transcription factors.[9,11-14] As cardiovascular pathologies involve these myriads of pathways, Ang II and LPS activated pathways will be a good model for testing the possible actions of KV in cardiovascular dysfunction *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Reagents used in this study were Dulbecco's modified Eagle's medium (DMEM), MTT, Anti-biotic and anti-mycotic consisting of 100 U/mL penicillin G sodium, 100 mg/mL streptomycin sulphate, 2.5 mg/mL amphotericin B and Trypsin-EDTA. They were purchased from Sigma-Aldrich, St Louis, MO. Matrigel (BD Biosciences, Franklin Lakes, NJ. All other chemicals and reagents were of pure analytical grade.

Extraction of *Garcinia kola* and isolation of Kolaviron

Kolaviron was extracted from the seeds of *Garcinia Kola* according to the method of Iwu with slight modification.[1] The seeds were sliced, air-dried and powdered. The powdered seeds were defatted by extraction using n-hexane in a Soxhlet extractor apparatus for 24 hours. The defatted dried marc was repacked and extracted with methanol. Kolaviron was fractionated from concentrated methanolic extract using chloroform to give a golden brown solid which consists of Garcinia biflavanones – GB1, GB2 and kolaflavanone.

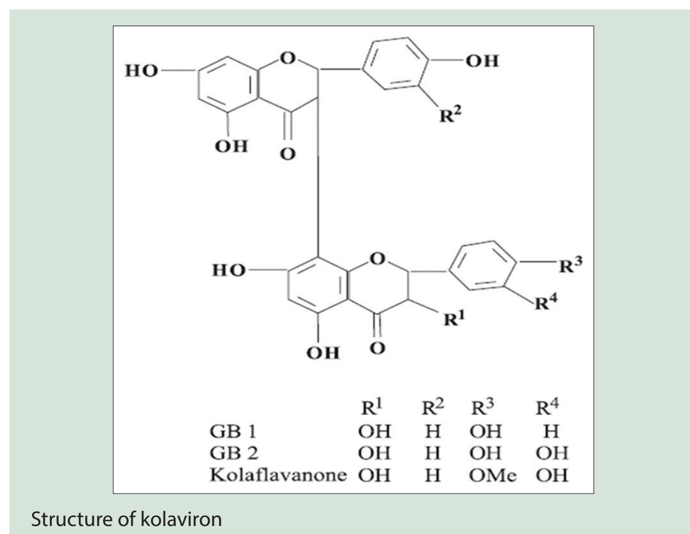
METHODS

Vascular smooth muscle cell culture

VSMC was a gift from Dr. Ranganna of the RCMI Core Lab at TSU, Houston. The cells were cultured and maintained as previously described.[15] Briefly, VSMC were culture in a culture flask T75 and maintained at 37°C in a humidified 5% CO_2 incubator in a 20% FBS conditioned DMEM plus anti-biotic consisting of 100 U/mL penicillin G sodium, 100 mg/mL streptomycin sulphate, and 2.5 mg/mL amphotericin B until confluent. Confluent cells were trypsinized and plated in a 96-well plates at a population of 7,000 cell per well for proliferation assays. For determination of treatment on NO production, cells were cultured in a 12-well culture plates.

Effects of KV on LPS VSMC proliferation

To determine effects of KV on cellular proliferation, 24 hours following cell seeding in 96 well plates, cells were treated with KV (25-100 μg/mL) and cell growth determined at 24, 48, 72, or 96 hours following treatments.



Structure of kolaviron

Effects of KV on Ag II- and LPS-induced VSMC proliferation

To determine the effects of KV on mitogen-induced VSMC growth, 24 hours following plating of VSMC in 96 wells, cells were exposed to Ag II (10^{-6}M) or LPS (100 μg/mL) in the presence or absence of KV (25-100 μg/mL). The treated plates were further incubated for 24, 48, 72, or 96 hours before effects of treatments on proliferation determined.

