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Article

The Vitamin k_2 improves endothelial progenitor cells vascular repair in rats' dyslipidaemia: an experimental study

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Abstract.*Background*: Vitamin k_2 (menaquinone-7) was reported to possess a vascular protective action against atherogenesis through reduction of vascular calcification.
Objectives: The present study investigated whether the augmentation of endothelial progenitor cells' (EPCs) reparative capacity could be an underlying mechanism behind vitamin $k_2$vasoprotective action, in a rat model of dyslipidaemia.
Methods: Forty-five Wistar rats were randomly assigned to normal control (15-rat) or dyslipidaemic rats (30-rat) fed on laboratory show or high fat diet (HFD), respectively. Dyslipidaemic rats were further assigned to receive either vehicle or vitamin k_2 (30 mg/kg) 5 days a week, orally for 8 weeks. At the end of the study, lipid profile was assessed. Thoracic aortae were dissected for histopathological examination, immunostaining for detection of EPCs markers; CD133 and vascular endothelial growth factor receptor-2 (VEGFR-2), and β-catenin expression, and for expression of NADPH oxidase 4 (NOX4). Vascular function was assessed biologically in-vitro. *Results:* Vitamin k_2 supplementation conferred an endothelial protection and anti-atherogenic potential when compared to vehicle treated HFD fed rats evidenced by improved lipid profile; serum TG (mg/dl): 109.4 ± 16.56 versus 153.60 ± 9.88, serum LDL: 53.56 ± 12.76

versus89.00 ± 9.80 and atherogenic index of0.52 ± 0.12 versus 0.69 ± 0.05, respectively. Moreover, The significant increase in EPCs numbers induced by vitamin k2 treatment in comparison to vehicle treated HFD fed rats; 33.50 ± 4.73 versus 22.30 ± 3.30, respectively, appears to play a pivotal role in the vaso-protective action of vitamin K_2 that could be mediated by interplay between vascular Wnt/β-catenin signalling and NOX4 expression.

Conclusion: 8-week treatment with vitamin k_2 increases EPCs count as well as confers anti-atherogenic potential in rats with dyslipidemia and whether continued vitamin k_2 supplementation encounters sustained EPCs endothelial regeneration warrants further investigation.

Keywords: endothelial dysfunction, atherogenesis, CD133, VEGFR-2, NOX4

Introduction

Endothelial dysfunction (ED) is a hallmark of cardiovascular diseases and is considered the earliest event in pathogenesis of atherosclerosis. To establish endothelial homeostasis, endogenous repair mechanisms are operating in parallel to the endothelial constant insult. Endothelial progenitor cells (EPCs) play a vital role in vascular regeneration after injury and inhibition of atherosclerotic lesion progression. EPCs contribute to the pro-angiogenesis in ischemic tissue and vascular regeneration and remodelling.(1)

Dyslipidaemia, as a risk factor, switches the endothelium quiescent state into activated one which alters endothelial cell signalling leading ultimately to ED.(2) Accumulation of the lipid-containing lipoproteins in the intima activates the endothelium and initiates an atherosclerotic process and cardiovascular events.(3, 4)In dyslipidaemia, EPCs profile is altered and their circulating numbers are reduced which collaborate into the induction of ED,(5) though the underlying mechanisms are still not clear.

Oxidative stress could be one of these mechanisms. In fact, NADPH oxidases (NOXs) are major sources of endothelial reactive oxygen species (ROS). Although ROS are mainly involved in the physiological vascular homeostasis, excess ROS is pathological and results in oxidative stress leading to ED and vascular disease progression.(6)The endothelial physiological homeostasis is thought to be maintained by the constitutively expressed NOX4 (7).

The EPCs reparative function process remains obscure and needs further investigations. One of the master regulators of EPCs enrolment and differentiation is the canonical Wnt signalling pathway.(8)An interaction between Wnt and ROS-producing NOX enzymes has been postulated; however the individual role of NOX4 is not clear yet.(9) NOX1 andNOX4 were demonstrated to activate the p38 MAPK pathway,**(10)** which phosphorylates and inhibitsglycogen synthase kinase (GSK-3β), thereby maintaining Wnt/β-catenin in a stabilized state during cellular differentiation.

Thus, interplay could be raised between NOX4 expression and Wnt/β-catenin signalling in vascular repair mechanisms. Being a marker for ED, EPCs became an attractive target for vascular repair. Therefore, it is mandatory to explore more effective EPC-based therapies.(11)

In this context, vitamin k2, as a nutritional supplement, was of concern. Vitamin K2 (Menaquinones) is obtained from meats, a variety of cheeses, and eggs. Besides reduction of coronary calcification and atherogenesis, vitamin K2 enrolment in maintenance of endothelial cells' survival by rescuing it from apoptosis has been raised.[12] In the present study, whether vitamin K2 can improve the detrimental effect of dyslipidemia on EPCs reparative capacity is investigated in a rat model of dyslipidaemia and its possible relation to the expression levels of NOX4 and β-catenin proteins is further unravelled.

