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Research Article
Caffeic Acid Phenethyl Ester Inhibits the Progression of Elastase Induced Aortic Aneurysm in Rats

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Abstract

Background and Objective: Caffeic acid phenethyl ester (CAPE) is the active constituent of propolis. The study evaluated the effect of CAPE on aortic aneurysm (AA) in rats induced with perfusion of Elastase. **Materials and Methods:** For the study 36 male Sprague Dawley (n = 12 each group) rats were divided into Sham operated, control and CAPE treated groups. The control and CAPE treated group were subjected to intra-aortic perfusion of elastase to induce AA whereas the sham group received perfusion of saline. The rats of CAPE group received dose of 10 µmol/day control and sham group received saline treatment throughout study. The aortic diameters were recorded before the elastase infusion, post-infusion and final. Blood pressure was also recorded twice before administration and on final day before sacrificing the rats. The aortas were harvested on 24th day and were evaluated for western blot analysis, immunohistochemistry followed by staining with Millers Elastin-Van Gieson. **Results:** The control rats showed significant increase of aortic size ($p<0.05$) versus the sham operated group after 24 days, whereas the CAPE treated group showed decrease in aortic size compared to control ($p<0.05$) with no alterations in blood pressure. CAPE treated rats exhibited decreased expression of metalloproteinase (MMP-2 and MMP-9) and monocyte chemotactic protein-1 (MCP-1). The treatment demonstrated significant decrease in levels of iNOS. Treatment also prevented loss of vascular smooth muscle cells (VSMCs) in aortic walls. **Conclusion:** The CAPE inhibited the progression of aortic aneurysm in rats by curbing inflammation, reducing oxidative stress and conserving VSMCs. The findings suggest a new therapeutic approach in managing AAs.

Key words: CAPE, aortic aneurysm, inflammation, metalloproteinase, VSMCs, Inos

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The occurrence of Aortic aneurysm (AA) also called as abdominal aortic aneurysm has been evidenced for mortality of upto 90% deaths in US alone[1]. The deaths are diagnosed to be due to rupture of aneurysm. The disorder is among the leading causes of deaths in United states of America (USA)[2]. The present treatment approaches for AA include invasive strategies such as surgery and implantation of stents[3]. A study reveling two randomized controlled clinical trials suggested that early surgery of AA with narrow diameters (diameter<5.5 cm) contributed no survival advantage[4,5]. The study hence established surgery as treatment mode for subjects with AAs having diameters of 5.5 or more. It was been found in a survey involving screening of AAs in USA that about 90% of patients reported for AAs suffered from small diameters (<5.5 cm) hence were unfit for surgical treatment and were only put for observation in clinics[6-8], moreover to convert small diameter AAs to acceptable larger diameters of 5.5 cm or greater for surgical operation require time[9]. Therefore a pharmacotherapy for treating AAs has been desired from long time. There are still no accepted and established prophylactic therapies for limiting growth of aneurysm preventing surgery.

Reports suggests correlation between aortic aneurism with destruction of both extracellular matrix and aortic wall structural integrity[10,11]. It has been evidenced that proteins such as elastin and collagen together contribute necessary tensile strength and resilience to aorta, demolition of these proteins is an important feature for expansion followed by rupture of the aneurysm. Destruction of vascular matrix of aortic wall in aneurysm is chiefly due to inflammatory response[12]. Proteolytic digestion has been found to be one of the mechanism by which the inflammatory response demolishes the matrix of connective tissue. Metalloproteinases (MMPs) group of enzymes have been one of the chief among the inflammatory infiltrates responsible for destruction of proteins[13,14].

Caffeic acid phenethyl ester also known as CAPE is reported to be among the major active medicinal moiety of propolis which is produced by honeybees of American and European countries. Propolis is reported to contain variety of chemical constituents such as flavonoids, esters of phenolic acids, terpinoids and many aminoacids[15]. The CAPE have been investigated for activities which include anti-bacterial, anti-inflammatory, anti-fungal, anti-cancer and anti-viral[16-22]. Anidjar *et al.*[23] while investigating the chemopreventive role of CAPE found that the molecule exerted anti-metastatic effect via inhibiting levels of both metalloproteinase i.e., (MMP-2, MMP-9). Since CAPE inhibits MMPs which contributes to be a factor in developing AAs the aim of work was to find the effect of CAPE on progression of AAs in direction of developing an alternative therapy for their management.