Effects of KV on LPS-induced NO production

To determine effects of LPS-induced NO production, VSMC were seeded on matrigel coated 24 well plates and incubated until 75-80% confluent before treatment with LPS (100 μg/mL) in the presence or absence of KV (25-100 μ g/mL) for 4 hours. At the end of the incubation, media was removed and stored in -80°C until needed for NO determination.

MTT assay

VSMC proliferation was determined using MTT assay. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay is based on the ability of life cell actively metabolizing to reduce MTT to purple formazan crystal in the mitochondria of living cells. The degree of purple formazan crystals formation is proportionate to the life cells. Following 24, 48, 72 or 96 hours incubation of VSMC with vasoactive agents –KV, Ag II, LPS, alone or in combination; 20 μl of 5 mg/mL MTT was added to the 96 well plates and incubated for 3 hours. At the end of the incubation, the purple formazan crystals were dissolved with the addition of 150 μL DMSO and absorbance determined at 540 nm wavelength using Bio-Tek Plate Reader (Model ELX 800, BioTek Instruments, Winooski, Vermont, USA). The proliferation assay was conducted in triplicates, experiments repeated at least 4 times and expressed as mean of % change in VSMC growth.

Determination of nitric oxide (NO)

NO level in the media was determined using the Griess assay as describe previously.[16] Briefly, assay samples were mixed with an equal volume of the Griess reagent [0.1% *N* (1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 3% H_3PO_4] and incubated to yield a chromophore. Using a Bio-Tek Instruments plate reader (model EL808UV; Uniooski, VT), absorbance at 540 nm was measured and nitrite concentration was determined using a nitrite standard curve. The efficiency was at least 95%. The assay was conducted in triplicate, experiments repeated at least 4 times and results were expressed as nM/mL.

Statistical analysis

Data are presented as mean ± S.E.M of n = 4-6. Differences between groups were assessed using one-way ANOVA followed by Turkey comparison tests. A value of $P < 0.05$ was considered significant.

RESULTS

Effects of KV VSMC proliferation

Figure 1 shows effects of treatment of VSMC with KV (25, 50, 100 μg/ml) for 24 hours. VSMC growth was reduced by 14, 6, and 15.4% following treatments with 25, 50, 100 μg/ml KV respectively. This reduction in cell growth was significantly ($P < 0.05$) different from the control [Figure 1, n = 4].

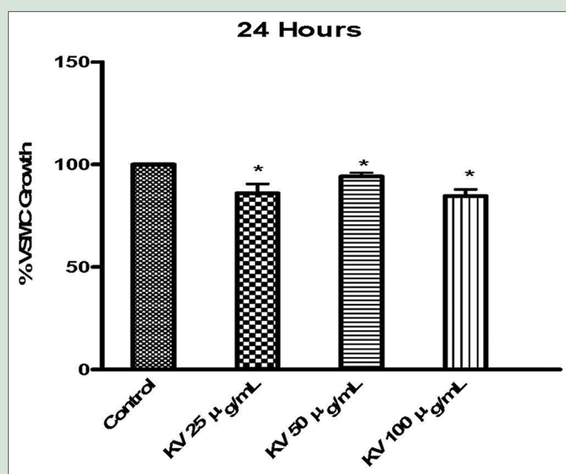


Figure 1: Effects of 24 h incubation of vascular smooth muscle cell with kolaviron (25–100 μg/mL) on cell growth

Effects of KV on Ag II- and LPS-induced VSMC proliferation

Figure 2a-d shows effects of KV (25, 50, 100 μg/ml) treatment on Ag II (10^{-6} M)-induced VSMC proliferation at 24, 48, 72 and 96 hours. The 24 hours incubation of VSMC with Ag II (10^{-6} M) significantly ($P < 0.006$) increased VSMC proliferation by 173.3% compared to the control (100%). The increase in cell proliferation induced by Ag II was significantly ($P < 0.05$) attenuated by KV, reducing cell growth from 173.3% to 84.8 ± 5.0, 94.2 ± 1.8, and 84.6 ± 3.3% of the control level for KV (25, 50, 100 μg/ml) respectively [Figure 2a, n = 4].

