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Original Article

# Effect of curcumin on the structure of thoracic aorta of adult male albino rat in experimentally induced calcification

Histology

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## ABSTRACT

**Background:** Vascular calcification is when calcium phosphate salts build up in the wall of blood vessels, making them stiff and less flexible, causing restriction of blood flow and making it harder for the body to move and breathe properly. **Objective:** To assess the possible role of curcumin in ameliorating the vascular structure in case of experimentally induced calcification of the thoracic aorta of adult male albino rats.

**Methodology:** An experimental study was conducted on forty adult male albino rats, each with an average weight of around 200 grams. They were divided into four equal groups: GA (control group). G B (curcumin group): Animals were given curcumin orally. G C (adenine group): Adenine was administered to rats with oral gavage for 4 weeks. Group D: (adenine & curcumin group): animals were given both adenine and curcumin orally for 4 weeks. After the experiment concluded, the animals were gently anesthetized. The thoracic aorta was carefully removed and split into two sections for further analysis. One part was studied under a light microscope, while the other part underwent a detailed examination using an electron microscope. Morphological measurements included the average area percentage of calcification in the thoracic aorta. The data obtained was statistically analyzed.

**Results:** The mean value of the calcification area ratio in the thoracic aorta of the adenine group showed a statistically significant increase in comparison with the adenine and curcumin group.

**Conclusion:** In the current experiment curcumin showed improvement of the ectopic calcification in the thoracic aorta, despite that curcumin cannot be considered the only specific line for treatment of this illness. In general, it is advisable to add curcumin to the diet daily because of its remarkable beneficial, curative and prophylactic effect on human health.

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**Keywords:** Adenine; calcification; curcumin; thoracic aorta.

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## INTRODUCTION

Vascular calcification occurs when calcium phosphate salts accumulate in vascular tissues in an inappropriate and pathological way. This can happen with normal aging, but it is accelerated in certain disease states, including chronic kidney disease. [1] The incidence of vascular calcification appeared during the fourth decade of life and was present in 12% of people under the age of 50. In older subjects, calcification is practically more common, reaching 100% after 65 years [2].

Vascular Calcification can occur in either the intimal or medial layers of the vascular wall. Both are associated with increased mortality [3]. Calcification of the medial layer can occur completely independently of atherosclerosis. It tends to be more diffuse and typically forms a sheet devoid of lipid or atherosclerotic changes [4]. Vascular calcification is affected by a variety of factors, including changes in mineral metabolism and systemic and local factors that can promote or prevent vascular calcification [5]. The etiology of vascular calcification is that vascular smooth muscle cells (VSMCs) undergo apoptosis and vesicle formation, are converted into osteoblast-like cells, induce matrix formation and are involved in the mineralization process [6].

Herbal products are widely used because they have few side effects, accessibility and affordability when compared with conventional medicine. [7] The use of curcumin in vascular protection may offer a safe, efficacious and cost-effective [8]. Curcumin reduces ectopic calcification by utilizing its antioxidant and anti-inflammatory properties. It can effectively reduce inflammation by interacting with various inflammatory

pathways [9]. The oral bioavailability of curcumin is approximately 1%. However, a significant issue with consuming curcumin alone is its low bioavailability [10]. This issue seems to be addressed by incorporating enhancers like piperine, which can boost the bioavailability of curcumin by up to 154% [11].