Materials and Methods

Animals

The study was conducted on male Wistar albino rats weighing 190-230g. Animals were housed under standard conditions with food and water ad libitum. All animals' procedures and treatment were conducted in accordance with the Research Ethics Committee - Faculty of Medicine, Alexandria University, in compliance with the "Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals Policy".

Experimental procedure

After acclimatization, 45 rats were randomly assigned into 2 main groups according to the fed diet for 8 weeks. Normal control rats (n=15) were fed on laboratory chow (1.29 KJ as fat) and a high-fat diet (HFD, 7.74 KJ/g as fat)was served to the dyslipidaemia-induced group of rats (n= 30)(13) The dyslipidaemia-induced group of rats was then randomly subdivided into two subgroups (15-rat each) based on the assigned oral treatment that started with the dietary regimen for 8 weeks; vitamin K2 (Sigma-Aldrich - 30 mg/kg/day, 5 days a week),(14) and vehicle (Gum acacia 2%) treated subgroup. Throughout the 8 weeks, rats were closely observed for any sign of humane endpoints. At the end of the study after an overnight fasting, rats were anesthetized by intraperitoneal thiopental sodium (50 mg/kg) and blood samples were collected by cardiac puncture on EDTA-containing tube and stored at 4 °C for lipid profile estimation. Then, animals were sacrificed by an overdose of anaesthesia. Multiple arterial sections from thoracic aortae were gently dissected and put in fresh Krebs solution for *in-vitro* isometric tension studies. Other aortic sections were frozen at - 80°C for later biochemical measurement or preserved in 10% formol saline for histopathological and immunostaining assessments.

Serum lipids measurements

Diagnostic kits for the lipid profile; Total cholesterol, High-density lipoprotein cholesterol, and triglycerides (TC, HDL-C, TG, mmol/L) (with the exception of Low density lipoprotein-cholesterol, LDL-C) were purchased from BioSystems (S.A Costa Brava, Spain). The assays were performed according to the manufacturer's instruction. The LDL-C and atherogenic index of plasma (AIP) were calculated usingFriedewald equation and logarithmic transformation of the ratio of TG to HDL-C, respectively.(15, 16)

NOX4 gene expression by qPCR

The abdominal aortic tissues were homogenised and total serum RNA was isolated using miRNeasy Mini Kit (QIAGEN), according to the manufacturer's protocol. The concentration of total RNA samples was quantified by a Nanodrop 2000 (Nanodrop, USA).The TaqMan MicroRNA Reverse Transcription (RT) Kit (Applied Biosystems, USA) was used for the RT reaction. The PCR was performed using a reaction volume of 20 μL. The plate was prepared and ABI prism 7900 sequence detection system (Ambion, USA) was used for amplification and detection by qRT-PCR. Amplification of both target and housekeeping genes (β-actin) was performed using TaqMan Universal PCR master mix (Applied Biosystems), 25 ngcDNA, and the pre-designed probe and primer sets for rat specific NOX4 genes (TaqMan Gene Expression Assays, Applied Biosystems).(17)

Histological sections and immunostaining

Histological examination

After processing into paraffin, thoracic aortic sections were sliced into5 ◎m transverse sections from each vessel and were stained with haematoxylin and eosin (H&E) stain and examined under light microscope for diagnosing and scoring of atherosclerotic changes according to the AHA classification of human atherosclerotic lesions.(18)

Immunostaining

Immunohistochemistry (IHC) of aortic sections for EPCs surface markers CD133andVEGF receptor-2, and for β-catenin was performed using prediluted (1:100 dilution) primary antibodies against CD133 (clone AC133; MiltenyiBiotec, BergischGladbach, Germany); VEGF receptor-2(clone KDR-2; Sigma-Aldrich, Missouri, USA), and β-catenin (Santa Cruz sc-7963). Immunoreactivity was developed using the streptavidin-biotin-immunoenzymatic antigen detection system (Neo Markers, Fremont, USA), which was performed according to manufacturer's protocol.The bound antibodies were detected using Ultra Vision Detection System Anti-Polyvalent, HRP/DAB (Dako REAL, EnVison™ Detection System, Denmark). Positive immunostaining was defined as a cytoplasmic and/or membranous staining of cells.Positive and negative controls were included in all runs. Staining density was semi-quantitatively assessed for each antibody separately and was defined as the percentage of positively stained endothelial cells to the total number of endothelial cells in ten randomly selected high-power fields (HPFs ×400).(19)The combined EPCs surface markers (CD133+/VEGF receptor-2++) reflect a subpopulation of EPCs with vaso-regenerative functions.(20)

Statistical analysis

One-way Analysis of Variance (ANOVA) followed by Tukey as a post-hoc test for multiple comparisons or Kruskal-Wallis test followed by Dunnett's multiple comparison for mean ranks between groups were used for parametric and non-parametric data, respectively. Data analysis was done using Statistical package: MATLAB Statistical toolbox (Matrices Laboratory

software-Math Works ®, USA) and data were expressed as means ± SD or medians ± interquartile range. Statistical significance was set at $p < 0.05$.