48 hours treatment of VSMC with Ag II significantly increased cell growth from 114 ± 5.9% (control) to 187.7 ± 37.9% (Ag II) ($P < 0.05$). This increased VSMC growth-induced by Ag II was prevented by treatments with KV (25, 50, 100 μg/ml) reducing cellular growth to 81.9 ± 9%, 73.7 ± 1.2%, 76.3 ± 3.5% of the control, respectively [Figure 2b, n = 4].

Also, 72 hours treatment of VSMC with Ag II significantly ($P < 0.05$) increased VSMC growth from 121.0 ± 6.6% (control) to 162.8 ± 9.2% (Ag II). This increased VSMC proliferation induced by Ag II was significantly attenuated by treatments with KV (50 and 100 μg/mL) reducing cellular growth from 162.8 ± 9.2% (Ag II) to 78.5 ± 7.5% (KV 50 μg/mL) and 79.8 ± 7.7% (KV 100 μg/mL) respectively but not KV 25 μg/mL (149.5 ± 10.0%) when compared to Ag II (162.8 ± 9.2%) and control (121.0 ± 6.6%) [Figure 2c, n = 4].

Similarly, 96 hours treatment of VSMC with Ag II significantly ($P < 0.05$) increased VSMC growth from 121.3 ± 6.6% (control) to 162.8 ± 33.6% (Ag II), this increased VSMC proliferation induced by Ag II was significantly attenuated by treatments with KV (50 and 100 μg/mL) reducing the growth from 168.0 ± 33.6% (Ag II) to 75.0 ± 12.6% (KV 50 μg/mL) and 79.8 ± 7.7% (KV 100 μg/mL) but not the lowest concentration KV 25 μg/mL with 169.7 ± 3.3% growth when compared to Ag II (168.0 ± 33.6%) and control (121.0 ± 6.6%) [Figure 2d, n = 4].

Effects of KV on LPS-induced VSMC proliferation

Figure 3 show effects of KV (25, 50, 100 μg/ml) treatment on LPS (100 μg/ml)-induced VSMC proliferation. 24 Hours incubation of VSMC with LPS resulted in significant ($P < 0.05$) growth of the cell to 262.7 ± 19.0% from the control (100%). The LPS induced growth was significantly ($P < 0.05$) reduced by the KV treatments to 91.7 ± 1.7% (KV 25 mg/mL), 102.3 ± 1.2% (KV 50 mg/mL), 90.8 ± 5.7% (KV 100 μg/mL) [Figure 3, n = 4].

Effects of KV on LPS-induced NO production

Figure 4 shows effects of LPS-induced NO production. 4 Hours incubation of VSMC with LPS 100 μg/mL resulted in significant increase in NO. NO levels in the media were significantly ($P < 0.05$) increased from 33.0 ± 0.3 nM/mL in the control to 36.4 ± 0.4 nM/mL in LPS treatment. LPS-induced increases in NO production was significantly attenuated by KV reducing the NO levels from 36.4 ± 0.4 nM/mL (LPS) to 32.4 ± 0.2, 31.2 ± 1.0, 31.4 ± 0.3 nM/mL for KV 25, 50, 100 μg/mL respectively [Figure 4, n = 4]. The KV-induced attenuation of LPS-induced increases in NO levels brought the NO levels to a level comparable to that observed in the control.

DISCUSSION

In the present study, we found that: (1) Treatment of VSMC with KV resulted in reduced VSMC growth, (2) KV attenuated Ag II-induced VSMC proliferation in a concentration and time-dependent manner, (3) 24 hours LPS treatment increased VSMC proliferation and NO production and were attenuated by KV treatment. Thus, these results demonstrated that KV possesses anti-mitogenic agents'-induced proliferation of smooth muscle cells as well as NO production via LPS mediated activation of inflammatory processes. Our findings suggest that KV possibly mediate its effects by regulating molecular signalling pathways that regulates diverse cellular functions.