## MATERIALS AND METHODS

An experimental study was conducted on forty adult male albino rats, each with an average weight of around 200 grams. Rats were carefully selected from the convenient animal house located at the faculty of medicine for girls at Al-Azhar University. They were kept in standard stainless steel mesh cages, at a room temperature of 24 ± 1 ◦C and a humidity level of 60 ± 5% and under a 12-hour cycle of light and darkness. Rats were acclimatized for a whole week and were allowed unlimited access to food and water. The experimental plan underwent rigorous scrutiny by the institutional committee of laboratory animal care and use. They were divided into four equal groups: Group A: (control group): Rats received ordinary diet alone without treatment. Group B: (curcumin group): Animals were given curcumin which purchased from Sigma-Aldrich Company (St. Louis, MO, USA) in the form of yellow powder. Curcumin suspension (freshly dissolved in saline solution) was administered to rats by oral gavage at a dose of 150mg/kg/day for 4 weeks from the beginning of the experiment [12]. Group C: (adenine group): adenine used to induce vascular calcification. It was purchased from Sigma-Aldrich Company (St. Louis, MO, USA) in the form of white powder. Adenine suspension (freshly dissolved in distilled water) was administered to rats by oral gavage at a dose of 334 mg/kg once daily for 4 weeks from the start of the experiment [13]. Group D: (adenine& curcumin group): animals were given curcumin and adenine for 4 weeks as the previously mentioned doses and route of administration. After the experiment conducted, the animals were gently anesthetized. The thoracic aorta, a crucial part of their bodies, was carefully removed and split into two sections for further analysis. One part was meticulously studied under a light microscope, while the other part underwent a detailed examination using an electron microscope. Special specimens were carefully selected for the light microscopic analysis and treated with various staining techniques such as Hematoxylin and Eosin, von kossa staining, and orcien staining to provide a clearer picture of the tissues. For light microscopy, the thoracic aorta was fixed with10% formalin saline for 3 days, dehydrated in ascending grade of ethyl alcohol, cleared with xylene, penetrated into molten paraffin in an oven at 60°C, paraffin blocks were prepared, left for 24 hours and then cut with a rotatory microtome (LEICA RM2 125 UK) at a thickness of 5µm.The section was mounted on clean albumenized slide glass and stained with the following stains: Hematoxylin and eosin are used to examine the general structure [14], Von kossa staining to show calcification [15], and orcien staining to see elastic fiber [16].

For electron microscopy, small pieces of the thoracic aorta (about 0.5 mm3) were taken while the rats were still alive under anesthesia and the specimen was placed on 5% glutaraldehyde. It was immersed in sodium cacodylate buffer (0.1 M) and held at 0-4°C for 24 hours. Post-fixation was performed in 1% osmium tetroxide in the same cacodylate buffer for 2 hours. Then, the dehydration of the test piece was carried out by ascending grade ethyl alcohol. All the steps from the beginning were done at 0-4°C. a mixture of epoxy resin and propylene oxide was used for impregnation. The specimens were then embedded in pure resin and left to harden in an oven at 40°C for 24 hours, followed by polymerization at 60°C for another 24 hours. Cutting of the sections was done using an LKB ultra-microtome with a glass knife. Initially, semithin sections of 0.5 µm were cut and stained with toluidine blue for light microscopic examination. Once the desired area was located, the block was trimmed, and ultrathin sections of 80 nm were cut and placed on copper grids. For the electron microscopic examination, the specimens were taken to the Regional Centre for Mycology and Biotechnology at Al-Azhar University. A Joel 100S transmission electron microscope operating at 60 KV was used for the examination.

Morphometrical study was performed using the image analyzer computer system (Leica Qwin 500; Leica, Cambridge, UK). Pathology Department, faculty of Dentistry, Cairo University. The study measured the thickness of the thoracic aorta wall in sections stained with H&E [17], as well as the average percentage of calcification in sections stained with von Kossa in the thoracic aorta [18].

### Statistical analysis

The collected data were analyzed and compared averages. expressed as mean ± SD, using one-way analysis of variance (ANOVA) followed by post hoc analysis and two-tailed t-test when necessary. We considered any results with a p-value <0.05 to be significant [19]. To help visualize our findings, we created a detailed statistical graph using the powerful software of Graph Pad Prism 5.0, developed by the talented team at Graph Pad Company in sunny San Diego, California, USA.

## RESULTS

### I. Light microscopic results of the aorta

Examination of H and E-stained aortic sections in both control group (A) and curcumin group (B) showed: endothelium (simple squamous epithelium with flat nuclei) within tunica intima. Tunica media formed of layers of smooth muscle cells (appearing spherical shaped with oval central nuclei) interspersed with elastic fibers. The outermost layer was the tunica adventitia made of loose connective tissue. in the adenine group (C) showed obvious distortion in the structures of the aorta, there were degeneration and calcification mainly in tunica media. There was separation in all layers and an apparent increase in the whole thickness of the aorta. In adenine and curcumin group (D) showed minimal distortion and degeneration

mainly in tunica media of the aorta as shown in figure (1).

