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허혈성 유리조직 접합술에서 Urokinase의 효용성
— 토끼 이개를 이용한 실험 —

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— Abstract —

The Use of Urokinase in Ischemic Free Tissue Transplantations
— An Experiment Using the Ischemic Replanted Rabbit Ear Model —

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장시간 허혈상태의 토끼 이개를 실험대상으로 하여 유로키나제와 헤파린을 병용 또는 단독으로 사용시와 또한 약물을 사용하지 않았을 때, 이들이 모세혈관의 개존성과 아울러 미세수술후의 조직 생존율에 미칠 수 있는 효과를 보기 위하여 허혈상태의 토끼 이개를 미세수술로 접합한 후 모세혈관으로의 혈류를 측정하기 위하여 레이저 초음파 혈류측정기(Laser doppler flowmetry)를 이용하였으며 방사선 구슬들(Cobalt-57 with plastic material with average diameter 15 micron)을 주입한 결과 유로키나제 조합에서 통계적으로 유효한 성적을 얻었다.

광학현미경 소견은 유로키나제와 헤파린을 병용한 조합에서 모세혈관내 내피세포의 배열이 유지되어 있었으며 헤파린을 사용한 조합에서도 유사한 소견을 보였으나 약물을 사용하지 않은 조합에서는 국소적인 내피세포의 배열이 결핍되어 있었다.

전자현미경 소견에서 유로키나제와 헤파린을 병용한 조합에서 내피세포가 혈관내벽에 배열되어 있었고 또 불규칙한 세포질이 돌출되어 있었다.

Key Words : Urokinase, Ischemic replanted rabbit ear

Introduction

Microvascular free tissue transplantation has improved along with better instruments, techniques, and post-operative monitoring systems. The success of microvascular repairs, however, still depend on variable perioperative factors and the prolonged ischemic time is the one which has decisive

deleterious influence.

Replantation of digit and free tissues are often performed after a relatively prolonged period of ischemia at room temparature from the time of injury. The prolonged ischemia which induces generalized deficient intrafree tissue flow markes reexploration unfeasible even after the vasculature is repaired, some of free tissues fail to have enough reflow, eventually resulting in failure.

To evaluate the effect of local perfusion of heparin, antithrombotic agent and Urokinase, fibrinolytic agent, alone and in combination on arterial blood flow in a prolonged ischemic tissue, the experiment was conducted with replantation of the rabbit ears with microvascular anastomosis and the results were assessed with laser doppler flowmetry and microsphere technique using radionculide-labeled particless and light and elecron microscopy.

Materials and methods

New Zealand white rabbits weighing 4.5-5.5Kg were selected. A simulated critical ischemia model was created in the ear. The rabbits were given intravenous anesthesia with 2.5% pentobarbital(50mg/kg body weight). Both ears were shaved and swabbed with chlorhexidine solution. A portion of left ear at 1.0cm distal to the central vein bifurcation was completely cut of with scalpel in a guillotine fashion. The proximal cut edge of the ear was sutured to stop bleeding using a sterile 3-0 Ethibond suture material and the rabbits allowed freely in a cage after coming out of anesthesia as usual. The amputated ear was placed in a sealed glass container to prevent dessication. It was kept at 4℃ for 8 hours and then brought in to a warm water bath at 20℃ for another 16 hours.

Microvascular anastomosis

At the end of the 16 hours of warm ischemia, the rabbit was put under anesthesia again. The amputated ear was revascularized to the central artery of the intact contralateral ear. End-to-end anastomosis of the central arteries of the amputated and intact ears was done by standard microvascular technique using 10-0 nylon by a single surgeon(JM LEE). Heparinized saline solution(100 units/ml) was only used topically to keep the field clear. The anastomosis was started at the last 20 minutes during the 16-hour warm ischemia period. No venous anastomosis was performed.

Pharmacologic regimen

In group A(five rabbits), before replantation, 2ml of heparinized saline(500U/ ml) were infused prior to revascularisation by hand infusion with an atraumatic 24 gauge catheter slowly over 8 minutes to washout remaining blood inside the ear.

In group B(five rabbits), 1ml of Urokinase (50,000IU/ml) and 1ml of heparin(500U/ml) in combination was infused in the same manner as the forementioned procedure.

In group c(two rabbits), no agent was infused prior to revascularisation.

