# Original Article Establishment of a new animal model for ischemic lumbar vertebrae

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Abstract: Degeneration and ischemia of lumbar intervertebral disc has become a more and more important issue for elder people. However the mechanism for this is still known, largely due to a lack of a suitable animal model. In this study, we constructed a new animal model for the study of ischemic lumbar vertebrae. 42 New Zealand white rabbits were chosen for the study. For each rabbit, two vertebrae were used. L<sub>e</sub> was set as the experimental group and L, was set as the control group. Percutaneous lumbar puncture needles were applied in vertebrae adjacent to endplate for L<sub>5</sub> and L<sub>4</sub>. For L<sub>4</sub> 1 ml saline was injected and for L<sub>5</sub> 1 ml pingyangmycin (2 mg/mL) was used. 1, 2, 3, 4, 5 weeks; 2 and 3 months after surgery, 6 rabbits at each time point were randomly chosen and underwent MRI, pathological test. The results in L<sub>s</sub> and L<sub>s</sub> were compared. Another 6 rabbits were used for DSA (Digital Subtraction Angiography) and vascular cast to study the length and diameters of the branches of lumbar artery. It was identified that since the third week, slightly hyperintense signal on T2-weighted image (T2WI) and fat-suppression T2-weighted image (FS T2WI) were detected. Lumbar vertebrae damage could be identified since the fourth week. Results of MRI and the size of pathological area were positively related (r=0.965, P<0.05). DSA and vascular cast could both clearly show the third level branches of lumbar artery. Our study suggested that injection of pingyangmycin via percutaneous lumbar needle could successfully induce ischemia in lumbar endplate. This method had little trauma, required a simple operation process and is highly repetitive. Besides, by vascular cast, the most important source of blood supply is the media branch of the lumbar artery. This branch could be a new therapy pathway for the degeneration of lumbar vertebrae.

Keywords: Ischemic lumbar vertebrae, vascular cast, rabbit model

#### Introduction

Nowadays, spinal disc degeneration has become a serious health issue for the older people [1]. However the mechanisms of how the degeneration occurs and develops are still poorly understood. Previous reports have indicated that microcirculation disturbance under the lumbar endplate plays an important role in this process [2-4]. However, further studies on this are restricted because of the lack of a suitable animal model. Currently, animal models of ischemic lumbar vertebrae are mainly constructed via physical or genetic methods. In these models, ischemia is caused by direct intervention, which is guite different compared to a normal clinical course [5]. No important changes like microcirculation disturbance are involved in these models. On the other hand, although lumbar artery is the main supplying artery for lumbar vertebrae, the anatomy of it remains unclear [6]. Thus a new animal model for studying ischemic lumbar vertebrae and vascular cast is required.

Rabbits are commonly used animals in this area [7]. The size of a rabbit is suitable for the complicated construction of microcirculation disturbance. Besides, there are already abundant choices of instruments for the detection of the lumbar vertebrae for rabbits. In this study, we used percutaneous lumbar endplate injection of pingyangmycin to conduct microcirculation disturbance in lumbar vertebrae. Pingyangmycin is an antitumor glycopeptide antibiotic belonging to the bleomycin family. It could

# <sup>A</sup> vertebrae puncture



# <sup>B</sup> lumbar vertebrae

- a plain radiographs
- b specimens
- c decalcified specimens



# <sup>c</sup> XperCT scanning of the location of injection

a the frontal film

b the lateral film

c vertebral angiography



Figure 1. A. Diagrammatic sketch of the puncture operation. B. Diagrammatic sketch of the lumbar. C. Scanning of the location of the injection.

induce apoptosis and fibrosis in local tissues [8]. With application of pingyangmycin, we aim-

ed to induce ischemia without direct damaging lumbar vertebrae. This is quite important for



<sup>B</sup> translumbar arteriography anteroposterior postition lateral postition

DSA image of the injection



А



<sup>c</sup> vasular casting anteroposterior postition

lateral postition



**Figure 2.** A. DSA image of the location for injection. B. DSA image of the translumbar arteriography in rabbits. C. The specimens of the vascular casting.

the study of the process of degeneration of lumbar vertebrae. Furthermore, we used Digital Subtraction Angiography (DSA) and vascular cast to detect the blood supply and anatomy in lumbar endplate. These studies help to figure out new pathways and methods for treating degeneration of the intervertebral disc.