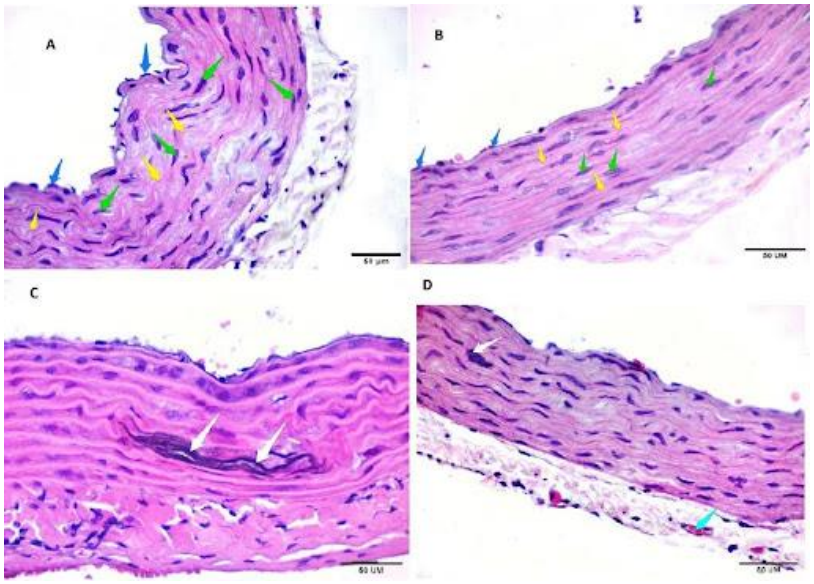


**Figure (1): Photomicrographs of H and E-stained aortic sections from experimental groups** showing (A and B): endothelium with flat nucleus (blue arrows). Elastic fibers (yellow arrows) and smooth muscle with central oval nuclei (green arrows), (C): dark purple deposits (white arrow) in the tunica media which decrease in the density in (D). **(H and E × 400).**

Examination of Von kossa stained aortic sections in both control group(A) and curcumin group (B) showed that elastic fibers and smooth muscle appeared pink, and nuclei appeared red with no calcification presented. In the adenine group (C) there was calcification in the tunica media which appeared black. In adenine & curcumin group (D) there was decrease in the density of calcification reaction in comparison to adenine group (C) as shown in figure (2).

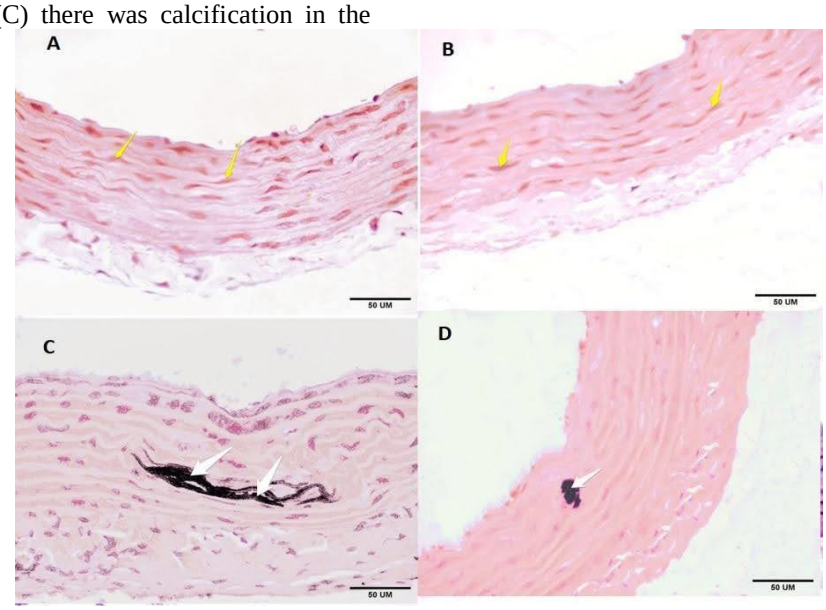


**Figure (2): Photomicrographs of von kossa stained aortic sections from experimental groups** showing (A and B): nuclei appear red (yellow arrow) with no calcification present, (C): Calcification in the tunica media (white arrow) decrease in the density in (D). (**Von kossa, x400**).

