

行政院國家科學委員會專題研究計畫 成果報告

心包膜腔內注入 Neuropeptide Y 以誘發缺氧心肌之血管新生－豬之動物實驗模式

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關鍵詞: 血管新生；側枝循環；心包膜穿刺；冠狀動脈病；心肌缺氧

有些冠狀動脈心臟病病人由於嚴重且廣泛之冠狀動脈阻塞，導致嚴重心肌缺氧，一般採行之血管再通術完全無法施行。目前發現有一些血管新生促進物資可加強側枝循環之形成，但至目前為止僅有 FGF 及 VEGF 在臨床上使用。

Neuropeptide Y (NPY) 已經證實為一種強力之血管新生因子。心包膜腔對心肌而言應是一個很適當的給藥途徑，可以長時間發揮治療效果。本實驗擬將 Neuropeptide Y 注入已誘發心肌梗塞的豬之心包膜腔內，以觀察其對缺氧之心肌誘發血管新生的功效。

我們進行三組實驗，即 NPY 組，對照組及心包沾粘組。首先在實驗豬以冠狀動脈堵塞法引發急性心肌梗塞，大多堵塞左前下行冠狀動脈。經 1-3 星期後，將藥物（NPY 或 minocyclin）或僅給生理食鹽水注入心包膜腔。NPY 組共有 10 隻豬，每隻打入 NPY 0.5 mg/5 西西無菌水。對照組有 10 隻豬，只打入 normal saline。心包沾粘組共有 9 隻豬，在 AMI 後 1-2 星期在心包膜腔打入 minocyclin。在 AMI 後 4-7 星期，將實驗豬犧牲，取其心臟作觀察，測量，並作顯微鏡觀察。

在本實驗中我們發現，在心包膜腔打入 NPY 並不能在心肌梗塞部位鄰近之心肌內誘發血管增生，但接受 NPY 之實驗豬其心臟重量較低，此為一有利之變化。我們也發現，左心室壁厚度及心肌梗塞大小並不因打入 NPY 或 minocyclin 而有所改變。但出乎意料的，我們發現，心包膜腔注入 minocyclin 可誘發血管之增生。此一發現值得更進一步之研究。

English abstract

Key word: angiogenesis; collateral circulation; pericardial drug administration; coronary artery disease; myocardial ischemia

There are patients with coronary artery disease who suffered from severe and diffuse narrowing or occlusion of their coronary arteries, in whom the conventional revascularization procedures can not be applied. One potential strategy for these patients is creation of new blood vessel channels in the region of ischemia, (therapeutic angiogenesis). A number of potential angiogenic agents have been identified to enhance the process of collateral development, but, to date, only two growth factors, fibroblastic

growth factor (FGF) and vascular endothelial growth factor (VEGF), have been clinically used in patients with refractory ischemia.

Neuropeptide Y (NPY) has been proved as one of the potent growth factors for angiogenesis. The pericardial space may potentially serve as a drug delivery reservoir which may render persistent effects of the administered therapeutic agents to the heart. In this experiment, we tested the effects of NPY administered through pericardial chamber on the angiogenesis of ischemic myocardium in a porcine myocardial infarction model.

We performed experiments on 3 groups of pigs, the neuropeptide Y (NPY) group, the control group, and the adhesion group. Acute myocardial infarction (AMI) was first induced in all experimental pigs by occlusion of a coronary artery, left anterior descending artery in most pigs. After 1-3 weeks, either drug administration (NPY or minocyclin) or non-specific drug (saline) administration (control group) was performed. The NPY group consisted of 10 pigs, in them NPY 0.5 mg in 5 ml sterile water was administrated through a catheter into the pericardium. In the control group, 10 pigs, after induction of AMI, only saline intrapericardial administration was applied. In the adhesion group, 9 pigs, minocyclin was administrated into pericardial sac 1-2 weeks after AMI to induce pericardial adhesion. Pigs were sacrificed 4-7 weeks after induction of AMI and the hearts were measured grossly and examined microscopically.