# Materials and methods

# Animals

From August 2008 to September 2009, 48 New Zealand Rabbits were randomly chosen for the study. The rabbits were male, had an average age of 2 months, weight 3200±30 g. All rabbits were bought from the animal center of Sun Yat-Sen University. (Permission Code: SCXK200-8-0002). The rabbits were housed under clean conditions in the animal center. The temperature was controlled at 25 degree Centigrade. All rabbits were feed separately. All animal experiments complied with the guidelines of Sun Yat-Sen University animal management program.

# Construction of the model

Among the 48 rabbits, 6 rabbits were used for the study of DSA/vascular cast and the rest were for the induction of the ischemic lumbar vertebrae. For the model, on each rabbit, two groups were set. The fifth lumbar vertebra ( $L_5$ ) was the experiment group and the fourth lumbar vertebra ( $L_4$ ) was the control group (**Figure 1B**). The induction

	Types T <sub>1</sub> WI		$T_2WI$	Histology				
	Modic I	Low signal	High signal	Fiber granulation tissue				
	Modic II	High signal	High signal/isointensity	Adipose tissue				
	Modic III	Low signal	Low signal	Sclerosis				

Table 1. Modic standard

methods were as follows; 3% pentobarbital was injected intravenously via ear vein. The dose is 1 ml/kg [11]. After anesthesia, the rabbits were fixed on the plate in the prone position. Hair on the back was removed and the skin in the operation area was cleaned and sterilized by povidone iodine. Then the puncture site was draped.

The operation was conducted under the direction of Computed Tomography (CT). The puncture site was located in 1 cm away right to the posterior midline, under 1/3 parts of the vertebrae. Medullo-puncture needle was used for the puncture. It was punctured with an inclination angle of 60 degree (Figure 1A). The needle point should reach the midline under the endplate. Via CT-guided localization, under lateral view, the needle should get to the position located between 2/5 of the anterior vertebra and 3/5 of the posterior vertebra. 1 ml ultravist (150 mg/ml) was injected into the local area via the puncture needle. Then, another 1 ml saline was injected to wash the needle. After 3 minutes, X per CT was used to analyze the location of the needle. Ultravist was used to help ensure the correct location of the needle point (Figure 1C).

For the control group, 1 ml saline was injected and for the experimental group, 1 ml pingyangmycin was injected. The injection was slowly and should last at least 5 minutes each time. After then, the needle was withdrawn and the puncture area was pressed for 2 minutes. The local operation area was sterilized again and bandaged up. After the operation, each rabbit receive intramuscular injection of 40000 U gentamycin for 3 days to prevent infection [11].

# Study of vascular cast

6 rabbits were randomly chosen from the 48 rabbits for the study of vascular cast. The rabbits were anesthetized by intravenous injection of 1 ml 3% pentobarbital. The sterilization and fixation procedures were the same

with the model-construction rabbits.

An incision was made in the right groin area. Arteria cruralis and femoral vein were isolated and lifted by 4/0 wire. Seldinger technique was applied for the

antidromic puncture of arteria cruralis. A balloon was sent to the place next to the aortaventralis under the guidance of the wire. The distal end of the balloon should be located 1 cm above the bifurcation of the two iliac arteries. The balloon was expanded to block the blood flow. The balloon pressure was 4-5 atm (1 atm=101.325 kPa). The blocking lasted for 60 seconds. Then 3 ml ultravist (300 mg/ml) was injected with a speed of 0.5 ml/s for the arteriography. The technique of digital subtraction angiography (DSA) was used to detect the form and structure of the artery. The length and diameters were recorded (**Figure 2A, 2B**).

After DSA examination, vascular casting was done on the rabbits. Skin preparation was done on the chest. Before the operation, 4000 U heparin sodium was injected intraperitoneally. A median incision was made along the chest and the abdominal wall. The descending aorta was isolated and lifted by the wire. Arteriopuncture was done on this site with a venous indwelling needle (22G). On the other hand, the two iliac arteries were also isolated and lifted. The proximal end of postcava was cut down. 250 ml heparin sodium was injected via the indwelling needle in the descending aorta. The lavage would last until the fluid in the postcava became clear.

Then both iliac arteries were ligated. The vessel casting agent was infused via the venous indwelling needle slowly until the resistance became very significant. The total dose was near 12 ml. The body would be placed in a ventilated room with the temperature of 25 degree centigrade for 8 hours. Then the local body including the chest and the abdominal cavity which contain the blood vessel was put into the cylindrical glassware full of hydrochloric acid (368 g/l). After 24 hours, the specimen was taken out and washed by clean water. The vessel casting specimen was then got and measured (**Figure 2C, 2D**).

