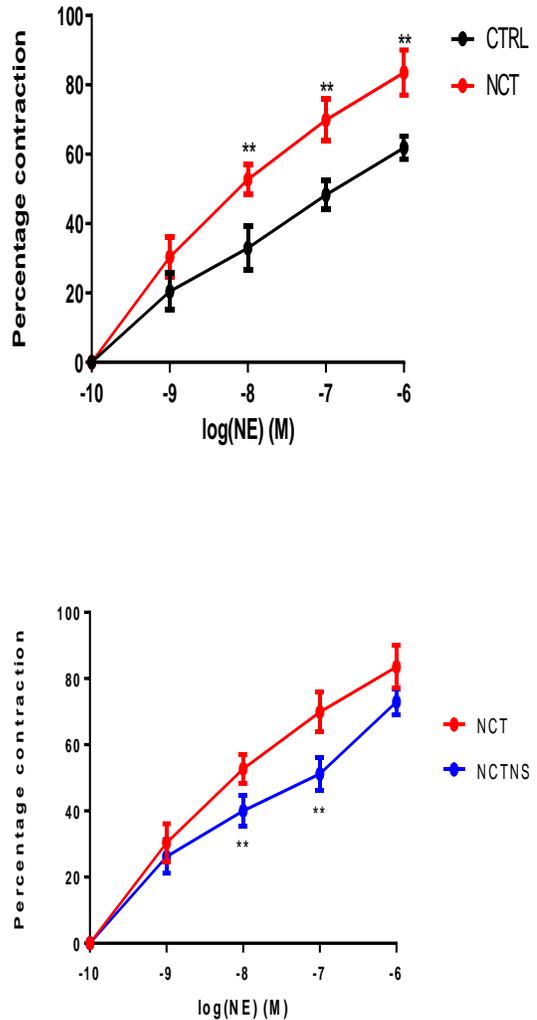

Figure 6a and b: Contractile response to norepinephrine in the abdominal aorta of nicotine-exposed rats. Contraction responses are expressed as percentages as elicited by NA (10^{-5}M). Each point on the graph represents Mean ± SEM. CTRL=Control, NS= *Nigella sativa*, NCT= Nicotine-exposed, NCTNS= Nicotine-exposed then administered *Nigella sativa*.

Relaxation response to acetylcholine (ACh) in the abdominal aorta of nicotine-exposed rats

There was a significant reduction in the maximum relaxation response to ACh in the NCT group compared to the Control (49.37±6.45 vs 67.17±6.59, $p < 0.05$). The maximum relaxation response was significantly higher ($p < 0.05$) in the NCTNS group (70.11±5.34) when compared with

the NCT group. In essence, oil administration caused a significantly reduction ($p<0.05$) on the relaxation responses of the rings (Figure 7).

inhibitions. NS restored the high percentage inhibition to L-NA and reduced the low percentage inhibition to 4-AP.
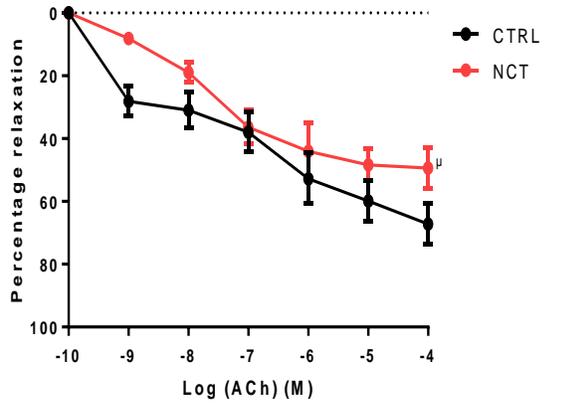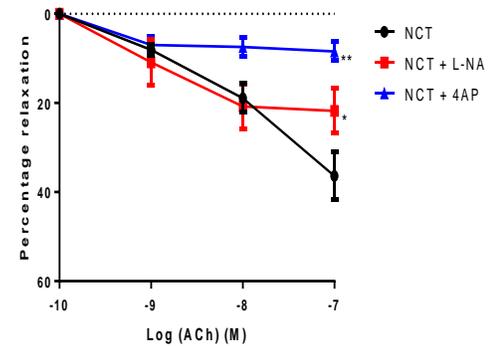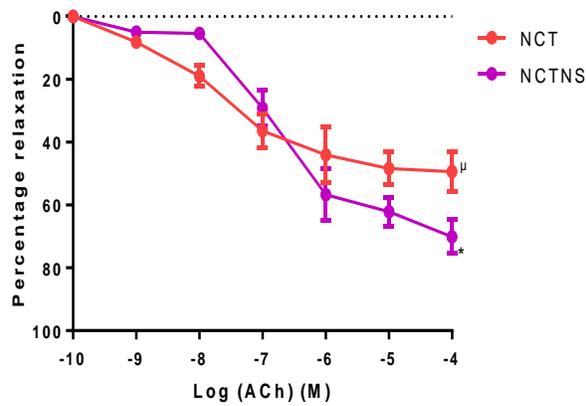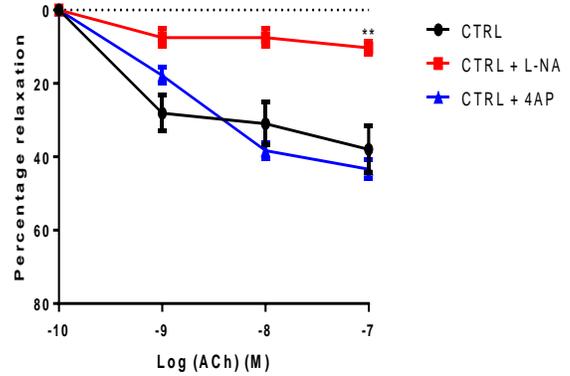