MATERIALS AND METHODS

Treatment groups in rats and induction of AA model: All the experimental protocols performed were in accordance with institutional ethical committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine, JiNan, China. (AEC/AHSUTCM/2016/13). For the study total 36 male rats aging 6 week were selected weighing approximately 175-210 g. The rats were selected indiscriminately and parted into three groups (12/group) CAPE, sham operated and control. All animals were put up by maintaining 12 h light and dark time cycle, the rats were fed with recommended diet followed by sufficient drinking water. Before subjecting the rats to laparotomy procedure described as per Anidjar *et al.*[23] and Petrinec *et al.*[24] rats were anesthetized. Briefly, aorta of rats was removed to the bifurcation at a level of left renal vein. A small incision was made at the site of the bifurcation of aorta followed by insertion of a polyethylene tube (No. 10, Smiths medical Int. UK). The aorta was then clamped with polyethylene tube just above the tip level and was ligated near the aortic bifurcation using a silk suture (4-0, Ethicon, J and J, New Jersey) and then subjected to perfusion of 27.2 U mL^{-1} of type 1 porcine pancreatic elastase (PPE) (Sigma) in all the animal groups. A 10 mL Saline perfusion charged with elastase (81.6 U) was loaded in aorta for time of 10 min with the help of infusion pump operated at 2 atmospheric pressure. All the clamps and tyings were removed along with tubing of polyethylene after completing perfusion after which the incison was closed using an suture (polypropylene 8-0) (Prolene, J and J). At last aortas of rats were removed and were processed for experiments 24 days post-perfusion. During protocol systolic blood pressure was recorded.

Treatment protocol: Dose of CAPE was fixed in accordance to published reports, 10 µmol/day was fixed as a dose for present work prepared by dissolving in isotonic saline solution. The selected dose of CAPE was administered to each animal in group (n = 12) via intraperitoneal route daily using a injection[25]. The dose administration of CAPE started one day prior to PPE perfusion until the rats were sacrificed. Similarly in sham and control group (n = 12 in each) saline solution was injected for same duration as for the CAPE treatment.

Measurement of aortic diameter: For measurement of aortic diameter the rats after 24 days of elastase perfusion were anesthetized by injecting sodium pentobarbital intraperitoneally. The Aortic Diameter (AD) was measured by reexposing abdominal aorta by laparotomy using microcalipers. Attention was made in measuring diameters preferably from areas having maximum aortic dilatation within the segment which was subjected to perfusion previously. In the process to harvest tissues the animals were sacrificed by injecting overdose of pentobarbital intravenously.

The AD was recorded for each animal in states of pre-perfusion, post-perfusion and final. The magnitude of aortic dilatation i.e., ΔAD was calculated as difference between pre-perfusion and final AD (ΔAD mm = final AD-pre-perfusion AD) and was represented as percentage change (ΔAD %) and individual animal served as its own control for statistical significance. The values of ΔAD (%) being 100% were considered to be significant (size twice the normal) which were in correlation to defined clinical values of AAs. All the three groups (control, sham and CAPE treated) were reported for values of AD which were mean±standard error of the mean for pre-perfusion, post-perfusion, final, DAD in mm and DAD (%). Finally the inhibition (%) of aortic dilatation was calculated by comparing the values of mean DAD (%) of each treatment group of rats verses the control rats. Statistical significance between the groups was established by performing analysis of variance (ANOVA) using Student-Newman-Keuls test for making multiple comparisons[26] or with the 2-tailed Student t-test for paired comparisons.