# <sup>A</sup> MRI examination of the model b sagittal view a coronal view sagittal view С FST2WI FST1WI T2WI E4 L4 L415 L5 .5

# Pathological examination of the model В

4 weeks after operation а

5 weeks after operation b



# <sup>c</sup> Final effects of the model

b endplate





**Figure 3.** A. MRI examination of the model after 4 weeks. B. Pathological examination of the model after 4-5 weeks. C. MRI and pathological examination of the model after 2 months.

#### MRI examination

1, 2, 3, 4, 5 weeks and 2, 3 months after surgery, MRI examination was done. At each time point, 6 rabbits were randomly chosen for the study. Fat Suppression T1 weighted image (FST<sub>4</sub>WI), T<sub>2</sub>WI and Fat Suppression T<sub>2</sub>WI (FST\_WI) were included in the MRI scanning. The scanning data was listed as follows; T<sub>1</sub>WI; TE 12 ms, TR 540 ms, FOV 200 mm, thickness 2-3 mm. T<sub>2</sub>WI, TE 100 ms, TR 1900 ms. The diagnosis was done by two independent MRI physicians [12]. Besides, 4 weeks after the operation, the size of the high signal area in the sagittal vertebrae was measured (length × width, mm<sup>2</sup>). For each vertebra, 4 sections were detected and measured. The degree of ischemia was evaluated by the Modic standard. Details of the standard were shown in Table 1.

#### Pathological examination

At each time point, the rabbits were sacrificed after MRI examination. The L<sub>4</sub> and L<sub>5</sub> vertebral bodies were taken out and then fixed in paraformaldehyde (4%) for a week. The fixed lumbar vertebrae were then decalcified by Plank-Rycholo liquid for 5-7 days. Then, these materials were disposed as following; triformol-fixed, dehydrated in a graded alcohol series, embedded in paraffin and then cut into slices. (5 mm tissue slice). Then slices were analyzed with hematoxylin-eosin (HE) staining. Site and range of the damage in vertebrae were detected at low magnification and the form of cells and structures were detected at high magnification by the microscope. In order to quantitatively analyze the effects of the model, the precise sizes of the lesion area were calculated by mea-



# <sup>B</sup> comparison of DSA and vasular casting



measurment of the arteries (mean±s, n=6, mm)



sure the length and width at high magnification (mm<sup>2</sup>). The index for comparing include the follows; form and structure changes of the lumbar endplate, disc material in both the experimental group and the control group. The experimenter and the observer were trained together and randomized to different responsibilities. They were blind to each other during the study.

#### Statistical analysis

The software of SPSS 17.0 was used for data input during the experiments. Chi-square test was used in comparing of enumeration data between different groups while student's t tests for measurement data. Pearson correlation analyze was used for the analyzing of relation between MRI and pathological examinations. P< 0.05 was set as the standard of a significant difference in a comparison.

#### Results

# Results for animals after model-construction

All rabbits survived after the puncture. The feeding behavior and activity situations of these rabbits are all normal. There was no hemorrhage or hematoma in the puncture area. 1 rabbits showed the sign of slight hemiplegia. MRI examination showed that there was hemorrhage inside the vertebrae (**Figure 3A**). The hematoma pressed the spinal nerve root. After 3 weeks, the symptom slowly remised.

## MRI and pathological examination

There were no significant changes in MRI and pathological examination in the control group. On the other hand, in the experimental group, there were also no changes for the first 2 weeks after operation. However, since the 3<sup>rd</sup> week, MRI examination showed low

signal of  $FST_1WI$  and slightly high signal of  $FST_2WI$ . The changes were more significant in the 4<sup>th</sup> week after operation (**Figure 3A**).

The pathological examination showed that since the 3<sup>rd</sup> week, there had been disorders in the structure of the bone trabecula. The number of the osteocytes also decreased. Adipocyte slowly began to gather and mixed together in this area. The chondrocytes began to disappear in the endplate and the structures also began to degrade. Since the 5<sup>th</sup> week, significant symptoms of intervertebral disc degeneration began to appear; apoptosis were detected in most of the chondrocytes in the endplate.

<b>Table 2.</b> Correlation analysis of $S_{T2WI}$ (the size
of $T_2$ WI area by MRI) and $S_{histology}$ (lesion area
by histology slices)

Items	S <sub>T2WI</sub>	S <sub>histology</sub>	
Maximum size	27.12	33.14	
Minimum size	4.17	6.32	
Average size	13.21±0.64*	17.03±0.57	
Data	r=0.965	p<0.01	

\*Pearson correlation, correlation is significant at the 0.01 level (2-tailed).

