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# Effects of Umbilical Cord Blood Stem Cells on Coronary Artery Damage in Mice with Kawasaki Disease By High-Frequency Ultrasound

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## ABSTRACT

**Objective:** to use lactobacillus casein cell wall extract (LCWE) to induce Kawasaki disease (KD) mouse model, and analyse the process of coronary artery damage and effects of umbilical cord blood stem cells by high-frequency ultrasound in small animals.

**Methods:** LCWE was prepared and 18 BALB/c pups were randomly divided into two groups: 15 in KD model group and 3 in the normal control group. KD model was induced by a single intraperitoneal injection of 0.5ml LCWE in the model group, and changes of the coronary artery were observed at 2d, 21d and 30d after the injection, respectively. From the 16th day of modeling, 300 μL PBS was injected intraperitoneally daily in the control group and model group. The hUC-MSCs 300 μL (105/mL) were intraperitoneally injected daily for 10 consecutive days in the stem cell group. The mice were sacrificed in batches on day 2, 15, 21 and 30, and the morphological changes of coronary arteries were observed by echocardiography and histopathology.

**Results:** the change of coronary artery diameter could be accurately measured by high-frequency small animal ultrasound. At 21d, the coronary arteries of the model group were widened compared with those of the control group. At 30d, there was no significant difference between the model group and the previous model group. Histopathology showed slight swelling of the epicardium of aortic valve, mitral valve, right ventricle and atrium, scattered infiltration of a few neutrophils, dilatation of the coronary artery lumen, necrosis and disintegration of a small number of myocardial cells, and significant hyperplasia of local fibrous connective tissue accompanied by solid calcium salt deposits. After hUC-MSCs intervention treatment, B-ultrasound showed a decrease in the main coronary artery diameter, histopathology showed multiple lymphocytes, eosinophilic granulocytes, and mononuclear cell infiltration in the left atrial appendage of mice, and no obvious vascular inflammatory reaction or other obvious abnormalities were observed.

**Conclusion:** high-frequency ultrasound can be used to clearly obtain the coronary artery image of KD mice and dynamically observe the evolution process of coronary artery diameter, which provides more diagnostic basis for the treatment of clinical KD. And hUC-MSCs intervention reduced the pathological lesion of coronary artery inflammation in mice compared with the model group.

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Kawasaki disease, also known as cutaneous mucosal lymph node syndrome (MCLS), is the most common acute self-limited vasculitis in childhood. It is common in infants and young children, mostly in children under five years old, with more males than females [1]. The clinical manifestations include persistent fever, conjunctival congestion, lip and oral symptoms, pleomorphic rash, hand and foot symptoms and non-suppurative cervical lymph node enlargement [2-3]. Most of the patients have a good prognosis, still some children may have coronary artery lesions, and the most serious complications include coronary artery dilatation, stenosis, coronary artery aneurysm formation, and even myocardial infarction [4]. In this study, through the mouse model of Kawasaki disease induced by LCWE, and use the high- frequency small animal echocardiography to measure the diameter of aortic annulus and the initial segment of left and right coronary artery. And observe the inhibitory effect of hUC-MSCs on coronary artery inflammation, The ventricular wall motion was recorded by M

ultrasonic group and combined with pathological results to study the damage process of Kawasaki disease to the coronary artery.

## Materials and Methods

### Animal preparation

Eighteen young BALB/c mice weighed about 15-20g ,and there were no specific pathogens, randomly divided into a model group (n = 15) and control group (n = 3).The model group was intraperitoneally injected with Lactobacillus casein cell extract, and the Kawasaki disease mouse model was induced by a single injection of 0.5ml, while the control group was not treated.

### Equipment selection

The Vevo 3100 high-resolution small animal ultrasound instrument of VISUALSONIC company of Canada, selected Mx400 single-chip mechanical fan sweep broadband head and fixed focus depth 1.2cm.

### Method

Remove the hair on the mouse chest with depilatory cream and put it in a sealed box. After inhaling the anaesthetic gas of enflurane, the mice were fixed on the examination table in the supine position after complete anaesthesia, and the heart rate and respiration were recorded synchronously. The heart rate was maintained at 300-400 beats/min. The chest was smeared with an ultrasonic coupling agent, and the high-frequency probe was fixed on the examination bracket for ultrasonic examination. By adjusting the angle of the examination table and the direction of the probe, the long axis of the left ventricle, the short axis of the aorta and the short axis of the left ventricle in mice were explored, to measure the internal diameters of the main opening of the left and right coronary arteries and the diameters of the aortic annulus. The amplitude of the left ventricular wall motion was recorded by M-ultrasound. All cross-section explorations continuously collect dynamic images for 10 seconds, store them in the background, and use an image analyser for offline analysis. This method was used to measure at 2 days, 21 days and 30 days after the establishment of the mouse model.