Results

Effect of vitamin K2 on serum lipid profile

Administration of HFD for 8 weeks exhibited a significant increase in atherogenic serum lipids and a significant decrease in serum HDL-C with a 50% higher atherogenic index compared to the normal control (**Table 1**). Treatment with vitamin k2 significantly improved lipid profile, yet its induced increase in serum HDL-C was not significant versus the vehicle treated HFD rats with an atherogenic index of about 20% lower.

Table 1: Effect of vitamin k2 on serum lipid profile in HFD fed rats

Lipid profile	Normal control	Vehicle-treated HFD	Vitamin k2- treated HFD
TC	115 ± 6.93	152± 8.37*	117.4± 8.53#
TGs	104.40 ± 13.17	153.6± 9.88*	109.4± 16.56#
LDL-C	45.22 ± 7.13	89 ± 9.80*	53.56 ± 12.76#
HDL-C	50.00 ± 5.52	31.60± 3.95*	33.56 ± 5.92
Atherogenic index	0.32 ± 0.04	0.69 ± 0.05*	0.52± 0.12#

TC; total cholesterol, TGs; triglycerides, LDL-C; low-density lipoprotein cholesterol, HDL-C; high-density lipoprotein cholesterol, HFD; high fat diet. Data are expressed as means ± S.D. *: significance difference versus normal control, #: significance difference versus vehicle-treated HFD.

Effect of vitamin K2 on NOX4 gene expression

Expression of NOX4 in aorta was significantly reduced in vehicle-treated HFD fed rats versus normal control, whereas, its expression was significantly increased after 8-week treatment with vitamin k2 (**Figure 1**).

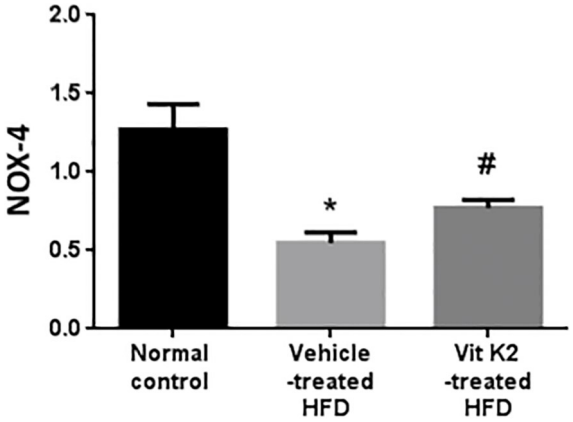

Figure 1: Effect of vitamin k2 on aortic NOX-4 expression. Data are expressed as means ± S.D. *: significant difference versus normal control, #: significant difference versus vehicle-treated HFD.

Effect of vitamin k2 on CD133/VEGFR2 *expression*

A significant decrease in percentage of $CD133^+$ cells was displayed in the endothelial layer obtained from vehicle-treated HFD rats versus normal control. Conversely, vitamin k2treatment resulted in a significant increase in $CD133^+$ cells percent (**Figure 2 A and Figure 3 A-C**).Regarding the percentage of $VEGFR2^+$ cells, it was non-significantly increased in vehicle-treated HFD rats versus normal control, whereas, vitamin k2-treated HFD rats showed a significant increase only versus normal control (**Figure 2 B, and Figure 4 A-C**). However, the percentage of combined EPCs surface markers (CD133/VEGFR2) was significantly decreased in vehicle-treated HFD rats versus normal control and it was significantly increased with vitamin k2 treatment compared to vehicle-treated HFD rats (**Figure 2 C**).

Effect of vitamin K2 on β-catenin⁺cells

A significant decrease in percentage of β-$catenin^+$ cells obtained from vehicle-treated HFD rats' aortae was observed versus normal control. However, 8-week treatment with vitamin K2 has significantly increased the percent of β-$catenin^+$ cells versus vehicle-treated HFD rats (**Figure 2 D and Figure 5 A-C**)

