Optimization of the model of abdominal aortic aneurysm by co-incubation of calcium chloride and collagenase in rats

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ABSTRACT

الأهداف: تحسين نموذج الورم بالشريان الأورطى البطني (AAA) في الفئران عن طريق استخدام كلوريد الكالسيوم ($CaCl_2$) والكولاجين معا.

الطريقة: أجريت هذه الدراسة في مستشفى الشعب التاسع– كلية الطب – جامعة جياو تونغ بشانغهاي – مدينة شانغهاي– الصين خلال الفترة ما بين يوليو 2008م حتى فبراير 2009م. تم تعرية الشريان الأورطى في 55 فأر ذكر بالغ من نوع سبارغو دوللي وتربيته لمدة 20 دقيقة في محلول ملحي جديد مع كلوريد الكالسيوم (0.4M) والكولاجين (w/v,4%) في (المجموعة A)، و مع كلوريد الكالسيوم مجرد في (المجموعة B)، و مع الكولاجين مجرد في (المجموعة C)، و مع محلول ملحي عادي مجرد في (المجموعة D). تم تقييم الشريان الأورطى المعالج بواسطة المقياس الرقمي، وتكنولوجيا التصوير الإشعاعي للأوعية الدموية، وفحص النسيج وذلك بعد أربع أسابيع.

النتائج: هنالك زيادة متوسطة في قطر 87.86%±69.49% (المدى 35.33-299.29%) في المجموعة A بعد 4 أسابيع منذ العملية الجراحية، أما تكرار AAA في هذه المجموعة فقد بلغ 83.3% (12/10). ظهرت حالة واحدة ل 1/13(AAA) في المجموعة C، بينما لم تظهر أي حالة في المجموعات الأخرى. كما ظهر في أنسجة AAA للمجموعة الأولى اختفاء أجزاء الغشاء الداخلي، وتمزق الإيلاستين، وترسب الكولاجين الشاذ، والذي يرتبط مع الورم الوعائي الفطري لدى البشر.

خاتمة: حسّنَ استخدامُ الكولاجين النموذج المقام الذي حُرض بكلوريد الكالسيوم $CaCl_2$ لدى الفئران، و قدم احتمالات عالية ل AAA في فترة وجيزة.

Objectives: To optimize the model of abdominal aortic aneurysm (AAA) in rats using calcium chloride ($CaCl_2$) and collagenase together.

Methods: This study was performed at the 9th People's Hospital, Shanghai Jiao Tong University, School of Medicine, Shanghai, China from July 2008 to February 2009. Aortas of 55 adult male Sprague-Dawley rats were exposed and incubated for 20 minutes with fresh normal saline solutions supplemented with $CaCl_2$ (0.4M) and collagenase (4%, w/v) (group A), $CaCl_2$ alone (group B), collagenase alone (group C), or normal saline alone (group D). After 4 weeks, the treated aortas were evaluated by digital measurement, angiography, and histological examination.

Results: In group A, there was a mean increase in diameter of 87.86% ± 69.49% (range, 35.33-299.29%) weeks after surgery. The frequency of AAA in this group was 83.3% (10/12). One (1/13) AAA occurred in group C and none in other groups. Partial endothelial loss, elastin disruption, and abnormal collagen deposition were noted in the AAA tissues in group A, corresponded well to native aneurysms in human.

Conclusion: The use of collagenase optimized the established $CaCl_2$-induced rat model, giving a high frequency of AAA in a short period of time.