Examination of orcien stained aortic sections in both control group(A) and curcumin group (B) showed that the elastic fibers appeared as dark purple brown fibers, forming continuous regular wavy corrugated well developed layer of elastic fibers. In the adenine group (C) showed that the elastic fibers appeared as dark purple to dark brown, with irregular discontinuous wavy corrugations. Some of the elastic fibers were fragmented. In adenine & curcumin group (D) there was decrease in the separation of elastic fibers in comparison to adenine group (C) as shown in figure (3).

**II. Electron microscopic results of the aorta**

Examination of ultrathin aortic sections of both control group (A) and curcumin group (B) showed that the wall of the aorta was formed of tunica intima, which was the inner most layer, it was formed of simple squamous endothelium facing the aortic lumen resting on elastic lamina of fibro collagen CT. the elastic lamina was of

regular uniform pattern. Tunica media was the middle layer of the aortic wall, it was formed of layers of smooth muscle cells with rounded or oval euchromatic nuclei interspaced by elastic membranes formed mainly of elastic fibers supported by collagen fibers. In the adenine group (C) showed the endothelial cells lined the aorta recognized by the flat nucleus, it was interrupted and at certain points separated from its basal lamina. The elastic lamina was also recognized. Apparently appeared irregular of unequal thickness, smooth muscle within the tunica media were also detected, electron dense bodies or masses of variable shapes and sizes were also seen distributed throughout the aortic wall in some sections. Some of these bodies had spiky and pointed edges, some others had rounded or blunt edges, in between these bodies mononuclear cellular infiltration was easily recognized. Examination of the adenine and curcumin group (D) showed that: the endothelial cells of the intima facing the lumen more or less showed broad similarity to the control. elastic lamina appeared regular of uniform pattern, some smooth muscles cells within the tunica media could be recognized by its oval euchromatic nucleus. Few electron dense masses were still noticed even at narrow scale if compared to the adenine group(C) as shown in figure (4).

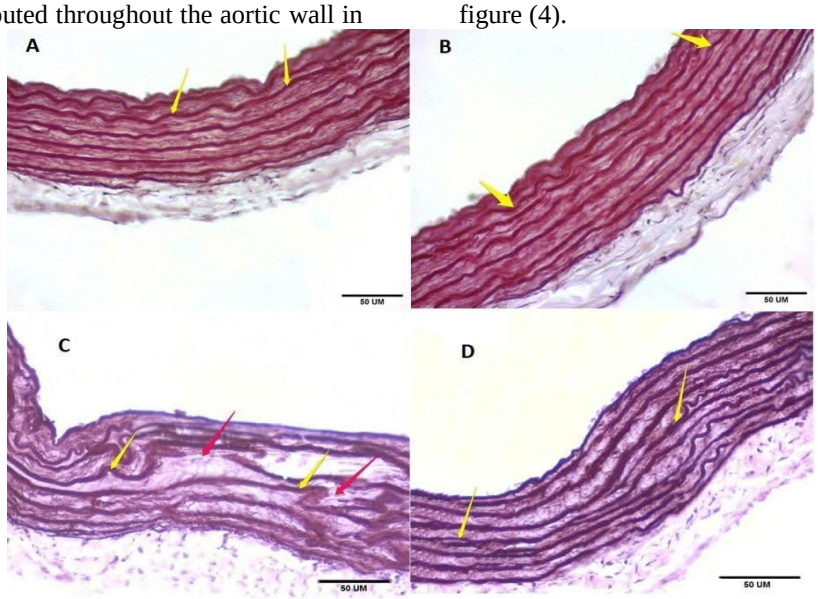


**Figure (3): Photomicrographs of orcien stained aortic sections from experimental groups** showing (A and B): elastic fiber (yellow arrow) appears wavy corrugated, and forming continuous deeply purple brownish lines, (C): the elastic fibers appears to lose their wavy appearance and are straighten at certain points, or even disrupted and fragmented at other points (red arrow), (D): decrease in their separation in comparison to adenine group (C). (**Orcien, x 400)**