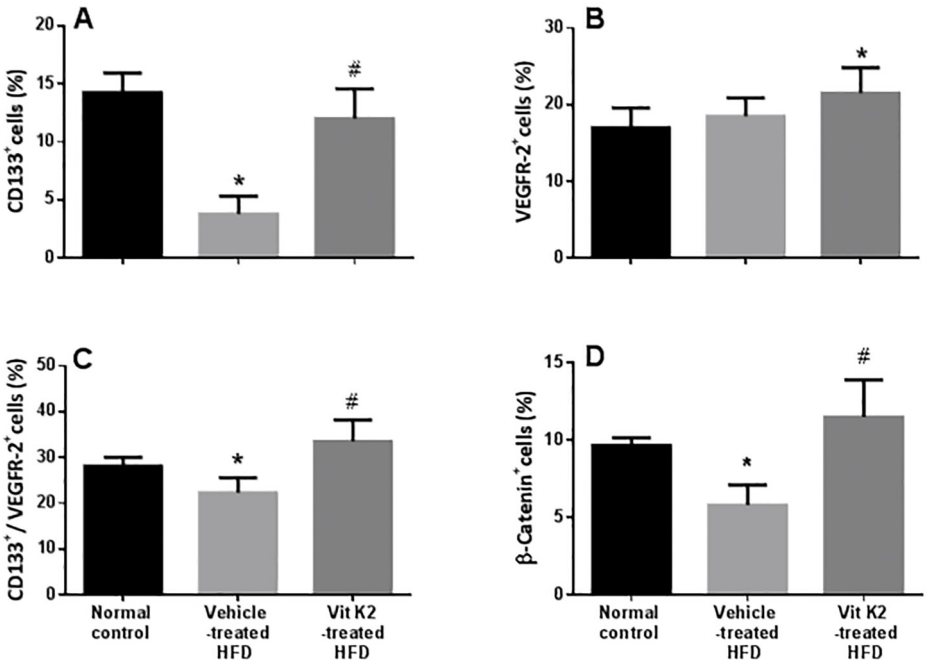

Figure 2:Immunohistochemical semiquantitative analysis of vitamin K2 effect on HFD changes in aortic $CD133^+$ cells (A), $VEGFR2^+$ cells (B), combined $CD133^+$/$VEGFR2^+$ cells (C), and β-$catenin^+$ cells (D) expression. HFD; high fat diet, K2; vitamin K2, VEGFR2; vascular endothelial growth factor receptor 2. Data are expressed as means ± S.D. *: significant difference versus normal control, #: significant difference versus vehicle-treated HFD.

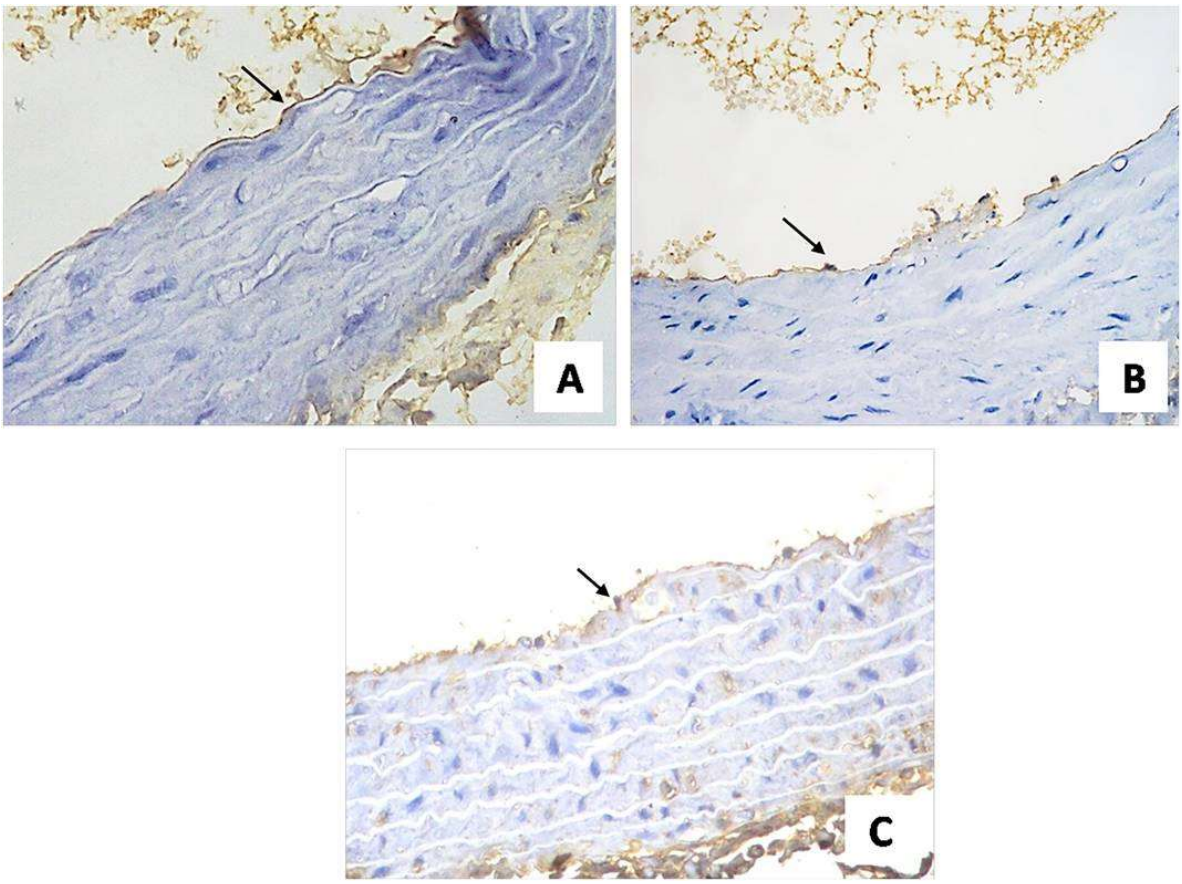

Figure 3: Photomicrographs of immunohistochemically stained sections of rats' aorta for CD133$^+$ cells. Normal control rat's aorta in (A) showing dispersed CD 133$^+$endothelial cells (↑) with brown stained nuclei, which were decreased in vehicle-treated HFD rats (B). (C) showed an increase in number of CD 133 $^+$endothelial cells in vitamin K_2 treated HFD rats. (×400)