Figure 7a and b: Percentage relaxation response to acetylcholine in the abdominal aorta of nicotine-exposed rats. Contraction responses are expressed as percentages as elicited by NA (10^{-9} to 10^{-5}M). Each point on the graph represents Mean ± SEM. *=significantly lower ($p<0.05$) vs CTRL, µ=significantly higher ($p<0.05$) vs NCT group. CTRL=Control, NS= *Nigella sativa*, NCT= Nicotine-exposed, NCTNS= Nicotine-exposed then administered *Nigella sativa*.

Relaxation response to ACh in the presence of LNA and 4AP in nicotine vapour-exposed rats

The inhibition to ACh caused by L-NA was reduced significantly ($p<0.05$) while the inhibition caused by 4-AP increased significantly ($p<0.01$). In essence, the percentage inhibition reduced significantly for L-NA but increased significantly for 4-AP. This is illustrated in Figures 8 a and b. Figure 8c shows the relaxation response in the rings of rats already administered with NS oil. NS reversed the
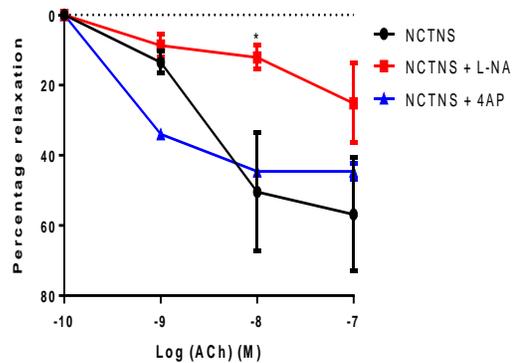

Figures 8a, b and c: Percentage relaxation response to acetylcholine in the control and nicotine-exposed groups. Relaxation responses are expressed as percentage of decrease in sub-maximal contraction elicited by NA (10^{-5}M). Each point on the graph represents Mean ± SEM. Significantly lower (*$p<0.05$, **$p<0.01$) vs Control. µ=significantly higher ($p<0.01$) vs SMK group

Relaxation response to sodium nitroprusside (SNP) in the presence of LNA and 4AP in nicotine vapour-exposed rats

In the control rats, relaxation response to SNP was significantly reduced in the presence of 4AP. Nicotine exposure did not significantly change both L-NA and 4AP-induced inhibition. NS caused no significant change in the inhibition by both L-NA and 4-AP. This is illustrated in Figures 9a, b and c.
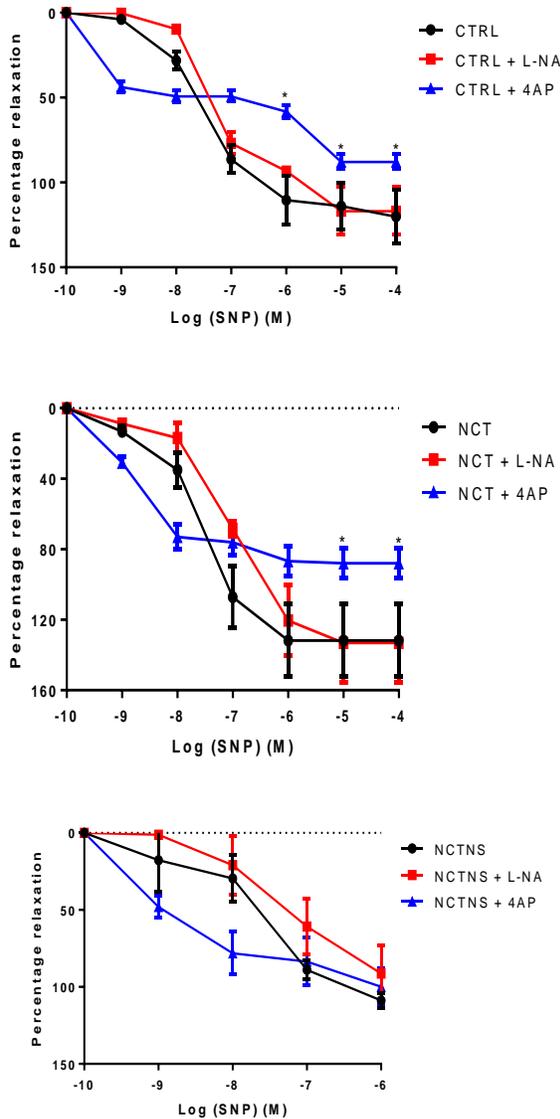
Figures 9a, b and c: Percentage relaxation response to sodium nitroprusside in the control and nicotine-exposed groups. Relaxation responses are expressed as percentage of decrease in sub-maximal contraction elicited by NA (10^{-5}M). Each point on the graph represents Mean ± SEM. Significantly lower (*$p<0.05$) vs Control.