Despite advances in knowledge and therapeutic drug development, cardiovascular diseases still remain a huge burden to individual and society. The processes that contribute to the initiation and maintenance of cardiovascular diseases become a target for therapeutic intervention. Cardiovascular pathologies are characterised by vascular cell proliferation, inflammation, and/or increased oxidative stress. Inflammation contributes critically to all stages of atherogenesis and cardiovascular remodelling.[17,18] Metabolic disorders such as dyslipidemia promote activation of circulating monocytes, endothelial cells and adhesion of these cell types leading to accumulation of macrophages.[13,17,18] Activation of macrophages can lead to the production of proinflammatory cytokines, NO, mitogens, and reactive oxygen species.[17,18] Cellular oxidative stress as well as other vasoactive agents can activate neighbouring cells including endothelial cells and further promotes monocyte recruitment. In addition, these processes can lead to production of mitogenic agents (Ag II, ET-1 etc.) and further propagating artherogensis. Such an uncontrolled amplification mechanism represents combined proliferative, inflammatory and oxidative stress aspects of atherosclerosis and cardiovascular disease pathologies. Numerous studies have been designed to investigate the involvement of inflammation, proliferation, and oxidative stress in cardiovascular diseases with the aim to developing agents that can prevent the development of atherosclerosis and its complications have resulted in unsatisfactory results.[13,19] This probably could be due to the multi-factorial nature of the pathogenesis of cardiovascular diseases; hence, single remedy focused on alleviating one of these factors will result in unsatisfactory outcomes. Therefore, the target should be the development or identification of possible therapeutic agents that possess a wide range of actions against this plethora of factors and possibly mitigating against activation of common mechanistic pathways. In the present study, we have evaluated the anti-proliferative and anti-inflammatory effects of KV in cultured VSMC. We found that KV attenuated VSMC growth and sequential cell proliferation induced by Ag II and LPS as well as LPS-induced increased NO production which could mediate inflammatory processes.

It is generally accepted that Ang II and LPS as well as other mitogens such as ET-1 could activate cellular processes involved in the production of growth factors, cytokines, chemokines, and adhesion molecules, which