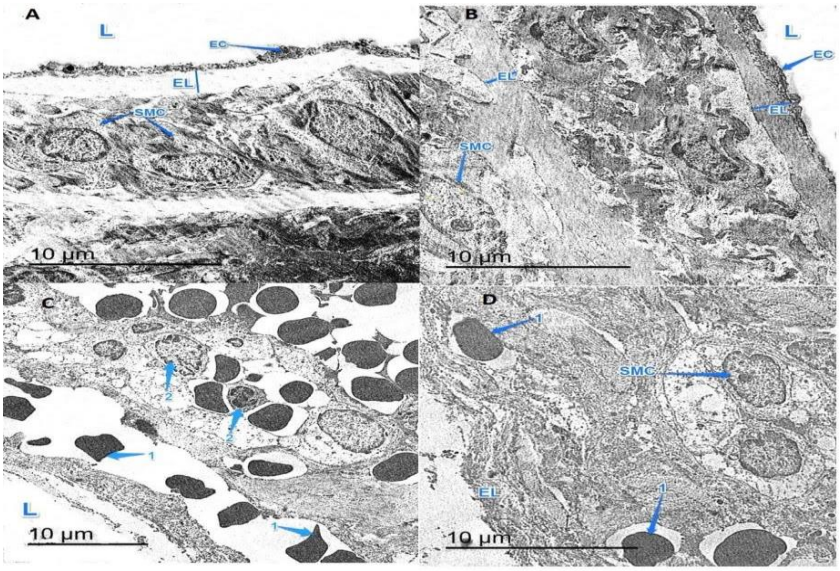


**Figure (4): Electron photomicrographs of a section in the thoracic aorta of the control group (A)and curcumin group (B)** showing: lumen of the aorta (L), endothelial cells (EC) in tunica intima. Elastic lamina of regular uniform pattern (EL). Smooth muscle cells (SMC). The adenine group (C) showing irregular elastic lamina. Sub endothelial deposition of electron dense materials, some of them are spikey (arrow1), also there are mononuclear cellular infiltration (arrow2). The adenine & curcumin group (D) showing: Few electron dense masses (arrows) still noticed even at narrow scale if compared to the adenine group(C), elastic lamina (EL) and Smooth muscle cells (SMC) also noticed. (**Uranyl acetate and Lead Citrate X 8000**).

**III. Area percentage of calcification in the thoracic aorta:**

The study calculated the mean value of the area percentage of calcification in the thoracic aorta for all groups. The adenine group exhibited the highest mean value at (13.68 ± 0.7), while both the control group and the curcumin group had the lowest mean value at (0 ± 0). There was a significant difference in the mean area percentage of calcification in the thoracic aorta among the control group, curcumin group), adenine group, and adenine & curcumin group (3.38 ± 0.7), as shown in table (1).

**IV. Aortic wall thickness in all groups**

The study calculated the mean values of aortic wall thickness for the control group, curcumin group, adenine group, and adenine & curcumin group. The adenine group exhibited the highest mean value (189.1±5.3), while the control group and curcumin group both showed the lowest mean value (127.47±2.2). there was statistical significant difference in aortic wall thickness among the groups: control group, curcumin group, adenine group and adenine & curcumin group (141.02±4.4), as shown in table (2) and figure (5).

**Table (1): Comparison of average area percentage of calcification in the thoracic aorta of all groups**

| Studied groups | Mean± SD | Stat. test |
|---|---|---|
| **Control (GA)** | 0±0 | t=7.6 |
| **Curcumin (GB)** | 0±0 | |
| **Adenine (GC)** | 13.68±0.7* | |
| **Adenine and curcumin (GD)** | 3.38±0.7* | |

SD: Standard deviation, t: Student t-test (adenine group vs. adenine and curcumin group), *: Significant p-value (≤ 0.05).

**Table (2): Comparison of average aortic wall thickness in all groups**

| Studied groups | Mean± SD | Stat. test | Post hoc test |
|---|---|---|---|
| **Control (GA)** | 127.47±2.2 | ANOVA test p < 0.001* | p1 =1.001 |
| **Curcumin (GB)** | 127.47±2.2 | | p2 < 0.001* |
| **Adenine (GC)** | 189.1±5.3* | | p3 < 0.001* |
| **Adenine and curcumin (GD)** | 141.02±4.4* | | p4 < 0.001* |
| | | | p5 < 0.001* |
| | | | p6 < 0.001* |

p1: Control group vs. curcumin group, p2: Control group vs. adenine group, p3: Control group vs. adenine and curcumin group, p4: Adenine group vs. curcumin group, p5: Adenine and curcumin group vs. curcumin group, p6: Adenine group vs. adenine and curcumin group, SD: Standard deviation, *: Significant p-value (≤ 0.05).