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Abdominal aortic aneurysm (AAA) is a common disease in aged populations, occurring mostly in men more than 65 years of age. Most AAAs have no obvious symptoms until rupture, which leads to death in 65% of patients.[1] Despite the high risk of mortality with AAAs, the etiology of this insidious disease remains unclear. Moreover, examination of the continuum of progression of AAA in human is not a practical option, which makes animal models of native aneurysms attractive on the pathophysiology of AAAs. Numerous AAA models have been established to elucidate the mechanism of development, growth, and rupture of AAAs. To be widely used, a model must be reproducible and easy to implement, and must histologically resemble human AAAs. As reported previously, the AAA model, induced by calcium chloride ($CaCl_2$), involves a natural process including local arterial injury followed by inflammatory cell invasion, matrix destruction, and a corresponding increase in vessel diameter; and it shares many features with human pathology.[2] However, in rats, the use of $CaCl_2$ alone does not produce the same outcome as in mice because of the longer induction period and the lower AAA frequency.[3] Previous studies had revealed that aortas treated with collagenase in vitro successfully altered the structure of the collagen fiber network within the aortic wall and reduced the thickness and tensile strength of the arterial wall.[4] Additionally, Hynecek et al[5] described an AAA model in swine by co-perfusion with elastase and collagenase. In view of the effects of collagenase, the present study was intended to test whether the combination of $CaCl_2$ and collagenase could optimize the model of AAA in rats.

Methods. This study was performed at the 9th People's Hospital, Shanghai Jiao Tong University, School of Medicine, Shanghai, China from July 2008 to February 2009. All animal experimental protocols were approved by the Animal Care and Use Committee of the 9th People's Hospital, Shanghai Jiao Tong University School of Medicine, and conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council, Chinese Version, 1996). Fifty-five male Sprague-Dawley rats, 8 weeks old (Shanghai Experimental Animal Center of Chinese Science College, Shanghai, China) were housed 3 per cage in a room with controlled temperature and humidity and 12-hours light-dark cycles. The rats were fed with standard rat chow and tap water ad libitum. After one week of acclimatization, the animals were randomly divided into 4 groups. Each rat was labeled by ear notch. Group A (n=15) was topically treated with a normal saline solution supplemented with $CaCl_2$ (0.4 M, Amoresco, USA) and collagenase (4% w/v, NB 4 standard Grade, Serva, Heidelberg, Germany). Rats (n=15) in group B were treated with a solution containing 4% (w/v) collagenase, while rats (n=15) in group C received a solution of 0.4 M $CaCl_2$. The sham group (group D, n=10) received normal saline. All buffered collagenase solutions were freshly prepared before surgery. The incubation was performed at room temperature (25°C).

Operating procedure. Rats were anesthetized by injecting a combination of ketamine (100 mg/kg) and 2.5% sodium pentobarbital (0.25 mL/100 g body wt; Shanghai 9th People's Hospital). A laparotomy was performed via a midline abdominal incision under sterile conditions. The abdominal aorta between the renal and iliolumbal arteries was freed from the inferior vena cava and the surrounding retroperitoneal structures. Any sheath or other connective tissue covering the vessels was gently trimmed. The diameter of the aorta was determined in images captured by a Moticam-2206 digital camera (Motic, Xiamen, China) and analyzed with a relative imaging program (Motic Images Advanced 3.2).

After base-line measurements, the arterial segment was dried up with gauze to eliminate remaining blood or abdominal fluid. The segment of interest was separated by applying a rectangular polyvinyl chloride (PVC) film (30 μm in thickness) transversely the aorta and surrounding tissues (**Figure 1a**). Then, a cotton carrier was soaked in one of the solutions described above and then applied to the external surface of the aorta under direct visualization (**Figure 1b**). After incubation, the arterial segments were rinsed 3 times in 0.9% saline solution before closing the abdominal cavity. The rats were returned to their cages, monitored daily, and given water and chow ad libitum. Four weeks later, measurements were repeated at the same location. A 50% increase over the normal diameter was considered aneurysmal dilatation.

Angiographic imaging. Digital subtraction angiography was used to confirm the changes in luminal diameter in the fourth week. For this analysis, the left common carotid arteries were exposed under general anesthesia as described above. All procedures were performed in a dedicated angiography suite using high-resolution imaging (Advantx; GE Medical Systems, Milwaukee, WI, USA). Contrast medium (Iopamidol, Solutrast 300, Byk Gulden, Konstanz, Germany) was injected into the ascending aorta by a intravenously catheter (Insyte 24G x 0.75 IN, Becton Dickinson Co., Ltd, Suzhou, China) inserted into the left common carotid artery, and angiography was performed in an anteroposterior view.