Histologic studies: For histological study the animals of all the three groups post operation (control, sham and CAPE treated) were sacrificed. Recovered aortas from rats were subjected to fixation in neutral-buffer (10%) and were further embedded in paraffin to obtain cross sections. The obtained cross sections (5 Mm) were submitted to staining of hematoxylin and eosin also the cross sections were exposed for Millers elastin-Van Gieson all the protocols followed standard established procedures. The results accounted area % filled by elastin-Van Gieson of total elastic fibers. All results were recorded by a MacScope morphometry syst. (2.2) (Tokiyo).

Expression of matrix metalloproteinase levels (MMP-2 and MMP-9) immunohistochemical analysis: To evaluate the levels of MMPs enzyme locally in aortic walls 24 days post operating animals, monoclonal antibodies of mouse were used for MMPs types (MMP-9 and MMP-2). For α-smooth muscle cells actin (Abcam) a mouse monoclonal anti-body was utilized mainly to evaluate the reduction of SMCs medially. A complex of immuno-peroxidase avidin and biotin was used to perform immuno-histochemical staining. The sections were subjected to incubation after blocking them for activity of endogenous peroxidase in I^{gy} anti-bodies (1:100) for overnight at 4°C. Further in accordance to instructions from manufacturer (Vectastain, Vector Labo.). The tissue sections were incubated with anti-mouse IgG antibody (biotinylated) for time period of 30 min followed by incubation with a complex of avidin-biotinylated and enzyme horseradish peroxidase in PBS for next 10 min. The formed complex was viewed using 0.05% solution of 3,3'-diaminobenzidine and slides stained with hematoxylin.

Western blot analysis: After sacrificing all the animals of defined groups 24 days post operation the aorta were homogenized to extract total proteins from them. The target tissue samples (75 mg) were submitted to electrophoresis in polyacrylamide gel of sodium dodecyl sulfate applying 80 V, then channelized on membranes made of polyvinylidene difluoride, subjecting them at 300 mA followed by incubation at room temperature for 60 min in mixture comprising Tris-buffer saline, non-fat milk (5%) and Tween20 (0.2%). Further the membranes were incubated at 4°C for 24 h with anti-bodies such as mouse monoclonal anti-bodies for MMP-9 and MMP-2 in ratio 1:2000. Inducible nitric oxide synthase (iNOS) was used with dilution of 1:4000, rabbit anti-bodies (polyclonal) for MCP-1 i.e., monocyte chemoattractant protein 1 in dilution of 1:5000. All of them were subjected to rinsing with Tris buffer along with 0.1% solution of Tween20 and were incubated for 2 h with sheep anti-mouse IgG anti-body for two metalloproteinase i.e., MMP-2 and MMP-9, also with anti-body for iNOS in ratio 1:5000. The membranes were observed with the help of ECL chemiluminescent kit (Amersham Biosciences) following the procedure as given by manufacturer. Beta Actin was used as standard loading protein, density of bands was measured and compared using densitometry (Shimadzu).

Data interpretation by statistical evaluation: All the data were calculated as average values±mean standard error, the calculations were done by using graph pad software. Comparisons between groups were done by opting using one-way ANOVA, the established level of significance was $p<0.05$.

RESULTS

Changes in blood pressure due elastase perfusion: Effect of elastase perfusion to induce aortic aneurysm on systolic blood

pressure was assessed before treatment of CAPE and after 24 days post-perfusion followed by treatment. Observations showed no significant changes in systolic blood pressure (Table 1) of control, sham and CAPE treated group of rats ($p>0.05$) suggesting no role of CAPE treatment on blood pressure of animals.