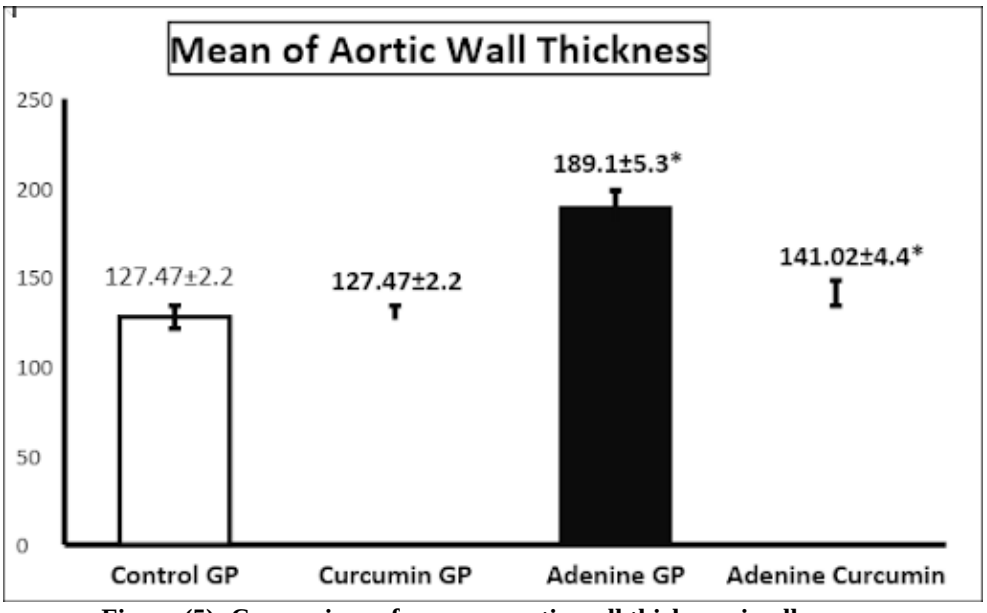


**Figure (5): Comparison of average aortic wall thickness in all groups**

Aortic wall thickness was measured in ten randomly selected fields and averaged per each section. Bars with error bars represented mean ± SD. Significant decrease in adenine and curcumin group (141.02±4.43) (P<0.05) compared to adenine group (189.10±5.33). *: Significant p-value (≤ 0.05).

## DISCUSSION

Depending on the location of occurrence, vascular calcification is divided into two types: arterial intimal calcification (AIC) and arterial medial calcification (AMC) [20]. Although the pathophysiology of vascular calcification is not absolutely understood, Systemic and local modifications in mineral metabolism can promote

or inhibit vascular calcification [21]. Curcumin, famous for its role in enhancing the color and taste of dishes, has gained worldwide acclaim for its many health benefits. Its antioxidant and anti-inflammatory properties make it a powerful weapon against vascular calcification [22].
In the present study curcumin was given daily for 4 weeks to normally control albino rats to detect any structural changes. At the same time curcumin was given daily for 4 weeks to rats suffering from ectopic vascular calcification due to adenine ingestion to assess any protective or curative effects. In the present study, calcification of the thoracic aorta induced by adenine administration to the rats through an oral route. Examination of H and E-stained sections of thoracic aortas from the adenine group (GC) revealed some disruption of the aortic medial layer. There was a significant increase in the mean thickness of the aortic wall in adenine group when compared to other groups. The dark purple deposits seen in the tunica media most probably calcification. These results agreed with Ou et al. [18] who detected that, adenine destroys the arrangement of elastic fibers that cause thickening of the vessel walls and cause aortic dysfunction. Also Nguy et al. [23] found that long-term use of adenine was related to increased blood pressure and thickening of the aorta. Chang et al. [24] demonstrated that calcification by adenine in the rat aorta revealed local calcification and calcium accumulation in the middle layer of blood vessels. This form of calcification has nothing to do with lipids and involves converting smooth muscle cells in the middle layer into cells similar to bone or cartilage, producing different bone-related proteins [25]. It is well established that pathological differentiation of smooth muscle cells is the main driving force of ectopic bone formation [26].