Histology. The rats were euthanized at 4 weeks for histologic evaluation. The abdominal aortas were harvested and perfusion-fixed with formalin. Five-

micron sections were stained using hematoxylin and eosin, Gomori's aldehyde fuchsin for elastic fibers, and Masson trichrome for collagen. Each staining cycle alternated between fixing and washing procedures. The slides were examined and photographed by light microscopy (Nikon, Boyce Scientific Inc., Gray Summit, MO, USA).

Statistical analysis. All data were presented in mean ± SD. The statistical evaluation was performed by means of Mann-Whitney U test and values of $p<0.05$ were considered statistically significant. Statistical Package for Social Sciences software Version 11.0 (SPSS, Statistical Software, Inc. Los Angeles, USA) was used for all statistical procedures.

Results. Three rats in group A died from ruptured aortas during incubation. One rat in group B and 2 in group C died during anesthesia. No death occurred postoperatively. In group A, oozing of blood and immediate dilation were observed 20 minutes after the co-incubation of $CaCl_2$ and collagenase during procedure (**Figure 1c**). This phenomenon was not observed in other groups. Angiographic confirmation showed increases in luminal diameter after 4 weeks as compared with normal adjacent segments which were not exposed to the solution (**Figure 2**). The gross view of dilated segments was consistent with angiography findings, showing a pronounced aneurysm located in the infrarenal region of the aorta (**Figure 3**). The external diameters of the aortas at 4 weeks were significantly

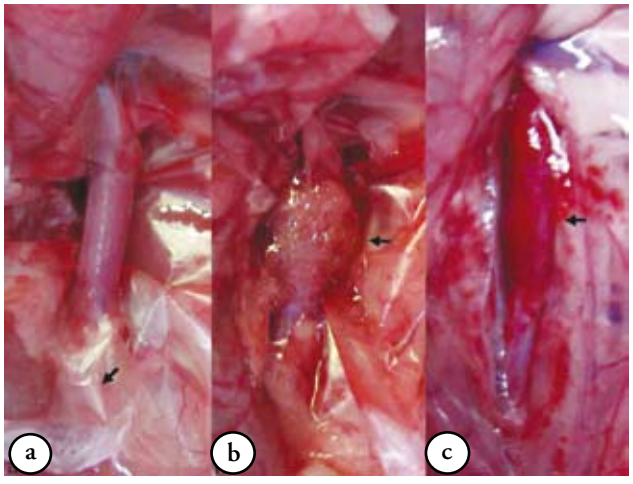

Figure 1 - Intraoperative photographs of the procedure to develop an infrarenal abdominal aortic aneurysm **a)** The infrarenal aorta was exposed and a 6 cm x 2 cm polyvinyl chloride film (arrow) was placed between the aorta and the surrounding tissues. **b)** A cotton carrier (arrow) was applied to wrap up the aorta. **c)** Slight dilation and oozing (arrow) were observed following incubation with calcium chloride and collagenase together.

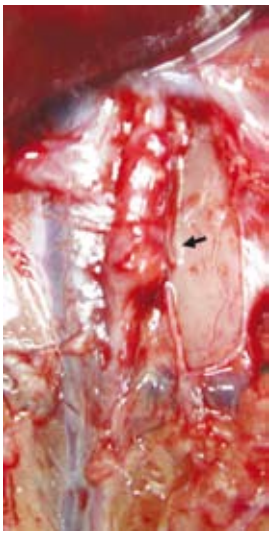

Figure 2 - Gross view of an infrarenal abdominal aortic aneurysm (arrow) 4 weeks after aneurysm induction.

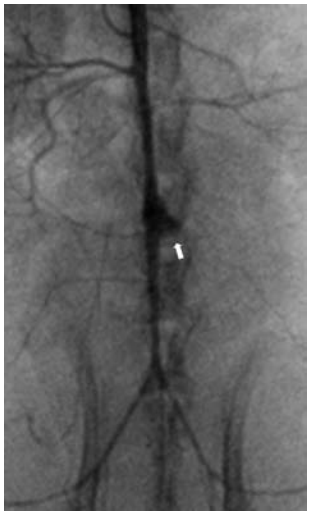

Figure 3 - A digital subtraction angiography showed a dilated segment (arrow) within an infrarenal aorta.