Development of aortic aneurysm by elastase perfusion method: In the study an aortic aneurysm rat model was created by elastase perfusion method, all rats were induced for AAs (total 36). Followed by elastase perfusion and operating the animals a total of 30 rats were found to survive on the next day, two animals from each group died; however no mortality was observed in the pre-operation period. Each group was left with 10 animals. Aortic diameters of control group were significantly ($p<0.05$) greater compared to sham operated group confirming successful induction of AAs in rats (Table 2). In the control group and CAPE group the mean aortic sizes in pre-infusion period were not significantly varied (control group 1.45±0.1 mm and CAPE group 1.45±0.1 mm). The aortic diameters recorded in post-infusion period also do not exhibit any difference between control group (2.35±0.2 mm) and CAPE group (2.38±0.3 mm). A high degree of significance was observed for pre and post-infusion aortic diameters of control and CAPE rats ($p<0.001$). After 24 days the rats were subjected to operation significant difference in aortic diameters were observed (Fig. 1). On the post-operative day the control rats were found to have aortic size 4.65±0.7 mm comparably significant results were observed with CAPE treated rats with diameters of 3.35±0.6 mm ($p<0.001$). The treatment of CAPE reduced the aortic expansion by 38% compared to control rats.

Table 1: Results of systolic blood pressure in rats before and after elastase perfusion followed by respective treatments

Groups	Before perfusion (BP mm Hg)	After 24 days post perfusion (mm Hg)
Control (n = 12)	108±2.3	109±1.9
Sham operated (n = 12)	107±1.9	108±2.8
CAPE treated (10 µmol/day) (n = 12)	108±2.5	110±1.9

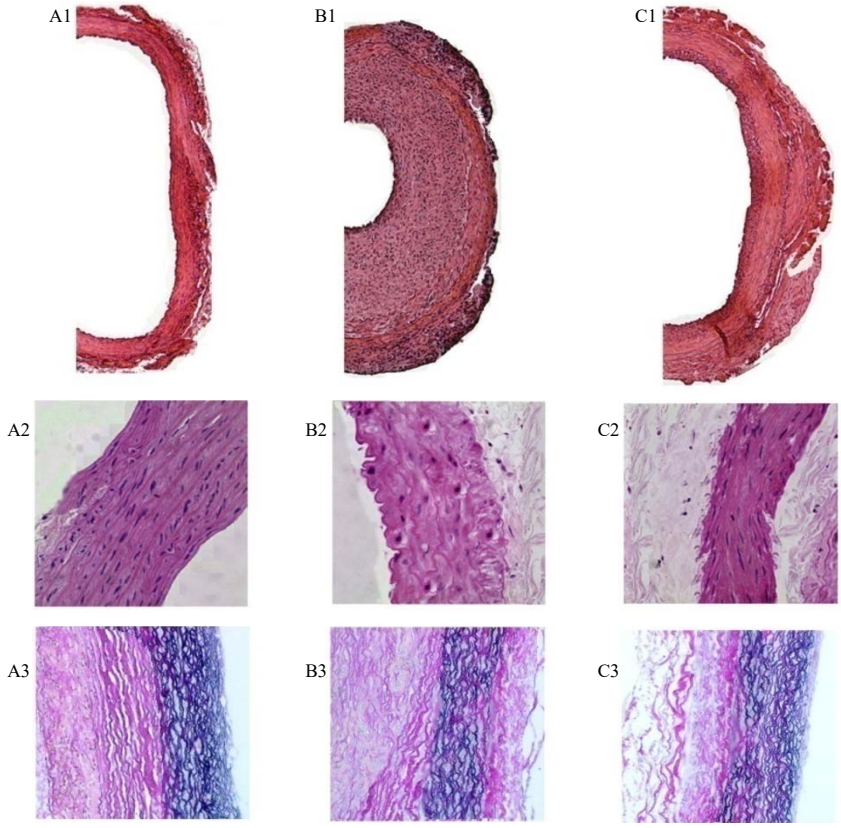

Fig. 1: Histologic sections of rat aorta stained with hematoxylin and eosin staining (A1-A3) Sham operated, (B-B3) Control and (C1-C3) CAPE treated

A1, B1, C1: Resolution 40x and A2, B2, C2: Resolution 200x. Staining of sections of rat aorta with Miller's elastin-Van Gieson staining (Resolution 100x), the dark purple color shows staining of elastin