### Statistical methods

Using SPSS 22.0 statistical software package, the measurement data were tested by normality test and variance homogeneity test, then use the t-test or rank-sum test. The enumeration data were compared by the $\chi^2$ test, the normal conformity distribution was expressed by x±s, and the nonconformity normal distribution was expressed by the median. The difference was statistically significant (P <0.05).

## Results

### Coronary artery shape and diameter

18 mice were examined by ultrasound, the aortic annulus and the opening of the right coronary artery could be clearly displayed in the long axis section of the left ventricle, and the opening of the left coronary artery could be clearly displayed in the short axis section of the main artery. And measured each result three times, take the average value. The ratio of the internal diameter of the coronary artery to the aortic annulus was recorded and expressed as "D". It can be seen in Tables 1 and 2.

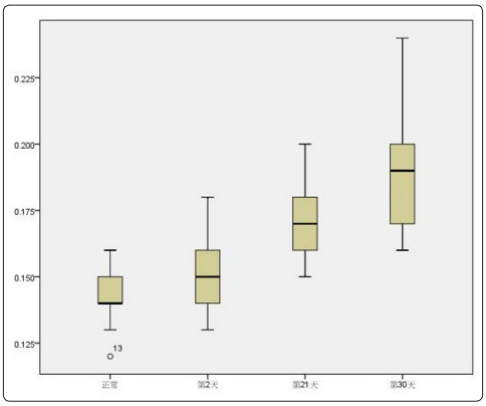



**Table 1:** The ratio of the internal diameter of the left coronary artery to the aortic annulus in the mode group.

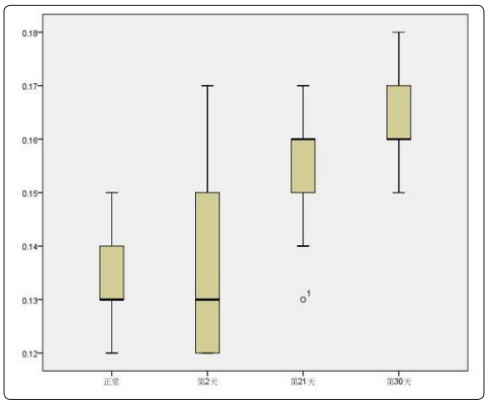



**Table 2:** The ratio of the internal diameter of the right coronary artery to the aortic annulus in the mode group.

### Omparison of D Value Between Kd Model Group And Normal Control Group

Two days after the establishment of the model, the ratio of left coronary artery diameter to the aortic annulus ((D) in the KD model group (0.136 ±0.021) was not significantly different from that in the control group (0.135 ±0.121), the difference was not statistically significant (P=0.568). But the D value of the right coronary artery (0.140 ±0.195) was slightly higher than that in the normal control group, and the difference was statistically significant (P=0.014).In the two groups, the echo of the coronary artery wall was fine, the intimal surface was smooth, and there was no obvious tumour-like dilatation.After establishing model 21 days, the D value of the left and right coronary artery in KD model group increased significantly (left 0.153 ±0.025, right 0.143 ±0.031), which was significantly higher than that in the normal control group (P<0.05).In the control group, the echo of the coronary artery wall was fine ,and the intimal surface was smooth. Compared with the control group, the D value of the model group increased significantly, especially in the left coronary artery, and the echo of the coronary artery wall was enhanced compared with the surrounding tissue, and the intimal surface was rough. As shown in figure 1. Then at 30 days, the D value of the left and right coronary artery in KD model group increased significantly (left 0.184 ±0.076, right 0.144 ±0.039), and the difference was statistically significant (P<0.05).

### Comparison of D values within KD model groups

After establishing model 2 days (left 0.136 ±0.021, right 0.140 ±0.195), compared with 21 days (left 0.153 ±0.025, right 0.143 ±0.031),the bilateral coronary arteries were thickened, the difference was statistically significant(P<0.05). The echo of the wall was significantly enhanced compared with the surrounding

tissue. After establishing model 30 days (left 0.184 ±0.076, right 0.144 ±0.039), compared with 21 days (left 0.153 ±0.025, right 0.143 ±0.031), the bilateral coronary arteries were thickened, the difference was statistically significant($P<0.05$). After establishing model 30 days, compared with the 21 days, the bilateral coronary arteries were thickened, especially in the left coronary artery, the echo of the wall of the coronary artery was enhanced, there was no obvious tumour-like dilation at the proximal end of the coronary artery, and the echoes of the coronary artery wall and surrounding tissue were enhanced.As shown in Figure 1 and Figure 2.

