行政院國家科學委員會專題研究計劃成果報告

中文計畫名稱：胎牛血清對小腸大量切除後，殘存小腸成長之影響

英文計畫名稱：Influence of Fetal Bovine Serum on Intestinal Remnant after Massive Intestinal Resection

計畫編號：NSC89-2314-B-002-210

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一、中文摘要

FBS(fetal bovine serum),因有成熟牛血清沒有的各種 growth factor,是做細胞培養時不可欠缺的 medium,本計劃之主要目的是研究 FBS 對 Lewis rat 做小腸大量切除後(80%切除後)小腸再生之影響。

採用之研究方法與原因：

使用公 Lewis rat (250g)

Test Solution; Fetal Bovine Serum

實驗方法：

實驗動物(n=20)公 Lewis rat(150~250g),於實驗之前 24 小時開始禁食，只給飲水，藉 Pentobarbital 及 ether 行全身麻醉，開腹，皮膚切開(1~2cm 長度)後，在腹壁左側皮下，創設皮下隧道，裝 Alzet osmotic minipump,同時在胃體部，造胃廔，胃廔和 Alzet osmotic minipump 用 PE-60polyethylene tubing (Clay Adams, Parsippany, NJ)連結，於實驗之前將 Alzet osmotic minipump 裏裝入 test solution(FBS)，minipump 裝在腹部皮下隧道後，閉鎖腹壁，讓 rat 醒來後用普通食料餵食 3 天，3 天後在同樣的麻醉下開腹。開腹後唯留下空腸口端 5 公分，迴腸末端 5 公分，切除全部小腸的 80% 後，空腸和迴腸做端端，一層吻合，使用 atraumatic needle, 7-0silk,吻合後經靜脈注入生理食鹽水 10cc,關閉腹壁，讓 rat 清醒，以後用普通食料養，三天後，六天後 kill rat,檢查吻合後小腸之變化。

從離開吻合部 1 公分的空腸，迴腸取得 2 公分的空腸及回腸，從切開腸管使用冷食鹽水(4°c)洗，使用吸取紙吸乾，用玻璃片取得 intestinal mucosa, DNA 是使用 Holchst 33258 staining, protein 是使用 Bradhord 之方法測量。

Alzet osmotic minipump(model 2002, Alza Corp Palo Alto, CA)之構造如下：

有三個 concentric layers (1) drug reservoir---放試驗藥品(FBS)

(2) osmotic sleeve---裏面有濃厚食鹽水

(3) rats-controlling membrane

作用機轉：

Alzet osmotic minipump 裝入皮下組織後，因 pump 裏面(最外側)有濃厚食鹽水，和組織內之滲透壓有差異，因 osmotic gradient 發生水份由動物組織滲入 osmotic sleeve 裏面，壓迫 flexible reservoir, 從 flow moderator, continuous 排出 test

solution(FBS)

Alzet osmotic minipump 排出量：
看 pump 的大小，其排出量有差異，一般來說一小時能排出 0.20~0.10ul 能使用 1 天到 6 週。在本實驗使用的是 model 2002, size 200ul, 0.5ul\hr.

關鍵詞：fetal bovine serum, 小腸大量切除,小腸再生

二、英文摘要

Keyword: Fetal Bovine Serum, Massive Resection of Small Intestine, Intestinal Growth

Fetal bovine serum (FBS) is known to have several growth factors and has been used as an important medium for cell culture. The objective of this study is to evaluate the effect of FBS to enhance the growth of remained intestine following massive intestinal resection (resection of about 80% of small intestine).
Young adult Lewis rats undergo subcutaneous implantation of Alzet osmotic minipump.
The perfusion methods of minipumps.Subcutaneous implanted minipumps will be connected to the gastrostomy tube for luminal perfusion. At 3th day following subcutaneous implantation of minipump, all rats undergo approximate 80% small bowel resection. At three-day or six-day following massive small bowel resection, biopsy specimens of small bowel mucosa will be obtained.
Growth indices will be evaluated by measuring protein and DNA concentration of mucosa.