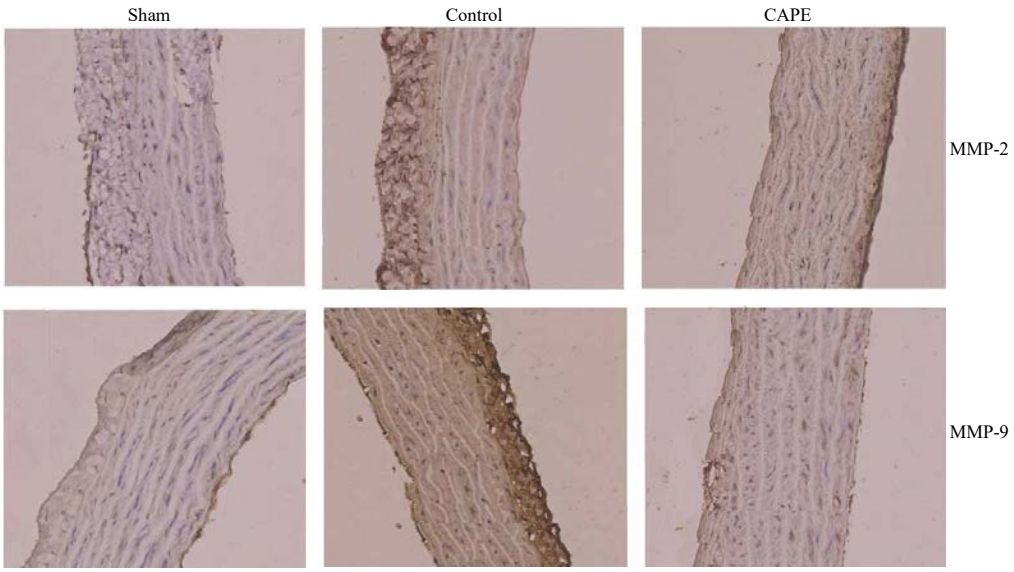

Fig. 2: Immunohistochemical staining for mapping MMP-2 and MMP-9 (400x) in aortic tissue

Table 2: Results of Aortic Diameters (AD) measurements of rats subjected elastase perfusion followed by CAPE treatment

Groups	Diameter of AA (mm) before elastase perfusion	Diameter of AA (mm) after elastase perfusion	Diameter (mm) of AA after 24 days of elastase perfusion	ΔAD (mm)	ΔAD (%)
Sham operated (saline treated) (n = 10)	1.42±0.01	1.40±0.01	1.41±0.01	0.01±0.01	0.70±0.1
Control (Saline treated) (n = 10)	1.45±0.01	2.35±0.2*	4.65±0.7**,#	3.2±0.9	220.68±0.8
CAPE treated (n = 10)	1.45±0.01	2.38±0.3*	3.35±0.6**,#	1.9±0.2	131.01±0.3

*p<0.05 Aortic Diameters (AD) after post elastase perfusion compared to control group diameters before perfusion, **p<0.001 Aortic diameters (AD) after 24 days post perfusion compared to control group diameters before perfusion, #p<0.05 Aortic diameters after 24 days post perfusion compared to control group diameters after elastase perfusion

Histological studies for changes in structure of aortic walls: The rats of all groups were sacrificed post 24 days of treatments; aortas were harvested and were submitted to hematoxylin and eosin staining. The staining distinctly reveled thinner aortic walls with increased mural thrombus in control group as compared to CAPE treated rats (Fig. 1(A1 ,B1, C1, A2, B2 and C3)). Staining of slides with Elastin-Van Gieson indicated significant degeneration of elastic lamellae in control group versus sham group (Fig. 1(A3, B3 and C3)). The CAPE treated and sham group however do not exhibited any degeneration and the medial elastic lamellae.

Immuno-histochemical studies for expression of metalloproteinase (MMP-2 and MMP-9): Immunohistochemical staining of aortic walls were done to analyze the activated MMP-2 and MMP-9 (Fig. 2). Strong staining of both activated metalloproteinase subunits was shown by aorta of rats of control group (Fig. 2) whereas a less accented stain was seen in aorta of CAPE treated rats which was stronger as compared to staining of sham rats. Results clearly indicated association of AAs with levels of both MMP-2 and MMP-9.