Discussion

Cardiovascular complications are well reported in smokers but the associated alteration of vascular function is yet to be fully characterized (Yanbaeva *et al*., 2007). Cessation efforts led to the development of electronic cigarettes (Alberg *et al*., 2014) which are assumed to be safer (Youth E-CUA, 2016). Unfortunately, both the traditional cigarette smoke and the e-cigarette has been reported to have many deleterious effects due to the presence of nicotine and other compounds (Farsalinos *et al*., 2015). This study sought not only to characterize the modulation of vascular reactivity in an animal model of traditional smoking and vaping but also to establish the mechanism for the possible ameliorative effects of NS oil on contractile and relaxation responses in the arterial vascular system.

In endothelial cells of most vascular beds, acetylcholine stimulates release of endothelial-derived relaxing and hyperpolarizing factors that had been reported to cause relaxation of vascular smooth muscle in an endothelium-dependent manner (Zhang *et al*., 2011). Rats exposed to cigarette smoke in this study had higher contractile response and increased sensitivity to norepinephrine which may be due to impaired endothelial function caused by increased basal tone (Potenza *et al*., 2009; Ahmad and Beg, 2013). At the same time, endothelium-dependent relaxation response to acetylcholine was significantly reduced. In agreement with our findings in this study, exposure to the traditional cigarette smoke had been reported to markedly impair endothelium-dependent relaxation response to acetylcholine in rats and in human smokers (Dalla *et al*., 2004, Takase *et al*., 2006). This was also in line with what was reported by Abbasnezhad *et al*. (2016). In support of this observation as well, long-term smoking was also associated with impaired endothelium-dependent coronary vasodilation (Zeiher *et al*., 1995). As a matter of fact, another study in human reported dose-dependent impairment of flow-mediated dilation in healthy young adults in response to second-hand smoke exposure (Raitakari *et al*., 1999). All these results put together further confirmed the ability of smoke exposure to alter vascular reactivity by markedly reducing endothelium-dependent relaxation without

necessarily damaging the vascular smooth muscle. These effects may not be unconnected with the presence of other toxic substances like carbon monoxide having direct damaging effects on vascular reactivity (Kozma *et al*., 1999). The smoke and nicotine exposure model used in this study was to mimic the sidestream smoke that had been shown to contain a lot of free radicals that are deleterious to the cardiovascular system (Valavanidis *et al.,* 2009).

Rats exposed to nicotine aerosol in this study had higher contractile response to norepinephrine which agrees with earlier studies (Olfert *et al*., 2018; Mayyas *et al*., 2020). At the same time, endothelium-dependent relaxation response to acetylcholine was significantly reduced. In line with earlier studies as well, exposure to nicotine aerosol in this study was used to mimic vaping via the use of electronic cigarettes in humans. Earlier studies had reported vascular endothelial damage and impaired endothelial function in human studies on vaping (Franzen *et al*., 2018, Caporale *et al*., 2019). Endothelial dysfunction was equally reported to be associated with cardiovascular morbidity in vapers (Münzel *et al*., 2020). In agreement with our observations as well, infusion of nicotine has been reported to cause impairment of endothelium-dependent vasorelaxation in the arterioles of rats (Mayhan and Patel, 1997). Nicotine in cigarette smoke has been reported to block potassium currents (Benowitz et al., 1990; Chowdhury et al., 1993) resulting in decreased vasorelaxation. Nicotine could also cause dual effects on the rapidly activating and slowly inactivating K+ current in rat artery smooth muscle cell (Hamon et al., 1997; Tang et al., 1999; Wang et al., 1999). The reduced relaxation may thus be linked to the nicotine in its pure form or as active constituent of cigarette smoke. Recently, Kuntic *et al*. (2020) reported impairment of acetylcholine-dependent vasorelaxation following e-cigarette use. Another study also reported arterial stiffness and endothelial dysfunction in isolated vessels exposed to e-cigarette vapour (Olfert *et al*., 2018). Our results and these previous studies indicate that nicotine vapour from e-cigarettes is as harmful as the traditional cigarette smoke (Staudt *et al*., 2018; Chaumont *et al*., 2019) or even worse (Qasim *et al*., 2019; Buchanan *et al*., 2020). A recent statement from the American Heart Association (AHA) also buttressed the fact that the acute cardiorespiratory toxicity of a single session of waterpipe smoking is worse than smoking a single tobacco cigarette due to the significantly higher levels of cardiorespiratory toxicants such as heavy metals and particulate matter (Bhatnagar *et al*., 2019). Contrary to these reports however, Li *et al*. (1994) reported no significant effect of chronic nicotine exposure on vascular reactivity in the rat. The different species of animals used, the different modes of nicotine exposure or the different arterial segments used to carry out vascular reactivity studies may be responsible for the contrasting results in the study by Li et al. (1994) and this study. Its worthy of note that nicotine could also diminish endothelium-independent vasodilation via interaction with ATP-sensitive K+ channels (Mayhan and Sharpe, 2002). However, we reported the voltage-sensitive potassium channels in this study.