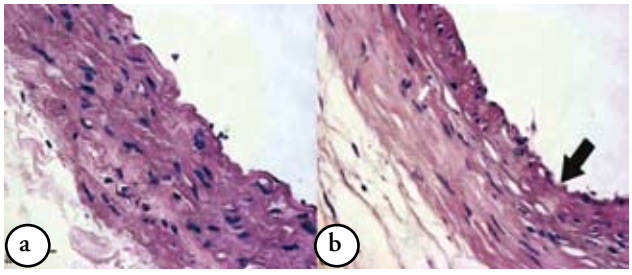

Figure 4 - Hematoxylin and eosin staining showed **a)** a smooth and continuous intima and normal shrunken smooth muscle cells (SMCs) morphology within the normal media (× 400) and **b)** an intermittent intima (black arrow) and abnormal SMCs (white arrow) in abdominal aortic aneurysm (x 400).

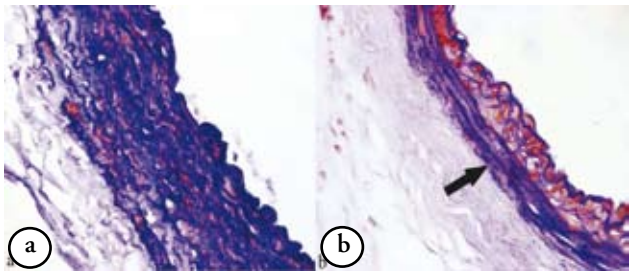

Figure 5 - Gomori's aldehyde fuchsin staining showed **a)** tightly coiled elastic fibers distributed regularly within the normal media (x400); **b)** straightening and fragmentation of the elastic fibers (black arrow) in abdominal aortic aneurysm (x400).

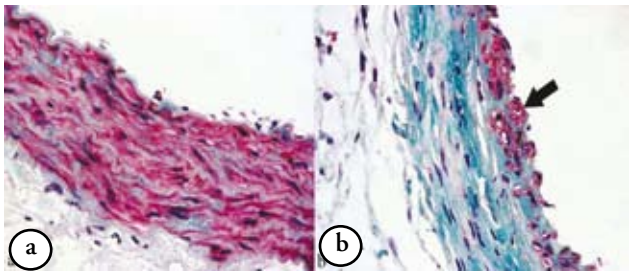

Figure 6 - Masson's trichrome staining showed **a)** integrated muscle fibers and a small amount of collagen between coils in normal aortas (x 400) and **b)** significantly diminished muscle fibers (black arrow) and a marked increase in collagen deposition (white arrow) in abdominal aortic aneurysm (x 400).

Table 1 - Average values of aorta sizes at the beginning and end of the experiment.

Group	Average values of aorta size at the pre-treatment ± SD (mm)	Average values of aorta size at the post-treatment ± SD (mm)	Average fold changes (Increase, %)
A	1.65 ± 0.34	2.94 ± 0.93	87.86 ± 69.49*,†
B	1.66 ± 0.29	1.71 ± 0.32	1.94 ± 2.73
C	1.63 ± 0.26	1.92 ± 0.23	21.41 ± 13.12
D	1.70 ±0.18	1.72 ± 0.19	1.38 ± 1.60

*p=0.0007 versus group D, †p=0.0004 versus group C

greater than before incubation in group A, with a mean increase in diameter of 87.86% ± 69.49% (range, 35.33-299.29%). The frequency of AAA in this group was 83.3% (10/12). In group C, with a mean increase of 21.41% ± 13.12% (range, 0-52.17%) in diameter, there was only one (1/13) AAA formed at 4 weeks (**Table 1**). No aneurysm was observed in groups B and D. Compared with normal aortas, which showed no irregular luminal contour with uniformly convoluted elastica (**Figures 4a, 5a, & 6a**), the light microscopic examination of AAA segments in group A revealed a rough and intermittent luminal surface, damaged endothelial cells and shrunken smooth muscle cells (SMCs) (**Figure 4b**). In sections stained for elastic tissue, the internal elastic lamina, which appeared virtually straight, showed areas of marked discontinuity in the dilated segments (**Figure 5b**). Corresponding sections stained with Masson's trichrome showed a prominent reduction in muscle fibers and a marked increase in collagen deposition within the media (**Figure 6b**).