Findings of western blot were parallel to results of immuno-histochemical staining, the results depicted over expression of both metalloproteinase subunits (MMP-2 and MMP-9) in control rats compared to sham rats (Fig. 3a). Expression levels of both MMP-2 and MMP-9 were significantly lower in CAPE treated rats compared to control ($p<0.01$, Fig. 3b), however when compared to sham operated rats the MMP-2 expression levels were highly significant ($p<0.05$, Fig. 3b) but differences in levels of MMP-9 were not significant ($p<0.18$, Fig. 3b).

Results for loss of vascular smooth muscle cells (VSMCs): Immunohistochemical studies suggested vascular smooth muscle cells (VSMCs) loss of aortic wall of rats belonging to control group, conservative role of CAPE on VSMCs was observed in CAPE treated rats (Fig. 4).

Effect of CAPE treatments on levels of MCP-1 and iNOS: Western blot analysis of MCP-1 and expression of iNOS in aortic tissues by densitometry was performed Fig. 5a. Levels of MCP-1 a pro-inflammatory cytokines along with iNOS was done from aortic walls. Expression of both MCP-1 and iNOS in

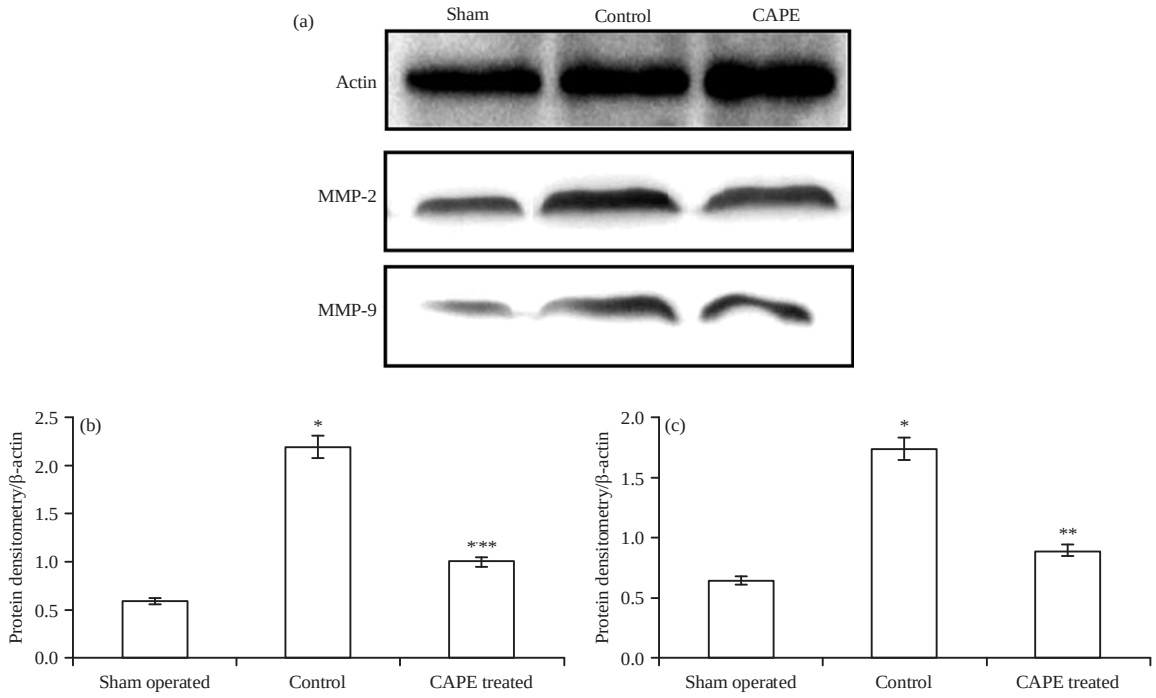

Fig. 3(a-c): (a) Western blot analysis for expression of MMP-2 and MMP-9 in aortic wall of rats and Quantitative results of densitometry for (b) MMP-2 and (c) MMP-9