三、計畫緣由與目的

從過去在國外所發表的論文及本人等五年前所發表的論文得知，用 newborn 或 fetal Lewis rat 小腸種在同種成鼠腹壁上，intestinal graft 會存活且成長，從 1995 年以後本人等，對其沒有血管吻合，使植入腹壁上的腸片會存活的原因一直探討，結果得到以下的假設：在母鼠懷孕後，母鼠的生理機構會有變化而產生某種物質，這種物質是存在母鼠的血液內，newborn 或 fetal rat 的血清內，或胎盤內，使 newborn 或 fetal rat intestine 種在同種成鼠的腹壁上，雖然沒有血液的供給，也能存活 3-4 日，當新生血管產生後，便能很快的恢復其機能，從 1997 年以後，我們陸續利用 fetal bovine serum 利用 luminal perfusion,來觀察 FBS 對 intestine ischemia 之影響，現在還在實驗中。
下年度之實驗目標爲 FBS 對大量小腸切除後，小腸再生之影響，一般而言，動物大量小腸切除後，小腸都會過速度之生長，我們 expect 使用 FBS 利用 Alzet

osmotic minipump 做小腸之 continuous luminal perfusion,小腸大量切除後能 enhance 其生長速度。

我們使用 FBS 之原因有三個：

(1) 從過去在國外發表之文獻及筆者等過去之實驗得知 newborn 或 fetal rat 之血清內或胎盤內有某物質，此物質可能會保護小腸在無血液供給之下能生存，且小腸無血液供給之狀態恢復後能很快的恢復其機能。

(2) FBS 是細胞培養液不可欠缺之 medium。

(3) 在過去我們的細胞培養之結果，發現 placenta extract 對細胞培養不是好的 medium,所以不使用 placenta extract。

本人等目前還未查到 FBS 對小腸大量切除，小腸再生機能之研究。

四、Final Results

P4

No	Pump	Jejunum mg/g	Ilium mg/g
619A	0	10.05	.
619B	0	4.20	18.65
619D	0	.	15.02
619E	0	15.24	4.84
627A	0	16.28	18.55
627B	0	.	12.15
628C	0	.	4.20
628D	0	14.42	16.38
628E	0	9.98	13.24
1023A	0	15.37	16.32
1023B	0	5.73	15.89
1023C	0	7.74	19.34
1026A	0	13.78	11.17
1027B	0	23.47	17.45
716A	1	14.64	7.34
716B	1	5.24	.
718E	1	20.40	2.90
1016A	1	14.31	16.06
1016B	1	14.54	20.45
1016C	1	20.80	35.14
1016D	1	13.60	21.28
1016E	1	19.17	12.10
1016F	1	16.88	12.70
1019A	1	12.95	5.76
1019B	1	3.24	20.80
1019C	1	10.50	5.46
1019D	1	15.63	5.92
1019E	1	15.13	16.35
1019F	1	12.69	13.03

	DNA	Mean	SD	p-value
no pump	Jejunum	12.39	5.53	0.4444
with pump	Jejunum	13.98	4.89	
no pump	Ilium	14.09	4.92	0.9588
with pump	Ilium	13.95	8.67	

Protein

有無pump	日 期	編 號	(ug/mg) 值
x	6月19日	C10AJ	58.8320
x	6月19日	C10BJ	33.2096
x	6月19日	C10EJ	64.2372
x	6月27日	C10AJ	130.9484
x	6月28日	C10DJ	58.0842
x	6月28日	C10EJ	49.9476
x	10月23日	C10BJ	212.1962
x	10月23日	C10CJ	169.6693
x	10月26日	C10AJ	97.6673
x	10月27日	C10BJ	188.6156
x	6月19日	C10BI	27.5807
x	6月19日	C10DI	71.8084
x	6月19日	C10EI	157.2598
x	6月27日	C10AI	127.8577
x	6月27日	C10BI	139.6303
x	6月28日	C10CI	106.6346
x	6月28日	C10DI	52.8831
x	6月28日	C10EI	53.0421
x	10月23日	C10BI	222.2789
x	10月23日	C10CI	164.8904
x	10月26日	C10AI	77.8279
x	10月27日	C10BI	201.2760
△	10月23日	C7AJ	141.2016
△		C7AI	189.6225

有無pump	日 期	編 號	(ug/mg) 值
v	7月16日	C10AJ	158.6984
v	7月16日	C10BJ	141.2090
v	7月18日	C10EJ	179.5574
v	10月16日	C10AJ	148.0988
v	10月16日	C10BJ	177.0776
v	10月16日	C10CJ	212.5128
v	10月16日	C10DJ	162.0313
v	10月16日	C10EJ	123.5714
v	10月16日	C10FJ	208.8087
v	10月19日	C10AJ	173.2264
v	10月19日	C10BJ	152.0660
v	10月19日	C10CJ	181.2228
v	10月19日	C10DJ	158.9778
v	10月19日	C10EJ	184.8289
v	10月19日	C10FJ	121.0511
v	7月16日	C10AI	40.0313
v	7月16日	C10BI	159.7140
v	7月18日	C10EI	137.2664
v	10月16日	C10AI	142.6539
v	10月16日	C10BI	159.5525
v	10月16日	C10CI	195.4421
v	10月16日	C10DI	122.6377
v	10月16日	C10EI	121.4969
v	10月16日	C10FI	119.8156
v	10月19日	C10AI	143.0982
v	10月19日	C10BI	153.5759
v	10月19日	C10CI	180.1786
v	10月19日	C10DI	117.0852
v	10月19日	C10EI	163.6563
v	10月19日	C10FI	178.0871