Examination of von kossa stained sections of the thoracic aorta of adenine group (GC) displayed calcification in between elastic fibers and smooth muscle fibers in tunica media. The mean area percentage of calcification showed a significant increase compared with the control group. Sriram et al. [27] found that von kossa staining of the aorta in adenine-treated rats revealed remarkable micro-calcification and disorganization of the tunica media. Similar findings were observed by Ou et al. [18] who demonstrated that adenine administration caused thickening of the vessel wall, aortic dysfunction and significantly increased aortic calcification. Aleksandra et al. [28] also found that with notch signaling and activation of intercellular contact, endothelial cells can promote smooth muscle calcification.

Examination of sections of the thoracic aorta stained with orcein in the adenine group (GC) revealed a disruption of the internal elastic lamina. The tunica media showed areas where elastic fibers were completely lost, while other areas displayed irregularly condensed elastic fibers. Ou et al. [18] also reported similar findings who demonstrated that adenine administration broke the arrangement of elastic fibers. This fragmentation of the elastic lamina leads to an increase in the stiffness of the arteries [29]. This agreed with Chang et al. [30] who stated that the decrease in elastin synthesis by smooth muscle cells is due to the damage of medial smooth muscle cells in calcification. Hou et al. [31] also stated that curcumin was a potent inhibitor of cardiovascular calcification, and low levels of curcumin were associated with cardiovascular calcification.

In the present study, examination of electron microscopic sections of the thoracic aorta of adenine group (GC) displayed disruption of endothelial cells and lost corrugation of tunica intima, Naito et al. [32] found that the loss of wave form in the intima was a result of vascular endothelial inability to repair extensive damage to endothelial cells. The smooth muscle cells in the medial layer below were able to create a thinner pseudo-endothelium lining the inner surface. These pseudo endothelial smooth muscle cells showed an inhibitory effect on the regrowth of vascular endothelium. Wang et al. [33] clarified that when endothelial function is impaired, the balance of autocrine-paracrine factors, which are essential for maintaining normal vascular function, is disrupted. This disruption leads to the release of vasoactive substances that encourage proliferation, migration and phenotypic transformation of smooth muscle cells. As a result, endothelial cells can induce the migration of smooth muscle cells to osteogenic cells. In addition, there is evidence of micro calcification and a decrease in the amount of smooth muscle in the medial membrane, along with unorganized elastic fibers. Micro calcification appears as dense spherical particles clustered in various sizes.

Examination of H and E-stained sections of the thoracic aorta from the adenine and curcumin group (GD), we revealed that the tunica intima remained intact with a thin, wavy, corrugated endothelium. Additionally, there was a marked decrease in the thickness of the aortic wall in the adenine and curcumin group when compared to the adenine group. According to Memarzia et al. [34], they found that consuming antioxidants such as curcumin can have a positive impact on calcification, ultimately helping to reduce the progression of calcification lesions, and curcumin exerts anti-mobility and anti-proliferative effects on VSMCs, further studies have shown that curcumin can inhibit cellular apoptosis [35].

Examination of von kossa stained sections of the thoracic aorta of adenine and curcumin group (GD): calcification deposition was significantly reduced compared with the adenine group. It can be explained by the antioxidant effect of curcumin, which inhibits fibrotic activity by a significant reduction in high serum levels of p-III-P (Procollagen III peptide), a marker of fibro genesis [36]. This agreed with Hou et al. [31] that found that curcumin treatment significantly attenuated bone formation differentiation and calcification by suppressing apoptosis of rat vascular smooth muscle cells (VSMCs). Pharmacological inhibition of VSMC apoptosis significantly reduces VSMC calcification.