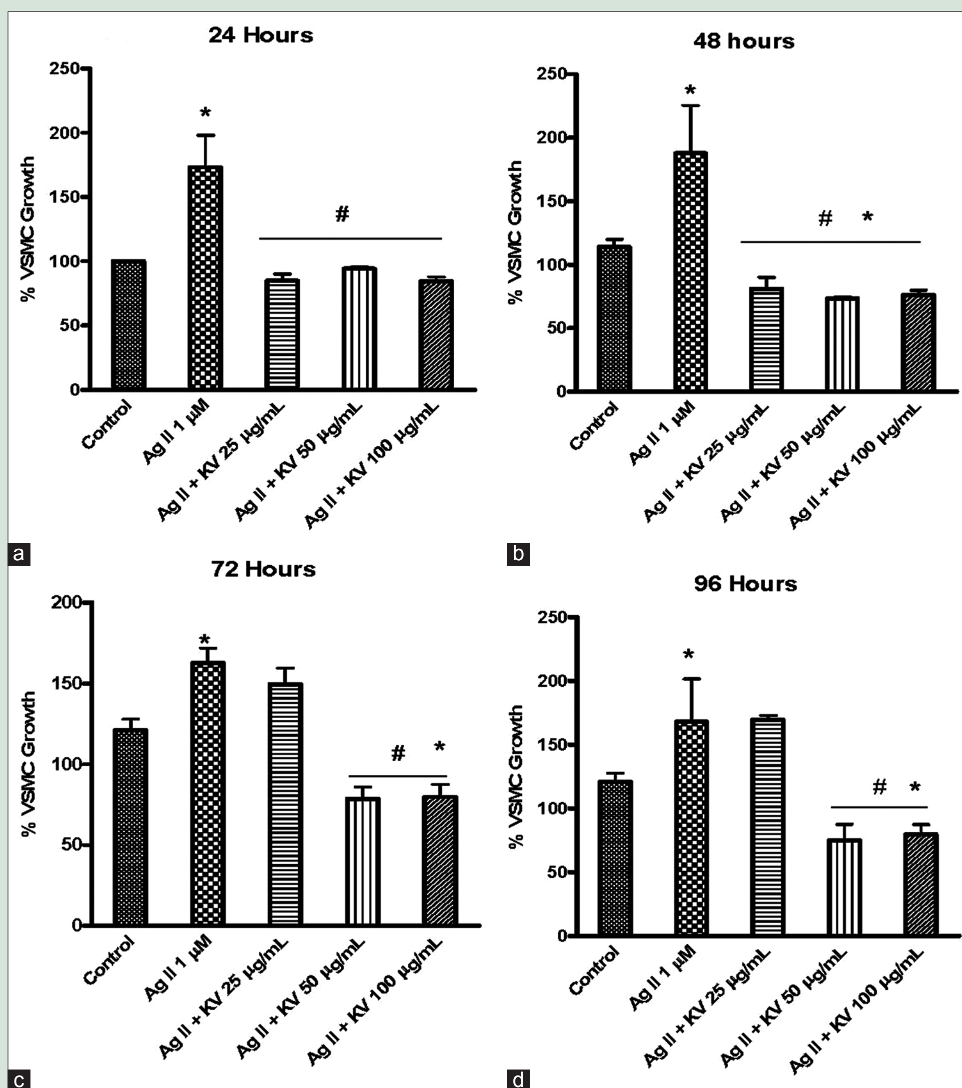


Figure 2: (a-d) Effects of 24, 48, 72, or 96 h incubation of vascular smooth muscle cell with kolaviron (25–100 µg/mL) on Ag II (1 µM)-induced vascular smooth muscle cell proliferation

are involved in cell growth/apoptosis, fibrosis, and inflammation.[2-4] Arterial wall production of Ang II is important in the normal regulation of arterial tone as well as its involvement in the pathogenesis of atherosclerosis. Ang II regulates many processes implicated in vascular pathophysiology, including cell growth/apoptosis of vascular cells, migration of vascular smooth muscle cells, inflammatory responses, and extracellular matrix (ECM) remodelling.[2-4] As a result of its important role in the regulation and pathogenesis of cardiovascular diseases, drugs that block Ang II actions, such as ACE inhibitors or Ang II receptor antagonists, are currently employed in the treatment of hypertension, heart failure, atherosclerosis, and other cardiovascular diseases.[2-4] Despite the widespread use of Ang II agents in clinical practice, its' mechanism (s) of action is not completely defined by which of the multiple pathways Ang II exerts its effects in the vasculature. Similarly, LPS exert its action through series of interference with vascular signalling pathways involving activation of different kinases culminating in the systemic dysfunctions observed.[2-4,20,21] However, we have shown in the present studies that KV possesses both anti-proliferative and anti-inflammatory properties in addition to its well known anti-oxidant effects as Ang II and LPS are pro-oxidants. The role of oxidative stress in the pathogenesis of vascular diseases is well recognized. Ang II and LPS stimulates the production of reactive oxygen species (ROS) via induction of vascular NADH oxidase mediated by gp91phox of NADPH oxidase and other subunits are mainly responsible for stimulated vascular oxidative stress and smooth muscle cells growth *in vivo*.[20-22] Furthermore, LPS is known to modulate pathological conditions through activation of ROS, cellular proliferation and inflammatory processes mediated by excessive production of NO. In the present study, we have shown that KV treatment prevents mitogen induced VSMC proliferation and attenuated LPS-induced generation of NO and by extension, reduction cellular stress. These actions of KV indicate that it can be useful in conditions that involve cellular proliferation, enhanced oxidative stress, and proinflammatory processes. According to our present findings, LPS-induced nitric oxide production was quenched by KV, clearly demonstrating the anti-oxidant and anti-inflammatory properties of KV.