Protein

沒有pum	(ug/mg) 平均值	有pump	(ug/mg) 平均值
C10 J	106.3407 (n=10)	C10 J	165.5292 (n=15)
C10 I	116.9142 (n=12)	C10 I	142.2861 (n=15)
總 和	223.2549		307.8153

五、計畫成果自評

本人 1994 年台大退休以後，也繼續收受行政院國家科學委員會專題研究經費之補助。但本計畫開始不久，國科會決定 89 年 8 月以後對退休人員停止經費之補助，助理對不安定的工作條件而影響其工作態度。本人多年在接受國科會經費補助期間，最難處理之問題是助理在進行之際的轉換，助理本身背景不同，影響到結果的正確性。

本計畫相關的研究在國際上尚無發現有研究者在進行，在 87~88 的專題研究我們也觀察到 FBS 對小腸黏膜之 ischemia-reperfusion injury 有保護之作用，在本研究也觀察到 FBS 對小腸大量切除後，對殘存小腸之成長有良好的效用。我們將在繼續確定實驗結果之正確性後，提出論文發表。87~88 年度之研究計劃中 DNA 因機器的老舊暫時擱置的部分，今年度繼續進行實驗，89 年 11 月在美國 Michigan 大學舉行的 13 期 international symposium on pediatric surgical research 會中報告，附在附件，請做參考。

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Protection of Small Bowel From Ischemia and Reperfusion Injury Using Luminal Fetal Bovine Serum Perfusion

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Running title: Fetal Bovine Serum, Ischemia-Reperfusion Injury

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Abstract:

The fetal or newborn organ (intestine, kidney, skin or lung) transplanted into a syngenic adult host without vascular anastomosis is able to survive [1-7]. We hypothesis that fetal tissue possesses an ability to prevent ischemic injury, the factors elicited during pregnancy. We created an experimental model to induce total vascular occlusion in a 15-cm long ileal segment. Using an osmotic minipump transplanted at the subcutaneous space of the abdominal wall, the FBS luminal perfusion was conducted. Three days before the 3-hour total vascular occlusion, the luminal perfusion using FBS was started. On day 0, day 3 and day 6 after release of vascular occlusion, the rats were sacrificed and tissue samples were obtained for morphological study and intestinal DNA and protein content measurement.

On day 0, the mean grades of morphological figures were both 5 in the control and experimental groups. The mean grades changed to 3.1 and 2.1 on day 3,and 2.6 and 1.5 on day 6 in the control and experimental groups, respectively.

DNA and protein content in each group were listed below:

		Control Group (μg/mg)	Experimental Group (μg/mg)
DNA	Day-0	2.26 ± 0.52	2.79 ± 0.78
	Day-3	3.46 ± 0.68	4.54 ± 0.76
	Day-6	3.50 ± 0.72	4.49 ± 0.49
Protein	Day-0	31.52 ± 2.96	57.93 ± 5.48
	Day-3	54.85 ± 4.16	74.69 ± 9.50
	Day-6	56.12 ± 3.93	71.72 ± 7.02

From the data described above, luminal perfusion using FBS could protect small bowel from ischemia and reperfusion injury and increase intestinal mass.

Key Words:

Fetal Bovine Serum, Ischemia-Reperfusion Injury,

Introduction:

The fetal or newborn rat intestine transplanted into the abdominal wall of a syngenic adult host without vascular anastomosis will vascularize, grow and maintain histological integrity [1-4,6]. In 1971, Zinzar indicated that the fetal transplant, although initially devoid of blood supply in the host, would become vasculized in 3-4 days, and maintain as differentiated intestinal tissue [1,8]. On the other hand, the turnover of intestinal enterocytes is rapid, and after 5 hours of complete interruption of blood supply the intestinal tissue becomes necrotic [9]. There is a

considerable time interval between the tissue necrosis and new vascularization. The current experiments do not provide information regarding the mechanism and the point of time of revascularization in transplanted fetal organs. For the mechanism, we have developed a hypothesis that fetuses possesses factors regulating growth and differentiation of enterocytes to prevent ischemic damage until new vascularization, which will be assumed by day 3 to day 4 after transplantation. These factors are related to components elicited during pregnancy. In fetal blood, pregnant maternal blood and placenta contain these elements.