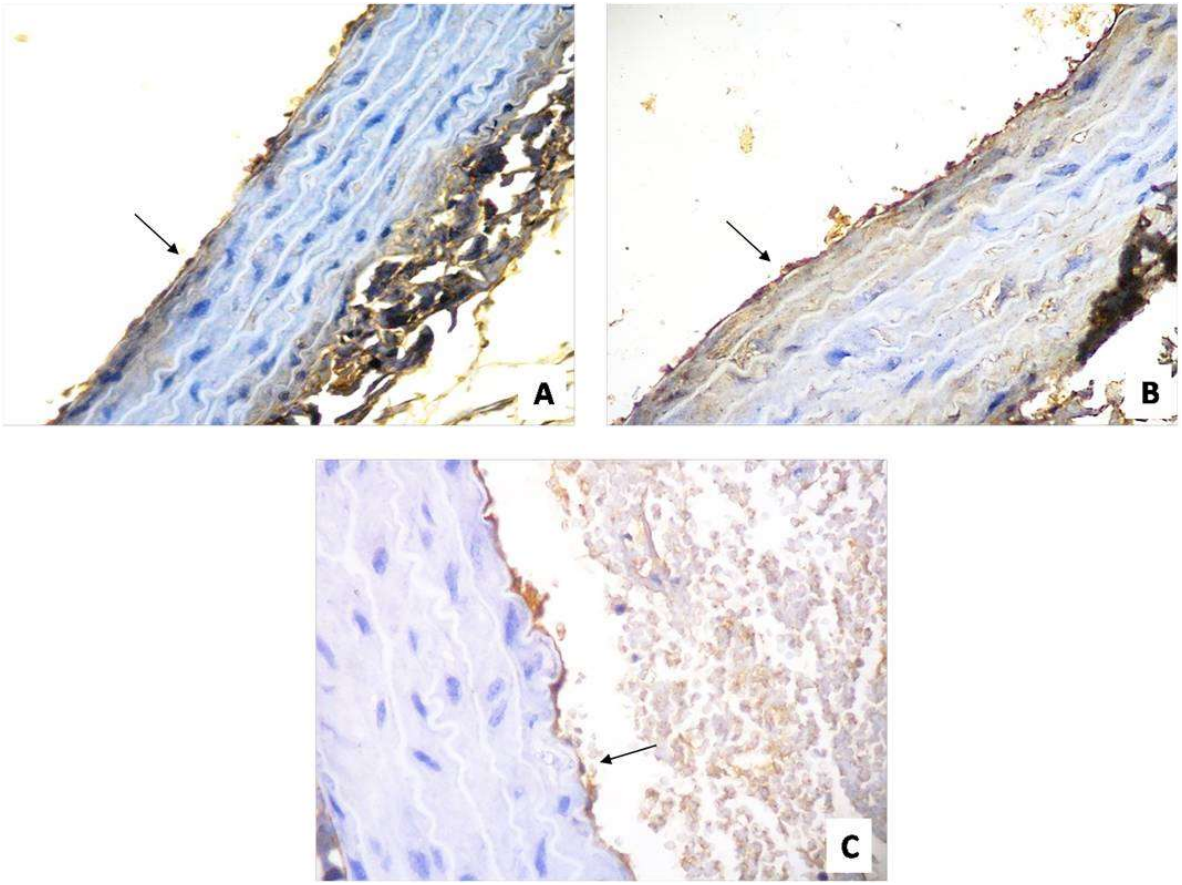

Figure 4: Photomicrographs of immunohistochemically stained sections of rats' aorta for VEGFR2⁺ cells. Normal control rat's aorta in (A) showing dispersedVEGFR2⁺ endothelial cells (↑) with brown stained nuclei, which were decreased in vehicle-treated HFD rats (B). (C) showed moderate increase in number of VEGFR2⁺ endothelial cells in vitamin K_2 treated HFD rats.(×400)