The choice of heparin alone or Urokinase and heparin in combination was made by sigle blinded, randomized selection. The solutions were prepared and numbered in the syringe by one author(HLS) so that the surgeon(JML) was unware of the drug content. The solutions were mixed immediately before use with the drugs in a quantity sufficient to make 2ml of solution.

Monitoring of blood flow

(1) Laser doppler flowmetry (Periflux PF Id Laser Doppler Flow Meter® Perimid KB, Stockholm, Sweden) was applied at the most distal central artery branching into the marginal capillaries near the tip of the ears with standard settings of gain (×10) and frequency 4 kilohertz and recorded the tracings (1cm/min) after release of the clamps.

(2) Microspheres technique with radionuclide-labeled particles :

After 20 minutes reperfusion, radioactive microspheres (Cobalt^{57} in plastic beads of an average diameter 15 microns) suspended in normal saline with 0.01% Tween^{80} by sonication and vortexing was injected. A total of 100,000 microsheres were injected in each case through the central artery proximal to the anastomosis via a catheter so as not to disturb the anastomosis. The most distally-defined capillary area was assessed by Gamma counter for microcirculatory radioactivity.

Histology

Light microscopy

Specimens selected from around central arterioles were prepared for hematoxylin and eosin staining.

Transmission electron microscopy

Specimens from the same area were prepared for electron microscopy.

Microangiography

In some cases, microangiography was performed with 50:50 Barium sulphate and 10% formaline solution.

Results

Immediately after clamp removal at the anastomosis, laser doppler output signal showed high level initially, reaching a peak, and then settled on an average and slowly decreases. In urokinase/heparin combined group, laser doppler output signal tends to show higher signal than other groups and the duration of constant blood flow of two

Table 1. Laser Doppler Output Signal(%)

	Control mean		Uro+Hep mean		Hep mean		p value
Maximum reading	24		66		62		
	34	29	56		42		
			46		32		
			36		24		
			28	46.4	21	36.2	p=0.34
Minimum reading	14		28		18		
	18	16	23		24		
			15		12		
			22		16		
			12	20	10	16	p=0.32

N.B. p values are computed with the SPSS for PC using unpaired t-test

Key : Uro+Hep : Urokinase and heparin combination group

Hep : Heparin only group

Table 2. Laser Doppler Flowmetry recording of duration of constant blood flow(minutes)

Control man	Uro+Hep mean		Hep mean		p value
10	16		8		
14	11		8		
	20		14		
	9		12		
	20	15.2	1	10.4	p=0.097

N.B. p values are computed with the SPSS for PC using unpaired t-test

Key : Uro+Hep : Urokinase and heparin combination group

Hep : Heparin only group

cases were maintained for twenty minutes (Table 1, 2).

Radioactive microspheres(Cobalt-57 with 100,000 inert plastic microspheres) were assayed for five minutes at the most distally defined capillary area by a Gama counter. The urokinase and heparin combination group and heparin group were compared in the count of radioactive microsheres. The count in the urokinase/heparin combined group was relatively larger than that in heparin group in proportion(Table 3).

Microangiography showed blockage of capillaries.

Table 3. Microsphere perfusion test(count of radioactivity, CPM/5 minutes)

Groups	Uro+Hep	Hep	p value
1	113	111	
2	1234	552	
3	5884	1887	
4	11799	7350	
5	2271	968	0.043

N.B. p value computed by SPSS for PC using nonparametric paired test

Key : Uro+Hep : Urokinase and heparin combination group

Hep : Heparin only group

CMP : Counts per minute

On Light microscopy study, in the Urokinase/heparin combined group, the capillaries had an intact endothelial lining as well as a good filling with barium sulfate particles. The heparin group showed similar microscopical findings to those in the Urokinase group. The control group showed capillaries filled with barium sulfate particles, but, focally, the endothelial lining was defective.

On transmission electron microscopy study, tissue damages caused by prolonged ischemia are demonstrated in all three groups. the damage was more marked in the control group than in the heparin or Urokinase/heparin combined group. The endothelial changes were similar in the heaprin(Fig. 1) and Urokinase/heparin combined groups, although ultrastructural preservation was seemingly better in the latter group. In the heparin and Urokinase/heparin combined groups, the endothelial cells completely lined the luminal wall. They had

Fig. 1. Although fine structural details are lost due to prolonged ischemic period, endothelial cells lining the vascular lumen are clearly demonstrated(arrow).
Heparin group × 8,000

irregular cytoplasmic projections and focally attenuated cytoplasm(Fig. 2). In the control group, the endothelial cells were lost almost completely, exposing the subendothelial connective tissue(Fig. 3).