The structures of the nucleus pulposus and the annulus fibrosus were significantly damaged. Ischemia in the endplate lasted ever since the model was induced (**Figure 3B**, **3C**). Correlation analysis showed that 4 weeks after the operation, the size of the  $T_2$ WI and the size of the lesion area in pathologic sections were positively correlated (**Figure 4A**), (r=0.965, P<0.01). Detailed data were shown in **Table 2**.

## Study of DSA and vascular cast

The processes of the application of DSA and vascular cast went smooth without any accidents. The DSA detection showed that in rabbits, the branches of the lumbar arteries were as follows; the trunk started from the aorta and divided into two branches to the right and left side. Each branch then divides again into two branches to the internal and external side. The internal branch went to the lower 1/3 parts of the endplate area and the external branch supply mainly the soft tissue area around the psoas (Figure 2A, 2B). The length and diameters of the branches were shown in Table 3. Vascular casting was used to directly and three dimensionally show the detailed form and location of the vessel (Figure 2C). Our findings revealed that the results of DSA and vascular casting were highly consistent (P>0.05, student's t tests) (Figure 4B).

# Discussion

Blood supplies of the intervertebral disc mainly come from the annulus fibrosus pathway and the endplate pathway, in which the latter one is the main resource [13]. Nutrients in the blood enter intervertebral disc via endplate and they are finally absorbed by annulus fibrosus and vertebral pulp. Previous reports showed that lumbar arteries in human would divide into 2

branches after entering the vertebrae and finally forming the vasoganglion in the endplate area [14]. Others further used the method of vascular casting and found that microvessel density in vertebral pulp was quite high but in the inner layer of the fibrosis rings it was quite low. Based on these findings, it was identified that nutrients in the capillary network in endplate could diffuse into the cavity of the disc. Thus, blood supply of the intervertebral disc mainly depended on the contact situation between blood vessels and the discs. For human body, it was reported that since the age of 20, the blood vessel density began to decrease. This time point was in accordance with that for the starting of intervertebral disc degeneration [14-16]. By now it has already reached an agreement that there was close relation between microcirculation disturbances in the endplate area and the degeneration of intervertebral disc [10, 16]. In order to suppress the process of degeneration, methods should be taken aiming at improving blood supply in the local area. Thus a good animal model of intervertebral disc degeneration is required for this study.

By now the most widely used animal model of intervertebral degeneration mainly included 2 types; induced model and spontaneous model [17]. Rats, rabbits and dogs are the preferred animal for constructing this model. Researchers induced degenerations by directly damaging the disc, changing the biomechanics in the local area or use genetic engineering technology to change the genetic background. Although these methods did induce degeneration, they did it directly and very soon. Comparing to the natural process, in these direct methods, there was an absence of microcirculation disturbance before the degeneration finally showed up. Ever since propose of the hypothesis of microthrombosis in the endplate, researchers tried to construct a new model of intervertebral degeneration by inducing microcirculation disturbance. By intravenous injection of lipopolysaccharide (20 µg/kg) and intramuscular injection of methylprednisolone (10 mg/kg), the rabbits could develop disc degeneration in 3 months [18]. However, this method would cost too much time to induce degeneration, besides, long period use of the medicine would be expensive and the long term use of glucocorticoid made the rabbits very easy to get infected

Items	Index	AA	TLA	BLA	IBLA	EBLA
length	DSA	-	6.42±0.66	1.40±0.09	3.82±0.79	6.65±0.94
	Vascular cast	-	6.83±0.75	1.48±0.08	3.67±0.82	6.50±1.05
	T**		1.016	1.548	-0.323	-0.260
	Р		0.334	0.153	0.754	0.800
Diameter	DSA	2.37±0.08	1.05±0.05	0.71±0.09	0.52±0.06	0.49±0.06
	Vascular cast	2.45±0.10	1.13±0.08	0.75±0.10	0.55±0.54	0.51±0.07
	T**	1.536	2.076	0.766	0.754	0.775
	Р	0.156	0.065	0.461	0.468	0.456

Table 3. Measurement of the aorta and lumbar artery (±s, n=6, mm)

\*\*2-Independent sample test,  $\alpha$ <0.05, DSA: Digital subtraction angiography; AA (abdominal aorta); TLA (trunk of lumbar artery); BLA (left or right branch of lumbar artery); IBLA (internal branch of lumbar artery). EBLA (external branch of lumbar artery).

and died. Iwahashi, et al tried to smear nicotine in the skin and kept a blood concentration of 110 ng/ml. They reported that after 8 weeks, micro-vascular reduction and loss of disc collagen were observed, finally leading to the degeneration [19]. However, a high blood concentration of nicotine could also cause many other symptoms, these affections are too complicated and could affect the results in the further application of this model. Besides, Xu, et al also used bone cement filling to directly damage the structure of the endplate and the disc and blocked the blood supply. After 4 weeks, the degeneration could be observed [20]. The lack of this method is that bone cement is rigid; it could significantly change the situation of spinal bearing, thus making the situation in the local area too complicated. Taking the previous trials together, an easier, highly repeatable, time saving method is required.