Examination of orcein stained sections of the thoracic aorta of adenine and curcumin group (GD) showed that there were remarkable internal and external elastic lamina. Tunica media appeared rich in parallel elastic fibers. Small areas showed lost elastic fibers. The relative improvement of elastic fibers in adenine and curcumin group might be explained by Yang et al. [37] who proved that a significant increase in the content of the intimal and middle elastic fibers of the adenine and curcumin group may be due to the antioxidant effect of curcumin, which protects smooth muscle cells in the medium from oxidative damage and ultimately leads to an increase in elastin synthesis.

Examination of electron microscopic sections of the thoracic aorta of adenine &curcumin group (GD) showed the tunica media had smooth muscle fibers distributed between the wavy elastic fibers. This could be due to the curative effect of curcumin which protects endothelial cells from injury. This explained by Memarzia et al. [34] who stated that curcumin has antioxidant and anti-inflammatory actions. This agreed with Cox, et al. [38] that showed that the antioxidant effect of curcumin can protect smooth muscle cells from damage or prevent oxidative damage to cell membranes. However, according to a study by Patrizia et al. [39], curcumin was found to have the same positive effect on endothelial function. It also helps to strengthen calcified areas and reduce oxidative stress and inflammation.

## CONCLUSION

In the current experiment curcumin showed an improvement in ectopic calcification in the thoracic aorta, but curcumin is not considered the only specific line for the treatment of this disease. In general, it is recommended to add curcumin to the daily diet, because it has a pronounced beneficial healing and preventive effect on human health.

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# الملخص العربي

## تأثير الكركمين علي تركيب البطين الايسرفى القلب و الأورطي الصدري علي الجرذ الابيض البالغ في حالة التكلس المستحدث تجريبيا

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**ملخص البحث**

**الخلفية:** يحدث تكلس الأوعية الدموية عندما تتراكم أملاح فوسفات الكالسيوم في جدار الأوعية الدموية، مما يجعلها متصلبة وأقل مرونة، مما يؤدي إلى تقييد تدفق الدم ويجعل من الصعب على الجسم التحرك والتنفس بشكل صحيح

**الهدف:** الهدف من هذه الدراسة هو تقييم الدور المحتمل للكركمين في تحسين بنية الأوعية الدموية في حالة تكلس الشريان الأورطي الصدري المستحدث تجريبيا في ذكور الجرذان البيضاء البالغة.

**الطرق**: في هذا التجربة البحثية، تم استخدام أربعين ذكرًا بالغًا من الفئران البيضاء، يبلغ متوسط وزن كل منها حوالي 200 جرام. تم تقسيمهم إلى أربع مجموعات متساوية: GA (المجموعة الضابطة). GB (مجموعة الكركمين): أعطيت الفئران الكركمين عن طريق الفم. GC (مجموعة الأدينين): تم إعطاء الأدينين للفئران عن طريق الفم لمدة 4 أسابيع. المجموعة الرابعةGD : (مجموعة الأدينين والكركمين): أعطيت الفئران كلا من الأدينين والكركمين عن طريق الفم لمدة 4 أسابيع. وبعد انتهاء التجربة، تم تخدير الحيوانات وتمت إزالة الشريان الأورطي الصدري بعناية وتقسيمه إلى قسمين :جزء تمت معالجته وفحصه بالمجهر الضوئى ، بينما خضع الجزء الآخر لفحص تفصيلي باستخدام المجهر الإلكتروني. شملت القياسات المورفولوجية متوسط نسبة مساحة التكلس في الشريان الأورطي الصدري. وتم تحليل البيانات التي تم الحصول عليها إحصائيا.

**النتائج:** أظهرت القيمة المتوسطة لنسبة مساحة التكلس في الشريان الأورطي الصدري لمجموعة الأدينين زيادة ذات دلالة إحصائية مقارنة مع مجموعة الأدينين والكركمين.

**الإستنتاجات**: في التجربة الحالية أظهر الكركمين تحسنا في التكلس في الشريان الأورطي الصدري، على الرغم من ذلك لا يمكن اعتبار الكركمين الخط المحدد الوحيد لعلاج هذا المرض. بشكل عام يُنصح بإضافة الكركمين إلى النظام الغذائي يومياً لما له من تأثير علاجي ووقائي مفيد على صحة الإنسان.

**الكلمات المفتاحية:** الأدينين، الكركمين، الشريان الأورطي الصدري.

**الباحث الرئيسي**
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