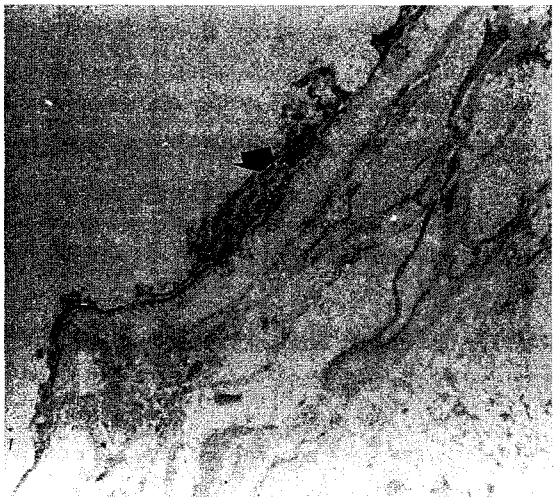

Fig. 2. The endothelial cells still line the luminal wall, but they have irregular cytoplasmic projections and, foxally, attenuated cytoplasm(arrow).
Urokinase and Heparin group × 9,200

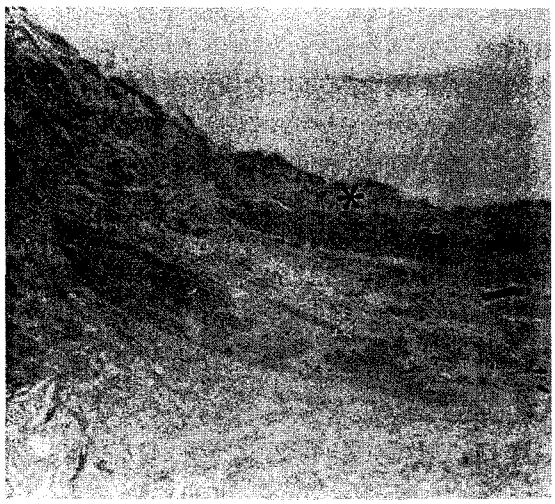

Fig. 3. The endothelial cells are lost almost completely, exposing the subendothelial connective tissue(asterisk).
Control group × 8,400

Discussion

Experimental works in impending failure of circulation, especially in the capillary level, after a prolonged period of ischemia has been extensively studied in animal models.

Ames[1] described no reflow phenomenon in 1968 and advocated combined factors including postischemic hypotension, an increase in blood viscosity, and a reduction in caliber of small vessels in rabbit brains. The impaired circulation was based on perfusion at normal pressure, the viscosity of the blood was directly related to the concentrations of the plasma proteins and of the formed element of the blood, and the obstruction in small vessels occured when metabolism was severely impaired leading to cellular swelling, the later causing localized perivascular edema and increased vascular resistance.

Several other different processes are connected in production of the no-reflow phenomenon. The denervation of the amputated limb may cause excessive arteriovenous shunting leading to poor tissue perfusion[2]. Stagnation, acidosis, and intimal cell damage may be responsible for the sludging of red blood cells and decreased reflow[3]. An impaired fibrinolytic system exists after prolonged ischemia in a rat groin flap[4]. Prolonged ischemia may change the balance of the thrombogenic-fibrinolytic system[5].

Harashina[6] studied perfusion with particular solutions(C-3) and cold heparinized saline solution before a period of cold ischemia in rat hind leg to determine the ultimate survival of transplantation in total five and half ischemic hours. he

observed that the irrigation group was less successful than that of no washout group in 10-day follow-up.

Rosen[7] observed perfusion in preischemia washout epigastric rats skin flap in 24-hour ischemia. He realized that heparinized saline perfused flaps has survived much below than that of nonperfused control groups or perfused groups with a chemicaly defined synthetic medium in 12-hour ischemia.