In this study we found that administration of NPY did not induce new vessel production in the myocardium near the infarction area. Yet, the administration of NPY seemed to result in reduced heart weight, a beneficial effect. We also found that the wall thickness of LV wall as well as the infarction size was not affected by the administration of either NPY or minocyclin. Incidentally, we found that intrapericardial administration of minocyclin could induce active new vessel growth in the myocardium near the infarction area. This finding may be worthy of further studies.

Despite the advances in revascularization therapy for coronary artery disease with either percutaneous technique or surgical bypass surgery, a certain number of patients are suffering from severe ischemia but are poor candidates for these therapeutic modalities (1,2). For these patients, the disability resulting from severe limiting angina is substantial. One potential strategy of therapy for these patients is the creation of new channels for blood supply to the ischemic region. Early series of transmyocardial revascularization (TMR) reported a high 5-9% perioperative mortality with even higher overall morbidity (3,4). An alternative option is the induction of growth of new blood vessels to the ischemic myocardium, or angiogenesis (1,2).

Although a number of potential angiogenic agents have been identified to enhance the natural process of collateral development, only two growth factors, fibroblastic growth factor (FGF) (5) and vascular endothelial growth factor (VEGF) (6) have been applied in clinical trials on patients with refractory ischemia. These agents have been administered to the patients through different routes, including intravenous, intracoronary, intramyocardial, and intrapericardial routes.

Although angiogenesis therapy using either systemic or local delivery of growth factors or surgical laser revascularization remains active areas of investigation, the results have been found somewhat disappointing to date (7). In one prospective, multicenter, randomized trial (8), in patients with class III or IV angina caused by nonrecanalizable chronic total occlusion of coronary arteries, percutaneous transmyocardial revascularization did not result in a greater reduction in angina, more improvement in exercise duration or survival free of adverse cardiac events, as compared with maximal medical treatment only.

Given the typically long course of new collateral vessel development, most attempts to stimulate myocardial angiogenesis have used methods of prolonged growth factor delivery, including gene therapy, continuous infusions, repeated injections, or sustained release polymers (9). The pericardial space may potentially serve as a drug delivery reservoir that may be used to deliver therapeutic agents to the heart (10).

Neuropeptide Y (NPY) is a 36-amino acid amidated peptide originally isolated from porcine brain (11). NPY is one of the most abundant peptides in the brain and heart (12). NPY is a potent mitogen for vascular smooth muscle cells and

endothelial cells, as potent as basic fibroblast growth factor (13). NPY has been tested in *in vitro* as well as *in vivo* systems for the induction of angiogenesis (14-21). Yet, to our knowledge, the angiogenetic effects of pericardially administered NPY for the ischemic myocardium has never been reported in the English literatures. We hypothesized that local application of the potent angiogenesis factor, NPY, may keep a longer effects on the ischemic myocardium to induce active angiogenesis and to render benefits for the symptom relief and function improvement for the affected myocardium. If this hypothesis is proven correct, there may be a novel therapeutic modality in the management of intractable myocardial ischemia and myocardial dysfunction.

Methods and Materials

A. Induction of acute myocardial infarction (AMI):

Mini-pigs with body weights between 10-30 kilograms were used in this experiment. Mini-pigs were anesthetized with intramuscular injection of ketamine (4-5 ml, 50 mg/ml) and atropine (0.5-1.0 mg). An intravenous route was set-up on ear vein for supplemental injection of propofol and succinylcholine during experiments, as needed. After endotracheal intubation, the pigs were connected to a Harvard respirator for artificial respiration with room air. The baseline electrocardiogram (ECG) was then taken. By skin cut-down, right femoral artery was catheterized with a 6F sheath. After blood pressure recording, through the sheath, a 6 Fr right Judkins catheter was advanced to engage the left coronary artery. After confirmation of the coronary anatomy by contrast medium injection, the catheter was further advanced into the left anterior descending coronary artery (LAD) to about the mid point between the orifice and the apex. There, through the catheter, a Gianturco coil (2 or 3 mm in diameter and 2- or 3 cm in length, Cook Group Inc.) was deployed to occlude the coronary artery (22,23). The pigs were then monitored for hemodynamics and ECG changes. VT/VF was treated with cardio-pulmonary resuscitation, drug administration (lidocaine and adrenaline), and DC cardioversion. The pigs were sent back to animal room for further care after stabilization. A second ECG was taken at the end of the procedure to document the ECG changes of AMI (Fig. 1).