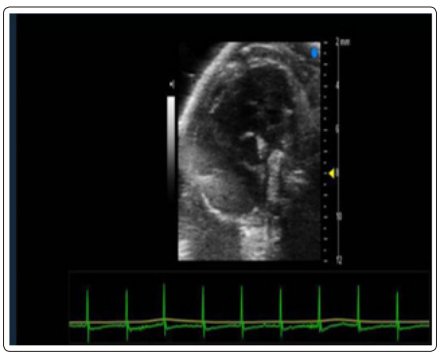


**Figure 1:** Left coronary artery diameter is 0.201mm in the model group at establishing a model for 21 days.

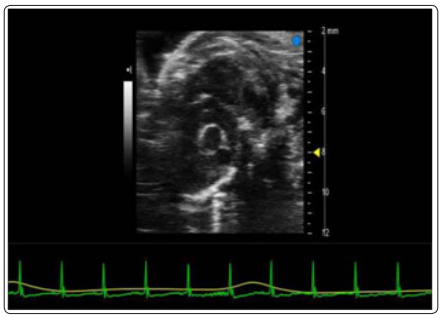


**Figure 2:** Left coronary artery diameter is 0.218mm in the model group at establishing a model for 30 days.

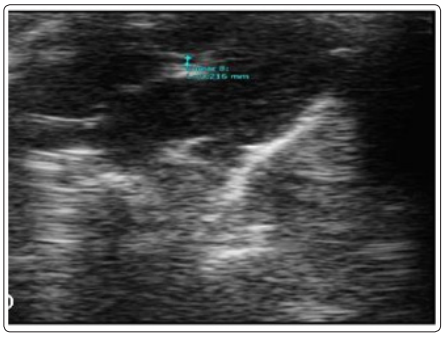


**Figure 3:** It was shown that the inner diameter of the main trunk of the left coronary artery decreased after 10 days of stem cell intervention.

**M ultrasound recording the amplitude of left ventricular wall motion**

After two days of modelling, no regional left ventricular wall motion abnormality was found in the model group and control group.After 21 days of modelling, the amplitude of left ventricular posterior wall motion was significantly decreased of two mice in the model group (13.3%,2/15 cases), while that of the control group did not change. As shown in Figure 3, Figure 4. After 30 days of modelling, the wall motion of the two mice in the model group did not change compared with the previous time and still showed a reduced state.

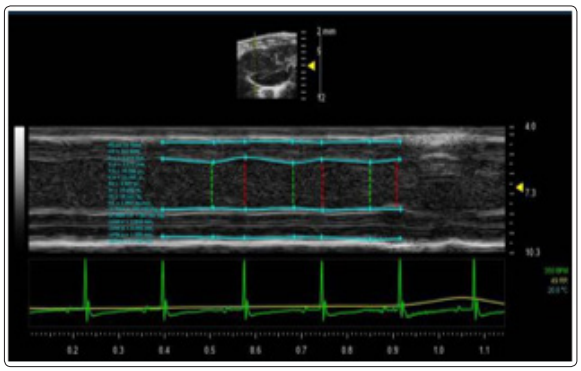


**Figure 3:** M ultrasound in the model group on the 15 day, the amplitude of left ventricular posterior wall motion was decreased significantly.

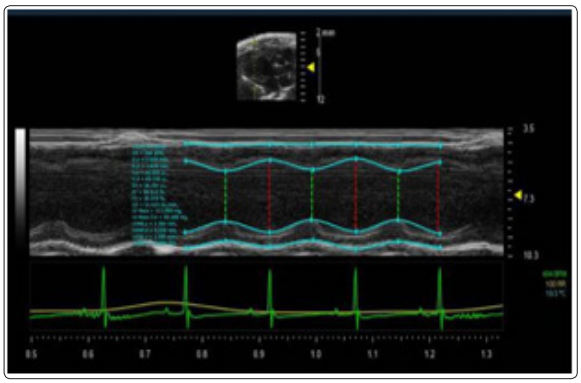


**Figure 4:** M ultrasound in the model group on the 10 day after stem cell intervention was normal

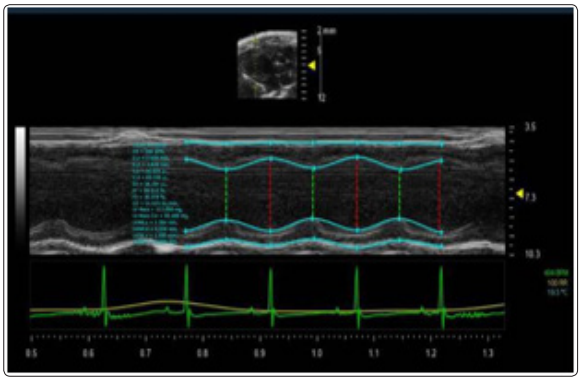


**Figure 5:** M ultrasound in the control group on the 21st day after modelling, the amplitude of left ventricular wall motion was normal.