Chait[8] observed that the perfused rabbit epigastric flaps after 8-hours and 12-hour ischemia with Hartmann, Mannitol, Solumedrol, and heparin mixed solutions showed more vigorous and diffuse arterial pulsation than non-perfused ones after anastomoes. He was also able to demonstrates than skin death was not infulenced favorably by any of the perfusion used and all the solutions were shown to reach all levels of the flap demonstrating the no-reflow phenomenon was no obstruction to peripheral blood flow, but not a total vascular blockade.

We choose the rabbit ear as an experimental model because of its constant an experimental model because of its constant anatomy in having paired dorsal arterial and venous supply of about 0.8-1.2㎜[9]. Its thinness, transparency and superficial vessels can be easily monitored and assessed by laser doppler flowmetry. The criticism is the lack of muscle, therfore the critical ischemic time is difficult to establish and the consequences cannot be directly equated to tissues which are more vulnerable to suffer from ischemia. Recently the emphasis is slowly shifted to study skeletal muscles and occasionally on skin flaps. However, it provides a relatively inexpensive, reporducible, easy visualized and quantitated model of digital revascularization.

Prolonged ischemia or transplantation has been known to change the function of the endothelial cells of the vessel, including fibrinolytic activity, and may be related to vascular thrombosis and late failure of replanted tissues[10]. In this sudy, to simulate the extremeischemia model, amputated rabbit ear which was wrapped in a moistened normal saline gauze was kept in glass container at 4 degrees Celsius for 8 hours by the similar method for replantatin[11] and was brought to be kept again in a warm water bath at 20 degrees Celsius for 16 hours.

In the laboratory studies, pharmacologic regimen, ranging from multiple agents to no agent, have been used to improve postoperative microvascular patency as well as survival of a microsurgical procedure, and success rate of impending prolonged ischemic transplantation. We experimentally set up the impending prolonged ischemic transplantation. We experimentally set up the prolonged ischemic flaps infused with heparin (500U/ml) alone and urokinase (50,000U/ml) with heparin in combination to be allowed 20 minutes prior to declamp of the anastomosis.

Heparin has an ability to inhibit fibrin strand formation[12]. Heparinized Ringer solution (40U/ml) with a perfusion pressure of 100cm H_2O infused prior to replantation in rabbit ear improved the immediate postoperative skin caillary circulation[13]. Heparin has an action preventing pedicle and microvascular clotting in prolonged low flow or stasis[14] and has been used in salvaging a failing flap transfer suffering from venous occlusion and in local flap perfusion to allow reestablishment as well as maintenance of good circulation[15]. When heparin alone was unsuccessful in improving flap perfusion, its continued administration following tissue-

type plasminogen activator-induced clot lysis has a sound rationale. Heparin prevents new fibrin formation and also prevents propagation and recurrence of thrombi once they are lysed, thus heparin is a natural adjuvant to thrombolytic therapy. Following clot lysis with tissue-type plasminogen activator, heparin would sustain vascular patency in the interim, allowing internal repair and biologic erasure of the thrombogenic focus[16]. The role of concurrent heaprinization during selective urokinase infusions will effectively prevent new thrombus formation without increased risk of a bleeding complication.

The fibrinolytic activation of Urokinase which is produced by renal parenchymal cells and is being extracted in human urine lyses existing clots and prevents fibrin-platelet deposition through its plasminogen-activating properties and enhances the recovery of the impaired fibrinolysis of tissue[17]. Urokinase has been shown to bind to specific cell receptors of a variety of cell types, including endothelial cells, platelets, and monocytes, and is able to bind to the intact, uninjured vessels. Thus urokinase may bind to both healthy damaged intima, and from these sites it may provide an additive effect toward the prevention of vascular occulusion[18].

In our study we found that after 16 hours of ischemia, perfusion by heparin lactate alone or in combination of Urokinase greatly improve the perfusion. This could be simply a mechanical "Washing out" effect. However the microsphere circulation assessment and laser doppler flowmetry both indicated a better perfusion in the Urokinase group. Under light and electron microscopy less endothelial damage is seen in the Urokinase group. This agrees with the findings by others that Urokinase adheres to endothelial cells and their fibrinolytic ability[17].

It appears that perfusion with Urokinase offers extraprotection and benefits to ischemic transplantation or replantation than heparin in alone and this funding can be of clinical importance although further study is needed.

Our emphasis has been on the early microvascular changes on reperfusion after prolonged ischemia, and our results compliment some previous studies on long term survival of the replanted rabbit ear after warm ischemia[11].

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