These plethoric actions of KV observed could not be possibly linked to actions on a single pathway mediated via activation of proliferative, inflammatory, oxidative processes etc. The actions of KV observed can be attributed to inhibition of a converging single pathway that all of these signalling processes recruit to mediate these actions – a

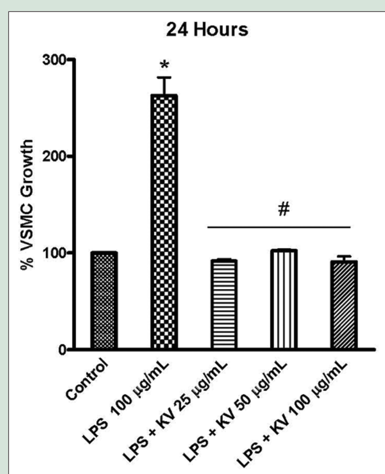


Figure 3: Effects of 24 h incubation of vascular smooth muscle cell with kolaviron (25–100 µg/mL) on lypopolysaccharide (100 µg/mL)-induced vascular smooth muscle cell proliferation

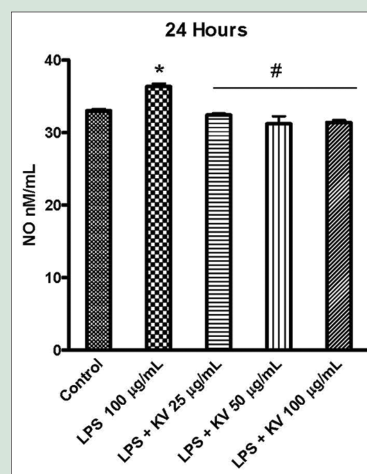


Figure 4: Effects of 4 h incubation of vascular smooth muscle cell with kolaviron (25–100 µg/mL) on lypopolysaccharide (100 µg/mL)-induced nitric oxide production

transcription factor probably. Nuclear factor-kB (NF-kB) consists of a family of transcription factors that play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival. Evidences suggesting the potential role of NF-κB as a mediator of proliferative and inflammatory processes are ripped. Increases in NF-κB activity in rat's vessels has been reported following systemic infusion of Ang II and LPS administration.[20-25] Ang II and LPS activates NF-κB in several cell types, including vascular smooth muscle, endothelial, renal, macrophages, and mononuclear cells in mediating oxidative, inflammatory, and proliferative processes.[19-24] Although, Ang II acts through binding to two main specific receptors, AT_1 and AT_2, both receptors share a common molecular pathway, the activation of NF-κB.[25] LPS has been suggested to activate NF-κB in regulating proinflammatory cytokines, NO, COX-2 etc., via series of kinase activation in pathophysiology of systemic shock.[25] There are possibilities that Ang II and LPS mediated vascular dysfunctions involves activation of proliferative and inflammatory responses via redox mechanisms and NF-κB pathways. Given the large number of signals that activate NF-κB, the list of target genes controlled by NF-kB; targeting NF-kB will be a viable opportunity for prevention and treatment of cardiovascular pathology. From our present results, KV-induced attenuation of LPS and Ang II-induced proliferation and NO production possibly involves inhibition of NF-kB activation. Consistent with this possibility, is the observed ability of KV treatment to significantly reduce activation and expression of NF-kB in a cancer cell line (unpublished observation). Thus, the actions of KV observed in this study may well be mediated via inhibition of NF-kB activation but further studies are warranted to understand the molecular mechanism involve in KV-induced anti-proliferative and -inflammatory with possible role of NF-kB.

CONCLUSION

Taken together, these results showed that KV inhibited cell proliferation and prevented the generation reactive oxygen species (ROS) and nitric oxide production mediated by LPS activation of inducible nitric oxide synthase (iNOS). In conclusion, KV possesses possible anti-oxidant, anti-proliferative and anti-inflammatory properties which would be useful in alleviating cardiovascular disease conditions and further studies are warranted to investigate the mechanism involved in the KV actions.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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