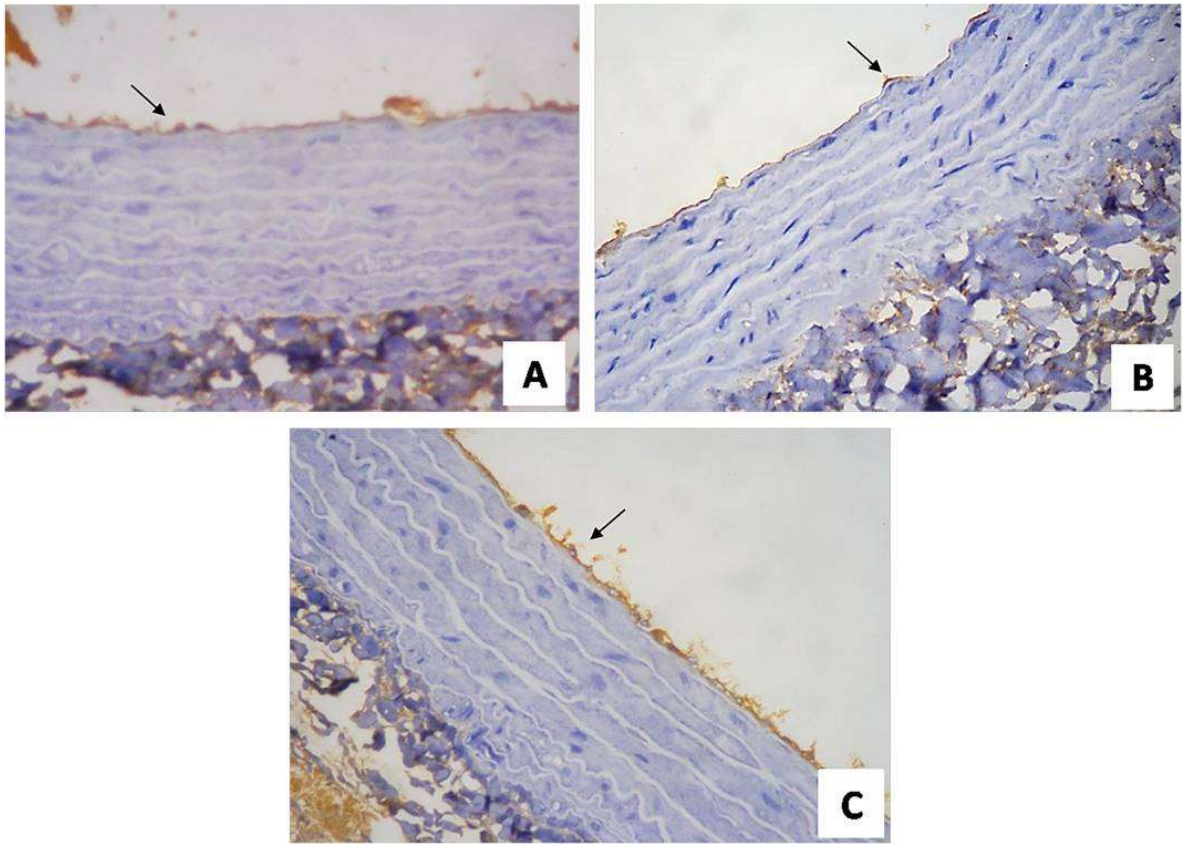

Figure 5: Photomicrographs of immunohistochemically stained sections of rats' aorta for beta-catenin⁺ cells. Normal control rat's aorta in (A) showing dispersed beta-catenin⁺ endothelial cells (↑), which were decreased in vehicle-treated HFD rats (B). (C) showed marked increase in number of beta-catenin⁺ endothelial cells in vitamin k²treated HFD rats.(×400).

Effect of vitamin k2 on the histopathological features in HFD aortae

Administration of HFD for 8 weeks revealed early atherosclerotic changes in comparison to normal control in H&E staining in the form of intimal irregularity with focal loss of the endothelium. The underlying tunica media appeared thin and showed fatty streaks formed of aggregates and dispersed foam cells. All these structural derangements were partly ameliorated after vitamin K2 treatment (**Figure 6 A-C**) as evidenced by the semiquantitative analysis, where a significant decrease in fatty aggregates in comparison to the vehicle-treated HFD rats' aortae was noted in vitamin K2 treated group (**Figure 7**).