B. Pericardial access

One to three weeks after induction of AMI, the pigs were again anesthetized and put on artificial respiration. Mediastinum was entered by a minimal subxiphoid skin cutdown. Under direct vision, a No. 20 intravenous catheter was inserted into the pericardial sac for administration of drugs. After drug injection, the intravenous catheter was removed and any leakage from pericardial sac was inspected. The skin wound was then closed surgically and the pigs were returned to the animal room for further care.

C. Intrapericardial application of NPY

NPY (Sigma) was prepared with 0.5 mg in 5 ml distilled water immediately before injection. NPY solution was injected slowly into pericardial sac through the catheter, avoiding leaking. Totally 10 pigs received NPY injection 2 to 3 weeks after induction of AMI. Some pigs without pericardial injection or with pericardial injection with other materials were included in the experiment as control.

D. Pathological examination.

At 5 -7 weeks after induction of AMI, the pigs were sacrificed and the hearts were examined pathologically. The weight and dimensions of the heart and the thicknesses of the free walls of left ventricle and right ventricle and interventricular septum were measured. The dimensions of left ventricle and right ventricle were also recorded. The location and size of the infarction were inspected. The hearts were then fixed with buffered neutral formalin for 24 hours and were cut along the horizontal plane (24). Myocardium at the margin of infarction area was examined and the numbers of small vessels were calculated under 200X microscopic examination (25). For each specimen, the number of small vessels were counted from 5 most proliferated areas at epicardiomyocardial junction and the average numbers were recorded.

E. Statistics

There were 10 pigs in the NPY group, 10 pigs in the control group in which no drug injection or non-specific injection into pericardium and 9 pigs in the adhesion group in which minocin was injected into pericardial sac to induce pericardial adhesion.

The data of mean and standard deviation were obtained for heart weight, thicknesses of left ventricle free wall, right ventricle free wall and interventricular septum. The corrected measurements (measurements divided by body weight) were obtained for heart weight, and the thickness of LV free wall, interventricular septum and RV free wall. The infarction area was calculated by multiplying the 2 perpendicular diameters and also corrected infarction size by dividing with body weight. The number of vessels in subepicardial myocardium near the infarction area were counted in 5 randomly selected areas and the average vessel number was obtained by dividing the total vessel number with 5. These data were compared between study groups by unpaired *t*-test. A *p* value <0.05 was regarded as statistically significant.

Results

As shown in Table 1, there were 10 pigs in the NPY group, 10 pigs in the control group in which no drug injection or only non-specific injection into pericardium and 9 pigs in the adhesion group in which minocin was injected into pericardial sac to induce pericardial adhesion.

The mean body weight was higher in the NPY group (25.61 ± 3.48 kg, range 18.4 to 29 kg) as comparing to the control group (16.64 ± 3.19 kg, range 12.2 to 20 kg, $p < 0.001$) and to the adhesion group (16.43 ± 2.42 kg, range 12 to 20 kg, $p < 0.001$). Body weights were not different between the control group and the adhesion group.

The heart weight was higher in the NPY group (177.1 ± 24.3 g, range 135 to 220 g) as comparing to the control group (142.2 ± 30.6 g, range 100 to 195 g, $p < 0.025$). There was no difference in heart weight between the NPY group and the adhesion group (158.6 ± 28 g, range 110 to 205 g) and also between the control group and the adhesion group. In comparing the corrected heart weights (heart weight divided by body weight, g/kg) among these groups, it was found that the NPY group had lower corrected heart weight than the control group (6.94 ± 0.61 vs. 8.75 ± 2.25 , $p < 0.025$) and also than the adhesion group (9.67 ± 1.12 , control group, $p < 0.001$).

The corrected thickness was also calculated for the LV free wall, interventricular septum and RV free wall, as shown in Table 1. There was no statistically significant difference between different groups for all of these measurements. LV size, corrected LV size, infarction size and corrected infarction size were also shown in Table 1. By

statistical analysis, there was no significant differences for all these data among the 3 groups.

Fig. 2 demonstrated gross observation of the heart specimen showing myocardial infarction area. Microscopic findings were shown in Figs. 3-5. It was noted that blood vessels were more dense in the adhesion group as comparing to the control group and the NPY group.