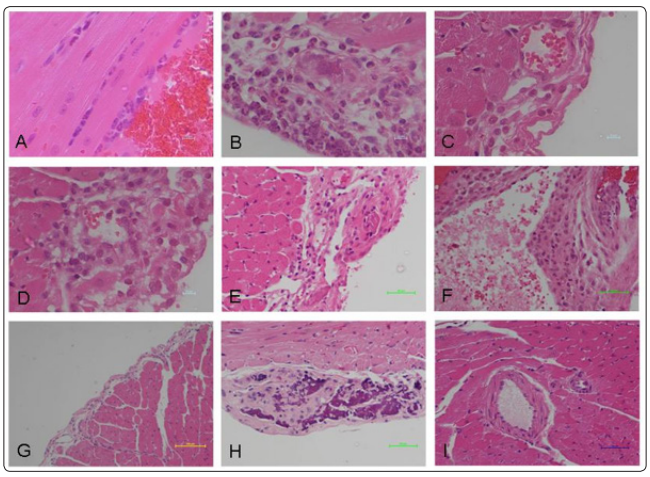
**Pathological results**

After 2 days of modelling, there was no significant widening of the coronary artery in the model group and the control group. Pathological results showed that a large number of neutrophils, a small number of monocytes, lymphocytes and other inflammatory cells invaded the coronary artery in the model group, as shown in figure 5. On the 21st day, part of the coronary artery in the model group was widened, and the pathological results showed epicardial interstitial oedema in the model group, with a small amount of lymphocyte infiltration and slight proliferation of fibrous connective tissue. Local fibrous connective tissue significantly proliferated with solid calcium deposition, as shown in figure 6.On the 30th day, the coronary artery in the model group was slightly wider than the previous time, and there was no obvious tumour-like dilatation. The epicardium of the right ventricle and atrium is highly swollen, the diffuse hyperplasia of a small number

of lymphocytes and fibrous connective tissue.The wall of the arteriole becomes thinner, and a small amount of solid calcium salt (thrombus calcification)is precipitated in the lumen,as shown in figure 7.

**Figure 5 Pathological staining results of mouse heart** (A-E, 40×; F, H, 20×; G, 10×; I, 200×)

After LCWE 4d injection, local endothelial cells in the intima of the left ventricle fell off, and white thrombus formed on the endothelium. A small number of neutrophils were occasionally observed locally between the intima and the muscle wall (A). The epicardium of the right ventricle and atrium is highly swollen. A large number of neutrophils and a small number of monocytes (B, C, and E) can be seen. The inner (right) coronary artery lumen dilates and a small number of myocardial cells necrotic collapse (C, D). The wall of a coronary artery was thickened and the lumen was narrowed, with white thrombus formation in the lumen (E); the aortic and mitral valve was mildly swollen with a few scattered neutrophils infiltrating (F). After 15 days of LCWE injection, epicardial interstitial edema was observed, with a small amount of lymphocyte infiltration and diffuses mild hyperplasia of fibrous connective tissue (G), and local significant hyperplasia of fibrous connective tissue accompanied by solid calcium salt deposits (H). After the hUC-MSCs intervention for 10 days, a large number of lymphocytes, eosinophilic granulocytes, and mononuclear cells were observed locally in the outer membrane of the left atrial appendage (I). No obvious vascular inflammatory reaction or other obvious abnormalities were observed.