Discussion. Gertz et al[6] first described the application of $CaCl_2$ in aneurysm production, and subsequently validated by other groups[2,7] as a reproducible method of inducing AAA. This model is most popular because it is simple to perform and promotes spontaneous expansion of the aorta.[8] However, periarterial incubation of $CaCl_2$ alone did not produce stable outcomes, with varied aneurysm frequency and induction time according to previous reports.[9,10] Several concentrations, including 0.5, 0.75, 1.0 and 1.25 M, have been tested in our laboratory; but the frequency of AAA was not directly proportional to the dosage of $CaCl_2$, and as the concentration increased, so the calcification and stiffness in the aortic wall was obtained. So far, no large animal models of AAA induced by $CaCl_2$ have been available for study. It is therefore desirable to make some improvements in this strategy. In the present study, we have shown that the use of collagenase optimized the established $CaCl_2$-induced rat model, giving a high frequency of AAA in a short time. In addition to the instant effects during co-incubation, we unexpectedly observed the formation of a marked expansion of the abdominal aorta in the infrarenal region at 4 weeks. The possible mechanism of action may be as follows: First, aneurysm formation involves a progressive degradation of the extracellular matrix (ECM), characterized by destruction of elastin and collagen within the media and adventitia. The ECM defines the load-bearing properties of the aortic wall,

and any alteration in amount and distribution of these proteins may lead to the formation of aneurysms.[11,12] An investigation by Dadgar et al[4] elegantly illustrated the digestion process that occurred during incubation of collagenase. It made a loss in wall thickness and caused weakness of the wall weakness by the digestion of the collagen fibers, which had an essential effect on the formation of AAA. Thus, the damaged wall cannot resist the mechanical pressure of the blood flow, resulting in dilation or even rupture of the aorta lumen. Second, $CaCl_2$ played an undoubted role in AAA formation as described in previous studies,[6] mainly through the following 3 aspects: a) endothelial desquamation; b) deposition of calcium within, and disruption of the internal elastic lamina; c) medial disorganization and inflammatory cell infiltration. Third, the results in our study showed that the single effect of $CaCl_2$ or collagenase could not achieve favorable outcomes within 4 weeks. This evidence supported the primary mechanism of action is unlikely the single, but the combination effects of the 2 agents. Due to the digestion of the 'protective' barrier (adventitia, lamina elastica interna) of the aortal wall by collagenase, penetration of the media became easier, which may speed up the calcium precipitation and inflammatory response. Simultaneously, Ca^{2+} played as an activator and a stabilizer in collagenase catalysis,[13] which could maximize the efficacy of the digestion. In this process, Ca^{2+} and collagenase made a concerted attack on the aortic wall, working jointly to promote the destruction of vessel wall. Several technical issues arose during the development of this model. Some authors applied a soaked applicator or sponge directly to the aorta; however, we found this gave a severe injury to surrounding tissues, resulting in organization of adhesion and calcification. We therefore introduced a PVC film, which can prevent the seep of solution and the direct contact to surrounding tissues. In addition, pieces of dry gauze were placed adjacent to the treated section of aorta, which may prohibit a dilution effect of the surrounding body fluid. Compared with the present experimental models, our results were proved more promising. The mean increase in diameter of 87.9% in our studies was higher than that in diameter of 42% at 28 days reported in other published studies using 0.5 M $CaCl_2$ alone.[3] The achieved AAA frequency of 83.3% was also higher than that reported AAA frequency of 60-80% in mice.[7,14] Furthermore, the histological changes in this optimized model corresponded well to native aneurysms in human. Although these results were encouraging, it must be taken into consideration that the sample size was relatively small. Therefore, further investigation is needed with more rats to confirm the effectiveness and efficiency.

In conclusion, we believe that creating an experimental AAA model through the effect of collagenase on the aortic wall in combination with $CaCl_2$ can be well used not only for further research into etiopathogenetic processes of aneurysm development, but also provide some implications to produce aneurysm models in large animals.

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