Discussion

In this experiment, we studied the effect of intrapericardial administration of neuropeptide Y (NPY) on heart changes in pigs after induction of myocardial infarction and comparing it with that of controlled pigs (MI without NPY treatment) and of pigs with chemically-induced pericardial adhesion after MI. Myocardial infarction was produced by acute coronary occlusion with a Gianturco coil by cardiac catheterization. In most of the pigs, anterior infarction was induced, as to our experience in previous studies, this infarction resulted in higher survivals.

At inspection of the excised heart, the induction of myocardial infarction was successful in all pigs in this experiment (Fig. 2). ECG also demonstrated acute changes of AMI (Marked ST-segment elevation, mostly in right precordial leads, V2 – V4).

As demonstrated in our experiment, the mean body weight was higher for the NPY group than the control group and the adhesion (both $p < 0.001$) while the latter groups were similar in mean body weight (Table 1). Heart weight was higher for the NPY group than the control group ($p < 0.025$). By correction with body weight, it was found that the NPY group had lowest heart weight among the 3 study groups ($p < 0.025$ in comparing with the control group, and $p < 0.001$ in comparing with the adhesion group). By body weight correction, it was found that there was no statistically significant difference among the 3 study groups for the thickness of LV free wall, interventricular septum, and RV free wall. The corrected LV chamber size and LV infarction size were also not different among the 3 groups.

It is interesting to note that the vessel number in the myocardial layer near infarction area was not increased after pericardial administration of NPY, suggesting no beneficial effects of NPY for the pigs after myocardial infarction. Of course, this conclusion may be applied only to our specialized experimental condition, that is,

anterior myocardial infarction, with drug administration through pericardial sac at 2-3 weeks after myocardial infarction. It is worthy to note that, parallel to our NPY study, we also did some adhesion study in which minocin was administrated through pericardial approach, We found that the heart size was not reduced, as contrary to our initial speculation. Yet, we detected much new vessel growth in the myocardium near the infarction area in the pigs treated with intrapericardial application of minocin. This finding is very interesting and needs further investigation.

In conclusion, in this experiment, we found that pericardial application of neuropeptide Y (a potent growth factors for angiogenesis) in pigs after myocardial infarction, did not produced increase in density of vessels in the myocardium near the infarction area. Yet, the corrected heart weight was reduced with this treatment. We also found that intrapericardial application of minocyclin to the infarction pigs may produce an active vascular proliferation in the myocardium near the infarction area.

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Table 1 Heart dimension measurements for the 3 different groups of pigs

Group	Pig No.	B.W. (kg.)	Heart wt. (g)	Heart wt.(c) (g/kg)	Thickness (mm)		
					LV	IVS	RV
NPY	10	25.61	177.1	6.94	1.49	1.43	0.54
		± 3.48	± 24.3	± 0.61	± 0.42	± 0.34	± 0.18
Control	10	16.64	142.2	8.75	1.11	1.08	0.51
		± 3.19	± 30.6	± 2.25	± 0.32	± 0.42	± 0.23
Adhesion	9	16.43	158.6	9.67	1.00	0.88	0.44
		± 2.42	± 28	± 1.12	± 0.30	± 0.20	± 0.24

Abbreviations: B.W.= body weight; Heart wt. (c)=corrected heart weight; LV=left ventricle

Table 1 Heart dimension measurements for the 3 different groups of pigs (Cont.)

Group	Thickness (c) (mm/kg)			LV size* (cm ²)	LV size (c)
	LV	Septum	RV		
NPY	0.068	0.057	0.021	9.0	0.355
	± 0.023	± 0.015	± 0.007	± 6.67	± 0.274
Control	0.069	0.069	0.031	10.53	0.642
	± 0.023	± 0.032	± 0.014	± 7.78	± 0.484
Adhesion	0.065	0.054	0.028	10.19	0.62
	± 0.029	± 0.014	± 0.021	± 4.05	± 0.227

*LV size calculated by multiplying 2 diameters

Table 1 Heart dimension measurements for the 3 different groups of pigs (Cont.)