**Discussion**

Kawasaki disease is an acquired heart disease, which often occurs in children under five years old. It is reported that Kawasaki disease has become the most common acquired heart disease in children in developed countries in the world, as well as in China. Yan Xiaohua et al. retrospectively analysed 170 children with Kawasaki disease who were hospitalised and diagnosed in the Children's Hospital of Shaanxi Provincial people's Hospital from January 2011 to January 2015 [5,6]. The results showed that 46 cases with coronary artery damage (27.1%, 46/ 170), include that 38 cases with coronary artery dilatation (22.4%, 38/170) and 6 cases with coronary artery aneurysm (3.5%, 6/170), and 2 cases with giant coronary artery aneurysm (1.1%, 2/170). Kawasaki disease has been studied by experts for about 30 years, but the aetiology and pathogenesis of Kawasaki disease are still unknown [7-8]. In 1979, American Lehman successfully induced the mouse model of arteritis with Lactobacillus casein cell extract (LCWE). The arteritis in this model was mainly manifested in the middle artery, coronary artery and the root of a large artery. At present, many studies tend to focus on the systemic inflammatory response of small and medium-sized arteries caused by the immune mechanism induced by infection factors [9]. Regarding the immune mechanism induced by LCEW, some scholars have carried out related studies, which have proved that the specific components of Lactobacillus casein cell wall are similar to some components of Streptococcus A cell wall, and have common antigenicity with some glycoproteins in human blood vessels, myocardium and other tissues. Under specific circumstances, the body may induce autoimmune response through molecular simulation mechanism, resulting in vascular, myocardial and other immune damage. Therefore, this model can be regarded as a relatively mature animal model for the study of coronary artery injury in Kawasaki disease.

With the continuous development of ultrasound detection technology in recent years, high-frequency animal cardiac ultrasound, as a non-invasive examination method, makes it possible to dynamically examine the coronary artery of KD mouse model. The mouse is recognised as an extremely valuable animal model for the study of cardiovascular disease. Because of the physiological characteristics of small size and high heart rate, high-frequency probe combined with small animal special ultrasound can obtain the high-resolution and clear anatomical structure of mouse heart [10].

Coronary artery damage in Kawasaki disease is a dynamic evolution process. As the mice grow up over time, so does the internal diameter of the coronary artery. Therefore, in this study, the ratio of the coronary trunk to the annular part was used to evaluate whether the coronary artery was widened or not. Combined with the pathological results, it can be observed that the ultrasonic coronary artery is similar to that of children with KD. At model 21 d,30d, the pathological results showed that part of the coronary artery in the model group was dilated.In contrast, the internal diameter of the coronary artery was broadened by ultrasound, which was consistent with the pathological results, but the degree was not typical. The reason is that the length of the coronary artery observed by ultrasound is limited to the initial segment. If the distal and middle segment of the coronary artery is dilated obviously, the general visual field is relatively limited.

The wall motion of mice was observed by M-ultrasound group, which indirectly reflected the left ventricular function. On the 21st day after modelling, a few KD mouse models showed abnormal wall motion. On the 30th day, the abnormal situation did not change, indicating that Kawasaki disease would affect cardiomyocytes in the acute phase, resulting in a decrease in the amplitude of ventricular wall motion, which may be related to myocardial swelling caused by acute inflammation [11].

According to the study, 72%-91% of patients with Kawasaki disease will have the manifestation of myocarditis [12]. Therefore, this study is consistent with the clinical manifestations of children. According to the above, the authors believe that Kawasaki disease can change the inner diameter of the coronary artery in KD mice and decrease the left ventricular function in a few mouse models.

In this study, high-frequency small animal echocardiography was used to dynamically observe the coronary artery of mice, and a clear image of the coronary artery could be obtained, but there were still some shortcomings. At present, there is no internationally recognised method to establish the arterial model of Kawasaki disease [13-14]. The existing animal models can only reflect some of the characteristics of several lesions of Kawasaki

disease and can not be completely simulated. Also, due to the influence of ultrasonic probe frequency and mice ' factors, the length of coronary artery observed by ultrasound is limited to the initial segment, and the observation of the middle and distal segment of the coronary artery is limited. However, because the coronary artery lesions caused by Kawasaki disease often occur in the proximal segment, this study can also accurately reflect the coronary artery lesions, which is in good agreement with the pathological results [15]. However, the sample size of this study is limited, and the mouse data indicators obtained have a certain reference value, so it is necessary to further improve the accuracy of the data indicators by increasing the sample size in the future.Stem cells are a kind of cells with strong self-renewal ability and multiple differentiation potential, and have unique biological characteristics. Human umbilical cord mesenchymal stem cells (hUC-MSCs) have gradually gained the favor of clinical researchers due to their wide sources, low immune response and multi-differentiation potential, and they have been able to differentiate into osteoblasts, lipids, fibroblasts, endothelial cells, cardiomyocytes and other cells [16].

In summary, the use of high-frequency ultrasound to study the process of coronary artery damage in LCWE-induced KD model in mice can objectively evaluate the changes of coronary artery in Kawasaki disease. At the same time, the measured value of mouse coronary artery can also provide the diagnostic basis for future animal research. But also proved that the intervention of hUC-MSCs could reduce the degree of inflammation of coronary artery lesions in mice.

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