All the results are calculated as Mean±standard error of mean, *p<0.05 compared to sham operated rats, **p<0.01

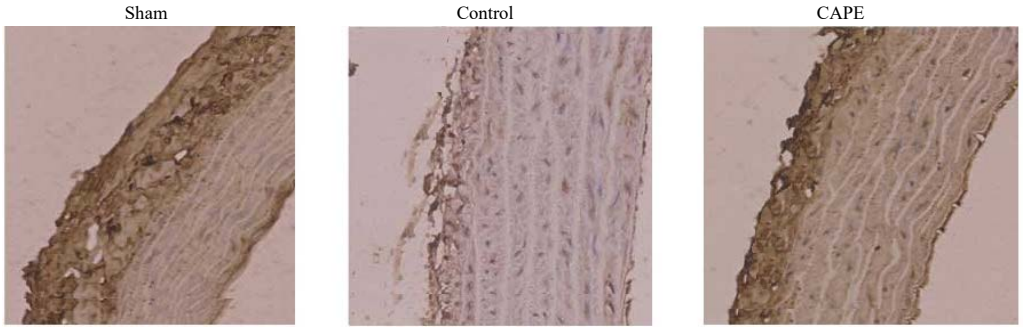

Fig. 4: Immuno-histochemical staining for preservation of vascular smooth muscle cells (VSMCs) in aortic tissues of AAs (magnification 400x)

control rats was on a significantly higher side versus the levels of sham group of rats (Fig. 5b, p<0.05), whereas the aortic tissues of CAPE treated rats exhibited lower levels against rats of control group (Fig. 5b, p<0.05).

DISCUSSION

The CAPE has been found to be the chief active constituent of propolis. Recently research shows potential ole of CAPE as anti-inflammatory, anti-bacterial, anti-fungal and anti-cancer agent[16-22] one major research have also proved the inhibitory effect on expression of MMPs[23]. Looking to the MMPs inhibitory effect of CAPE and role of MMPs in development and progression of AAs, CAPE could interfere and suppress the progression of AAs.

The study was initiated by inducing AAs in rats by elastase perfusion method as described by Anidjar *et al.*[23] and Petrinec *et al.*[24]. The animals were subjected to surgery then after by perfusion of elastase (27.2 U mL^{-1}) PPE in all the animal groups. The aorta was perfused for 10 min with 10 mL saline loaded with elastase in concentration of 81.6 U with the help of infusion pump operated at 2 atmospheric pressure.