Group	Infarct size# (cm ²)	Infarct size (c)
NPY	9.0 ±6.67	0.355 ±0.274
Control	10.53 ±7.78	0.642 ±0.484
Adhesion	10.19 ±4.05	0.62 ±0.227

#Infarct size calculated by multiplying 2 diameters

Fig. 1, A. ECG before coronary occlusion

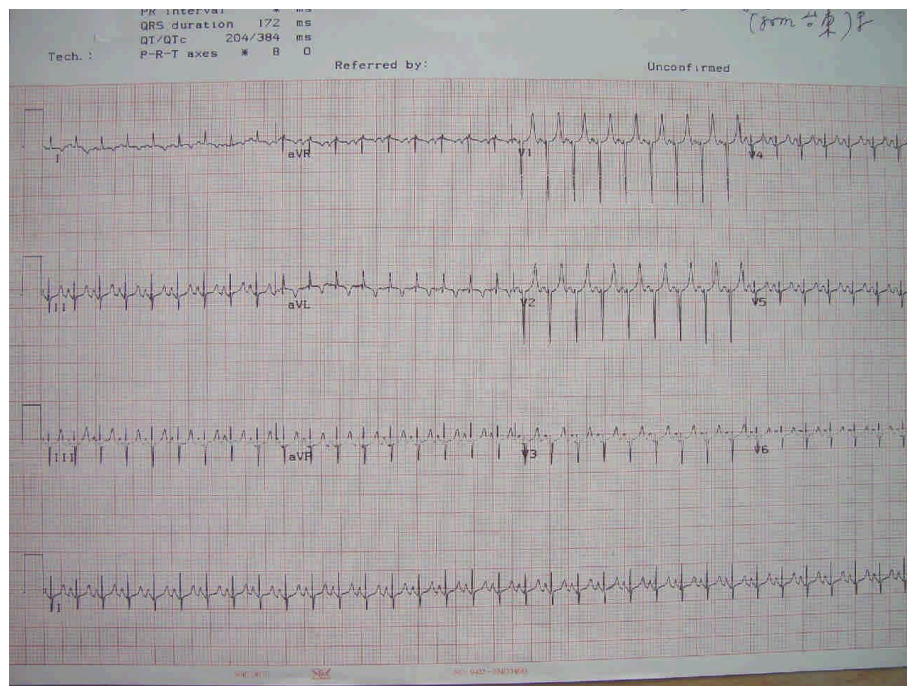


Fig. 1 B, ECG after coronary occlusion showing anterior myocardial infarction

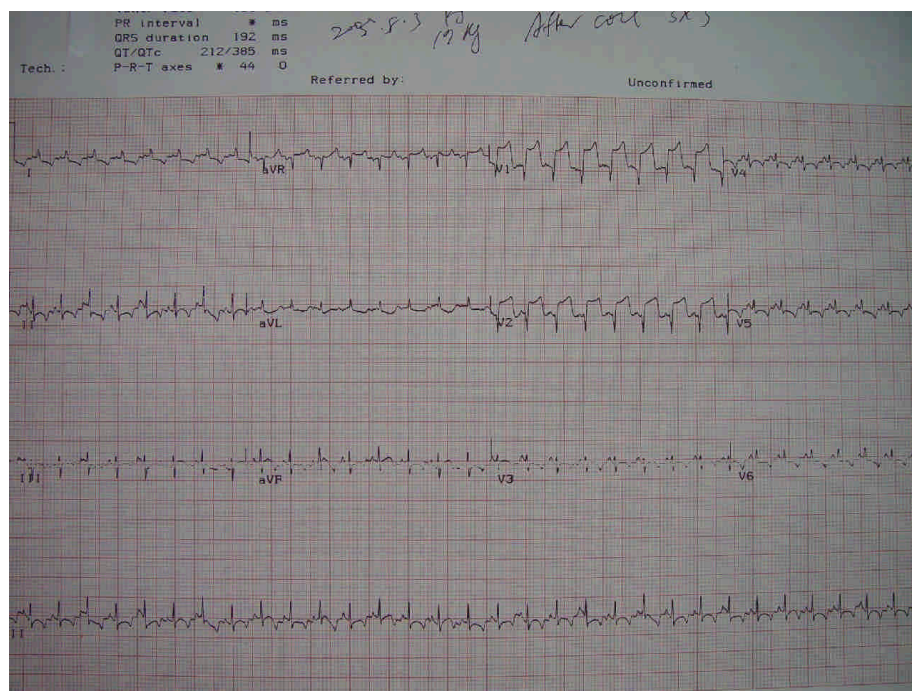


Fig. 2, Gross observation showing myocardial infarction

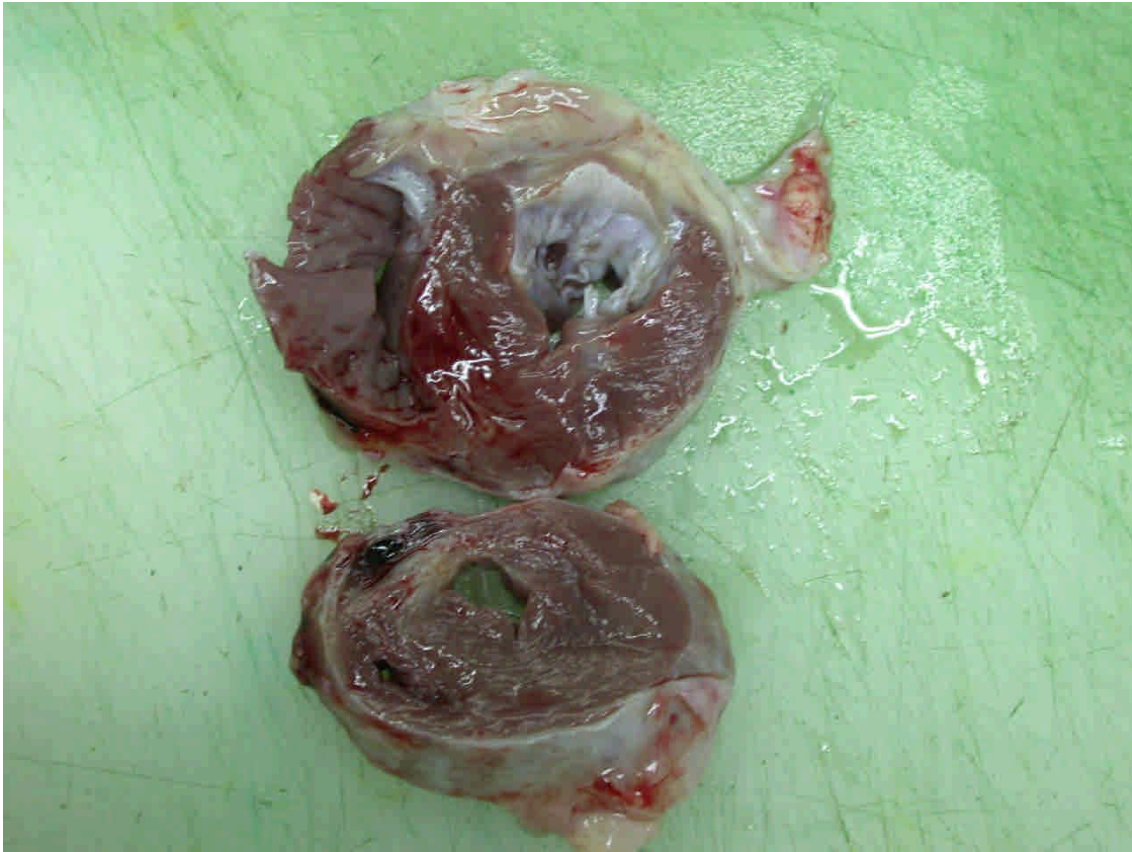