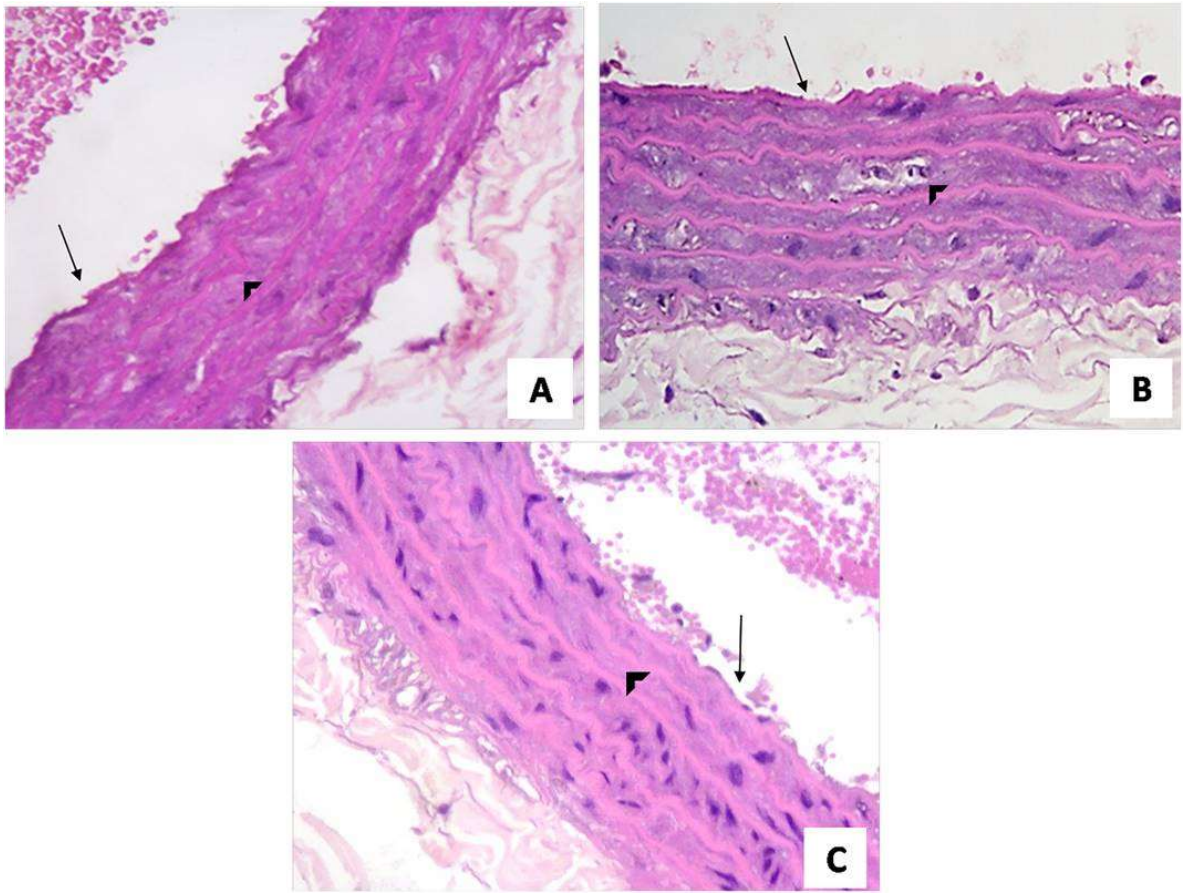

Figure 6:Photomicrographs of H&E staining of aortic tissue sections, showing in (A), normal control; a layer of flattened endothelial cells in the tunica intima (arrow) and the tunica media formed of several elastic lamellae with smooth muscle fibres in between. (B), vehicle-treated HFD rats, irregularity of tunica intima with focal endothelial shedding (arrow) and the underlying tunica media shows fatty streaks formed of aggregates and dispersed foam cells (arrow head). (C), vitamin K_2-treated group; minimal irregularity of the tunica intima (arrow) with fatty streaks in the underlying tunica media (arrow head). (×400).

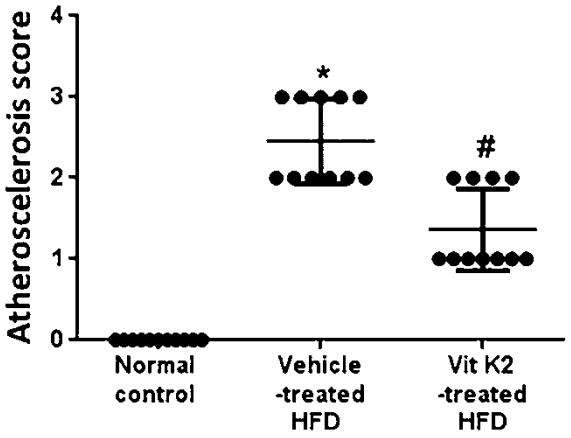

Figure 7: Atherosclerosis score. Data are expressed as medians ± interquartile range. *: significant difference versus normal control, #: significant difference versus vehicle-treated HFD. HFD; high fat diet, k2; vitamin k2.

Discussion

Cardiovascular morbidity and mortality are still rising despite the protection conferred by multiple therapies targeting major cardiovascular risk factors, which denotes the insufficient vascular repair process. An essential facet of this repair process is the EPCs signalling pathway in view of the cardiovascular risks as dyslipidaemia.

In the current study, administration of HFD for 8-weeks retrieved worsening in serum lipid profile. Instantaneously, the expression of composite surface markers of EPCs was reduced in association with endothelial NOX4 and β-catenin under-expression. These changes shared into vascular functional and structural damage, being evidenced by the altered histopathological features of the aortae and the elevated atherosclerotic score.