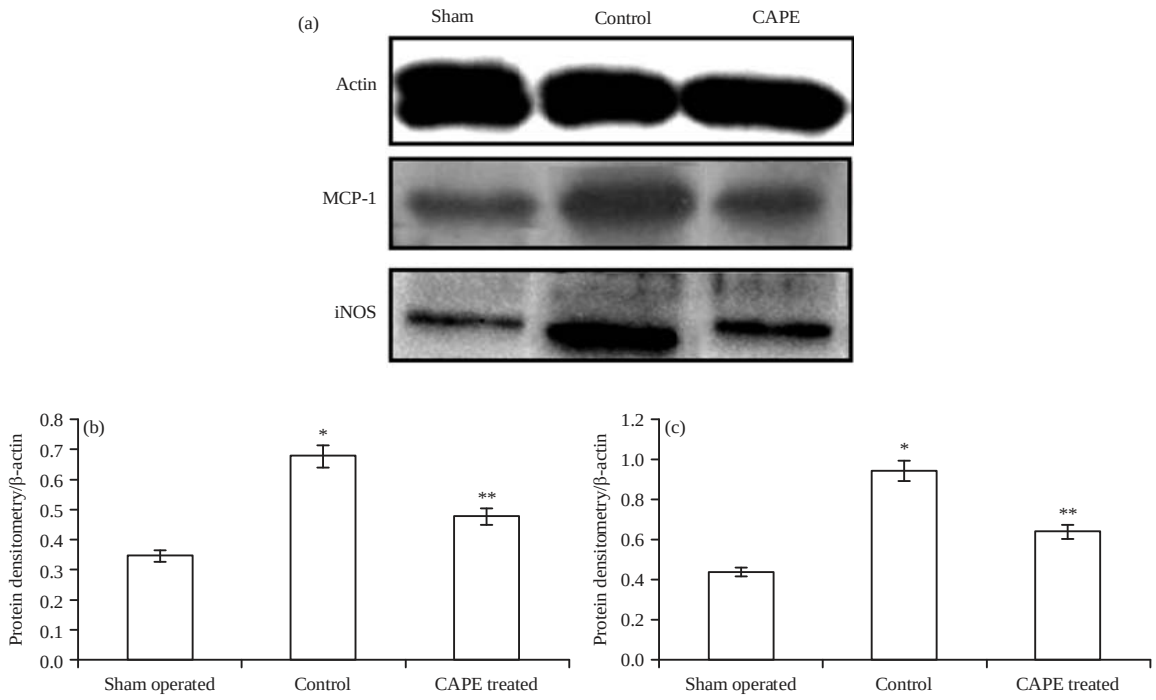

Fig. 5(a-c): (a) Western blot analysis of MCP-1 and expression of iNOS in aortic tissues by densitometry and Quantitative results for expression of (b) MCP-1 and (c) iNOS

*p<0.05 compared to sham operated rats, **p<0.05 compared to control

Development of AA is contributed by factors such as inflammation, demoliation of proteins and depilation of smooth muscle cells[27-32]. Inflammation leads to progression of AAs by causing infiltration macrophages and lymphocytes in the outer walls of aorta which further leads to secretion of MMPs and cytokines causing progression of AAs[33]. The enlargement of AAs has been found to contribute by degradation of elastin which is due to overexpression of matrix metalloproteinase[34]. All of above this few pro-inflammatory cytokines i.e., MCP-1 causes monocyte migration to the walls of developed aneurysm which further lead to secretion of MMPs and cytokines causing progression of aneurysm[35]. The outcome of the study documented over expression of both MMP-2 and MMP-9 in aortic tissues of rats subjected to AAs. The study confirmed attenuating role of CAPE towards inflammatory response by decreasing the levels of both matrix metalloproteinase (MMP-2, MMP-9) and also the levels of MCP-1 in AA rat models.

Destruction of smooth muscle cells is also one of the factors contributing to progression of AAs. Inflammation and oxidative stress mediated activation of nuclear factor-kB (NF-kB) are the reported mechanisms for destruction of VSMCs[27]. The results of immunohistochemical staining suggested loss of VSMCs in control group of rats, the cells were preserved in medial aortic wall of CAPE treated rats hence can contribute in inhibiting progression of AAs further by preserving VSMCs cells.

It has been reported that inflammation induced oxidative stress is associated with development of aortic aneurysm[36,37]. Studies have confirmed role of nitric oxide generated from iNOS in damaging aortic wall via oxidative stress mechanism hence confirming role of iNOS in vascular injury[38]. Results of the study suggested higher levels of iNOS in control rats where as the levels were on lower side in CAPE treated rats. The study confirmed role of CAPE in decreasing expression of iNOS and inhibiting development of AAs.

CONCLUSION

In the current study confirms that CAPE can inhibit expansion of elastase induced AAs in rats. The possible mechanisms could be by decreasing the expression of MMP-2, MMP-9 and MCP-1, decreasing the influx monocytes in aortic walls, preservation of VSMCs and modulating oxidative stress by inhibiting synthesis of iNOS. The findings of study have successfully established promising role of CAPE in managing aortic aneurysm.

SIGNIFICANCE STATEMENT

The present work is novel as there are no reports indicating use of Caffeic acid phenethyl ester in treating aortic aneurysm. This is the first report establishing usefulness of Caffeic acid phenethyl ester in aortic aneurysm.

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