Pingyangmycin has been widely used in the treatment of vascular disease. It could significantly inhibit DNA synthesis in the cells and interfere with cellular metabolism, resulting in cellular degeneration and apoptosis. Application of pingyangmycin in the local capillary network could cause formation of microthrombosis. Thus, in our study, we used injection of pingyangmycin in the endplate area to induce degeneration.

Considering the damaging effect of pingyangmycin, a precise location should be reached before injection to avoid side effects [21]. According to previous reports, the puncture point in the body surface was chosen at about 1/3 down the spine and 1 cm right to the midline. The guidance of XperCT was used during the puncture process [22]. With this technique, we could clearly locate the needle point in the endplate area. Before injection of pingyangmycin, the contrast medium ultravist was used to exclude existence of great vessels in the area. The use of XperCT for the guidance of the puncture and the application of radiography before injection of pingyangmycin were the key factors for constructing of the model.

Ischemia and degeneration are continuous processes. The time point for the show up of disc degeneration also required frequent examinations after the operation [12-14]. In our research, by MRI and pathological examination, we identified that since the fourth week after the injection, there had been signs of damages of osteocytes and capillaries. Since the fifth week, typical degeneration could be identified in the vertebrae. This finding not only proved the importance of ischemia in the development of degeneration but also showed that this method could successfully induce such a model.

However, detecting the pathological specimens required the experimenters to sacrifice the rabbits first. Thus, a new method which would not need to hurt the rabbits was needed. Previous studies on human revealed that, in the lesion site on the vertebral body, there were low signals in  $T_1$ WI and high signals in  $T_2$ WI. The changes indicated the existence of ischemia and necrosis. The MRI imaging characters could be explained as follows; In MRI imaging, the normal vertebrae containing more adipose tissue and less water would show a high signal in  $T_1$ WI and slightly high signal/isointensity in T2WI [23-25]. However, in the degeneration site, the osseous tissue including adipose and myeloid tissue slowly disappeared and was replaced by brous tissue and sclerotic bone. Thus in  $T_1WI$ the signals would get lower down and in  $T_2WI$ there would be opposite changes. According to this characteristic, we used MRI to detect the changes of vertebrae at different times after operation as well. The result showed that MRI could also be a good method for evaluating the results of the model. Moreover, this method did not need to hurt the rabbits after operation and thus more animal models would be available finally.

Digital Subtraction Angiography (DSA) is a technique combining both X-ray image and computer. It had been widely used in clinical examinations on coronary heart disease [26]. However, few reports on the study of DSA on lumbar arteries were found. In our research, we used both DSA and vacular casting to further study the anatomy and branches of the vessels in the endplate area. Both methods revealed similar findings that the internal branch of lumbar artery was the main blood-supplying artery. On the other hand, we noticed that in there were still a few differences in the data of the diameters. This may be caused by the different mechanism of the two methods. DSA imaging showed the inner diameter of the arteries but vascular casting showed the external diameter. In rabbits the differences may not be clear, but for those patients with diabetes or hypertension, thickness of the vessel may vary much. Vascular casting could help direct a more suitable intubation tube and DSA could help measure the actual situation of the artery. Considering the importance of the internal branch of lumbar artery in providing blood supply to the vertebrae, our findings by DSA and vascular casting may provide a new pathway for treating and preventing microcirculation disturbance in the endplate.

## Conclusion

In this study, by directly injection of pingyangmycin to the vertebrae endplate, we successfully induced an ischemia model in the vertebrae. This model was tested and certificated by further pathological examination and MRI detection. This new animal model did not require direct impact to the vertebra. The induction of ischemia was highly similar to the natural progress of vertebrae degeneration. Beside, this model was easy to construct and high repeatable. Thus the application value of this model should be very high. On the other hand, by vascular casting, we found that internal branch of lumbar artery was the main blood supply for the lumbar vertebra and intervertebral disc. Thus this may be a new pathway for cell or medicine therapies.

## Disclosure of conflict of interest

## None.

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