In this study as well, blockade of endothelial nitric oxide synthase enzyme with LNA caused a marked reduction in endothelium-dependent relaxation in rats exposed to cigarette smoke and nicotine vapour. This implies that the damaging effects of cigarette smoke or nicotine vapour exposure is mediated partly through disrupting the endothelial lining of the vessels (Chakkarwar, 2011; Kuhlmann *et al*., 2005). Interestingly, there appears to be a differential response to the blockade of the voltage-sensitive channels in the rats exposed to cigarette smoke and those exposed to nicotine vapour. Blockade of voltage-sensitive potassium channels caused a significant reduction in endothelium-dependent relaxation in only the rats exposed to nicotine vapour. This implies that these potassium channels are significantly involved in bringing about relaxation in the rats and by extension, possibly in vapers. On the other hand, blockade of voltage-sensitive potassium channels led to reduced endothelium-independent relaxation in only the rats exposed to cigarette smoke. By implication, these results indicate the ability of nicotine to disrupt both endothelium-dependent and endothelium-independent relaxation in the blood vessels of smokers or vapers. To confirm this, earlier studies had indicated the presence of alpha and beta subunits of acetylcholine in the vasculature (Boswijk *et al*., 2017).

NS oil administration had no significant effect on the increased contractile response to norepinephrine in rats exposed to cigarette smoke. It however significantly reduced the contractile response to norepinephrine in rats exposed to nicotine vapour. NS oil also significantly increased both endothelium-dependent relaxation response to acetylcholine in both rats exposed to cigarette smoke and nicotine vapour. NS oil administration had no significant effect on endothelium-independent relaxation response to sodium nitroprusside. In the presence of the inhibitors, the oil restored inhibition to acetylcholine in the presence of endothelial nitric oxide synthase blocker in both the rats exposed to cigarette smoke or nicotine vapour. The improvement in endothelium-dependent relaxation may be linked with the effect on nitric oxide production (Niazmand *et al*., 2014). In line with our observations also, NS oil had been reported to have relaxant effect on rat aortic smooth muscle contracted by both potassium chloride and phenylephrine (Suddek, 2010; Niazmand *et al*., 2014). The relaxant effect of NS oil observed in this study may be due to the opening of potassium channels in vascular smooth muscles (Boskabady *et al*., 2004).. Some studies had earlier reported the presence and expression of both K^{ATP} and nonselective K^+ channel (Côrtes *et al*., 2001; Jackson, 2000) in vascular smooth muscles. Another study reported the marked reduction in thymoquinone-induced relaxation following blockade of ATP-sensitive potassium channels by glibenclamide (Suddek, 2010) further buttressing the involvement of potassium channels in the vasorelaxant effect of NS oil. The downstream effect of the oil based on results from this study may involve opening of voltage-sensitive potassium channels (Keyhanmanesh *et al*., 2014) as the oil restored LNA-induced inhibition to acetylcholine in the rings. Findings from this study suggest that the use of NS oil has the potential to ameliorate the associated alterations in vascular functions in smokers and e-cigarette users.

In conclusion, both smoke and nicotine exposure alter vascular reactivity by increasing contractile response and reducing endothelium-dependent relaxation responses via nitric oxide pathway and partially through voltage-sensitive potassium channels. Black seed oil ameliorates nicotine or cigarette smoke-induced vascular dysfunction by increasing endothelium-dependent relaxation and improving the relaxation caused by the voltage-sensitive potassium channels.

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