In context of dyslipidaemia, the induced inflammatory reactions could play a role in changing EPCs microenvironment and inducing apoptosis, thus impairing their ability to contribute into vascular repair.(21) The endothelial inflammatory reactions induced by dyslipidaemia is essentially vasculo-protective in nature but if not maintained under strict homeostatic control, it will perpetuate a harmful redox imbalance that will eventually impact the EPCs differentiation and life span inducing an endothelial dysfunction.(22)

One of the major factors involved in the homeostatic redox balance is the NOX4. Indeed, in the present study, a down regulation of NOX4 expression was observed that was associated with a reduction in EPCs numbers and vascular structural damage. This could emphasize on the reported NOX4 important role in vascular protection that involves the activation of the stress responsive genes within the EPCs.(23, 24)

Being a master regulator of growth control pathway, β-catenin activation permits its interaction with relevant transcription factors, endorsing endothelial cells' survival and EPCs' proliferation.(25)The observed β-catenin downregulation by dyslipidaemia could support the observedreduction in EPCs numbers, though its mechanism is not clear. Studies reported a role of Dickkopf protein, an inhibitor of Wnt signalling pathway induced by dyslipidaemia, in β-catenin suppressionwithin the early atherosclerotic lesions.(26)Moreover, NOX4 downregulation with excess ROS accumulation can alter the downstream signalling of β-catenin diverting its positive contribution to EPCs differentiation into a pathological fate.(27)Consequently, the current findings could deduce how the altered EPCs' signalling induced by dyslipidaemia could induce an ED. This necessitates the emergence of drugs promoting the EPCs regenerative capacity.

In order to assess vitamin k_2 ability to promote EPCs repair, vitamin k_2 was given for HFD fed rats for 8 weeks. It exhibited a vascular protection against the injurious effect of HFD-induced dyslipidaemia. Vitamin k_2 increased EPCs' number and ameliorated vascular structural abnormalitieswith an increase in NOX4 and β-catenin expression.

In this context, vitamin k_2 induced increase in NOX4 expression that was associated with an anti-atherogenic potential and a vascular structural improvement emphasized on the previously reported NOX4 endothelial protective action.(28)Besides increasing NOX4 expression, vitamin k2 is reported to possess an antioxidant potential. It was recently demonstrated that vitamin k2 triggers the SIRT1 signaling pathway, resulting in increasing mitochondrial antioxidant superoxide dismutase deacetylation and decreasing mitochondrial ROS production.(29)

Likewise, the observed increase in β-catenin expression by vitamin k_2 could justify its induced increase in EPCs count. This could be in part achieved via activation of SIRT-1,thusfavoring the involvement ofβ-catenin signalling in an EPCs reparative potential.(30)Moreover, the reported anti-inflammatory and antioxidant potentials of vitamin k_2 could add to its EPCs beneficial effect. Besides its ability to downregulate several inflammatory genes,(31)its antioxidant potentials entail an ability to mediate cellular redox homeostasis. The latter is mediated via restoring mitochondrial dysfunction, aerobic glycolysis, and oxidative phosphorylation.(23)Also some of vitamin k redox-cycle enzymes; as vitamin K-oxidoreductase, do possess a free radical-scavenging potentials that can regulate NOX activity (32) and inhibit lipoxygenase; thus halting the cascade of oxidant and pro-inflammatory mediators.(33)

Moreover, vitamin k_2 can also act in a hormone-like manner fostering interactions between key microRNAs, transcription factors, sirtuins, and histone deacetylases that can all share in tempting cell fate.(34) This could explain the observed improvement in EPCs number and upregulation of NOX4 and β-catenin by vitamin k_2. This is in line with previous study,(35) which recorded an improvement in nitric oxide-dependent endothelial function in ApoE/LDLR−/− mice by Vitamin k_2 -MK-7.

On the other hand, vitamin k_2 improved the atherogenic profile of HFD fed rats. It thrived to reduce both the atherogenic index and the atherosclerotic score. This could be attributed to the reduction in LDL-C. This is consistent with previous studies, confirming variable antihyperlipidemic and antiatherogenic actions of vitamin k_2 that can eventually restrain the atherosclerotic progression.(36)

In agreement with the current work, vitamin k2 was stated to decrease total cholesterol and preventing its vascular deposition as esters (36, 37) rather than on an ability to modulate HDL-C, which was lacking in this work.

Nevertheless, one cannot neglect the reported vitamin k2 vasoprotective potential that pillars on its ability to promote post-translational carboxylation of matrix Gla-protein, a major endogenous calcification inhibitor.(38)This was found not only to inhibit vascular deposition of calcium crystals, but to further inhibit cholesterol deposition during the process of atherogenesis.(39)Overall, these raised vascular protective potential mechanisms of vitamin k2 supplement were translated into an evident vascular structural improvement that further corroborate the contribution of the currently observed increase in EPCs number into an observed repair.

In conclusion, vitamin k2 supplement was effective in preventing the injurious insults of the induced dyslipidaemia and inhibiting the progression of atherosclerosis. This is thought to be due to a possible interplay between vascular Wnt/β-catenin signalling and NOX4 expression that thrived to inducing EPCs repair signalling and increasing their number in addition to improving vascular structural integrity.

Declaration of interest

None.

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