Fig. 3, A pig received NPY intrapericardial injection, 200x

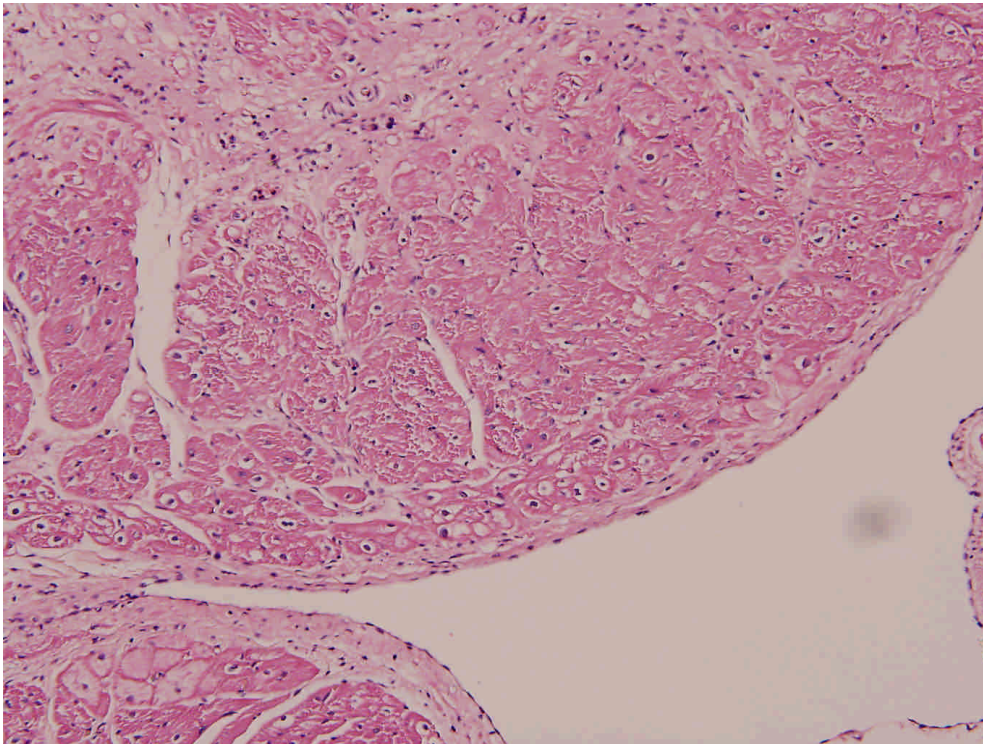


Fig. 4, A pig in control group, 200x

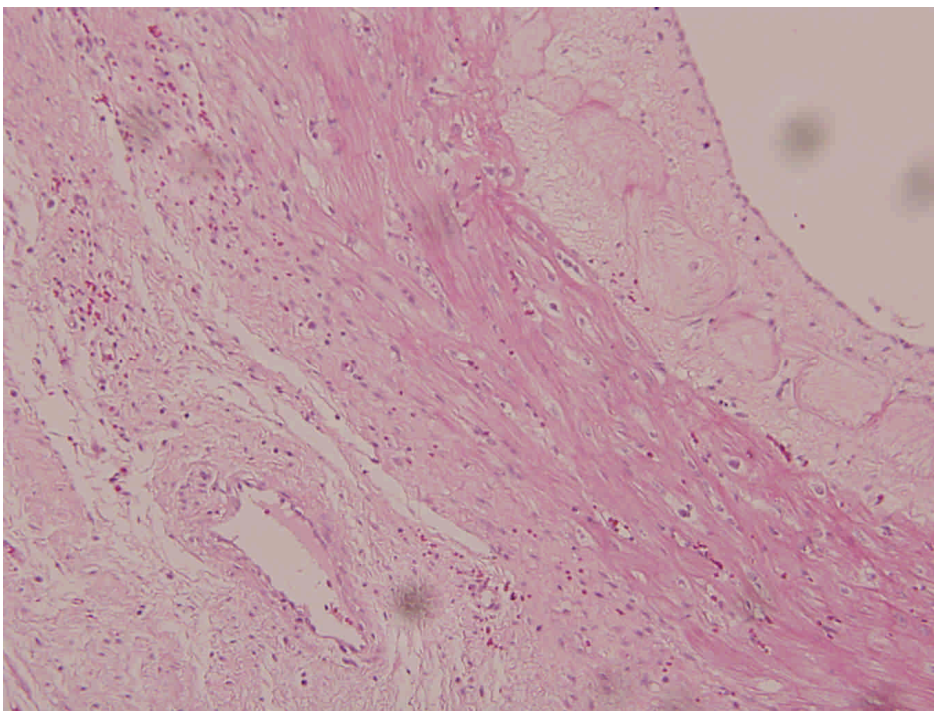


Fig. 5, A pig in adhesion group, 200x