Fetal bovine serum (FBS) is known for having several growth factors and has been used as an important additive for cell culture media. This study was designed to determine if FBS given luminally could protect small bowel from ischemia and reperfusion injury and increase intestinal mass.

Materials and Methods:

Male Lewis rats weighing 200-250 grams were used in this study.

Control group (n=15) : After an overnight fast, general anesthesia was induced with intramuscular injection of 25 mg/kg pentobarbital sodium, and anesthesia was maintained by ether inhalation. Following preparation of the abdominal wall with 10 % poridone-iodine solution, a laparotomy was done through a median incision. The distal ileum was displaced forwards and a 15 cm long loop of terminal ileum was subjected to 3-hour total ishemia by ligation of corresponding mesenteric arteries, veins, terminal ileum at both ends and the marginal vessels using no.3 silk sutures (Fig. 1). Loss of mesenteric pulsation and color change of bowels confirmed vascular occlusion. Then, the forwards displaced ileum was returned to the abdominal cavity and the abdominal wall was temporarily closed. After 3 hours of vascular occlusion the abdomen was reopened, and silk sutures were removed to release the occlusion. Reperfusion was confirmed by return of pulsation. After this procedure, the abdomen was closed. Then, the animals were allowed to recover and fed the usual rats diet.

At 0 (n=5) , 3 (n=5) and 6 (n=5) days after reperfusion, the rats were killed and 5 cm tissue samples were obtained from the middle of the ischemically damaged ileal loop. The excised intestines were used for morphological study and measurement of DNA and protein content.

Experimental group (n=15): After induction of general anesthesia using the same protocol and drugs for the control group, a midmedian abdominal incision was performed. An adequate subcutaneous space of the abdominal wall was created for placing an osmotic minipump (model 2002, Alza Corp. Palo Alto, CA.). This pump was connected to the stomach through a fine polyethylene catheter (PE60)(Clay Adams, NJ). The pump was designed to deliver FBS (Fetal bovine serum, tested for mycoplasma, Biochrom KG Berlin) at 0.5 ul per hour for 14 days. After this procedure, the abdominal wall was closed. Three days after initiation of luminal perfusion, total ischemia of an approximately 15-cm long ileal segment was induced by occlusion of inflow and outflow blood supply for 3 hours as the control group. Rats were fed

after awakening from anesthesia. Tissue samples were obtained at day 0 (n=5) , 3 (n=5) , and 6 (n=5) after the procedure.

The tissue samples obtained from the control and experimental groups were used for morphological study and measurement of intestinal protein and DNA contents.

Morphological study: The excised intestine was deprived of mesenteries, opened longitudinally, washed with ice- cold saline and fixed in formalin. Histological sections were prepared from paraffin blocks and stained with hematoxylin and eosin by standard techniques. Morphological changes of mucosa were evaluated by Chiu's Montreal University Grading System [10].

DNA and protein content: The excised intestine was immediately immersed in liquid nitrogen. Intestinal DNA content was determined using Hoechst 33258 staining [11] and the protein content was determined using Bradford method [12].

Results:

The rats tolerated the procedure well with no mortality.

Morphological study (Fig. 2): The morphological changes in the mucosa were graded into six grades. On day 0, just after 3 hours of total occlusion of blood supply, the median grades of mucosa damage were both 5, (the villi were completely denuded of epithelium and the villous stroma was necrotic, haemorrhagic and ulcerative) in the control and experimental groups. Three days after restoration of blood flow the median grade of morphological change recovered to 3.1 and 2.1 in the control and experimental groups. On day 6 after restoration of blood flow the median grade of morphological change recovered to 2.6 and 1.5 in the control and experimental groups, respectively. The recovery of mucosa damage from ischemia and reperfusion injury in both control and experimental groups was rapid, but the recovery of morphological damage was faster in the experimental group than that in the control group.

DNA and protein content: DNA and protein content in each group were listed in Figs. 3 and 4. On day 0, DNA and protein content of the ischemically damaged ileal segments of the control group were significantly lower than that of the experimental group. Then the level of DNA and protein contents in both groups elevated rapidly by 3 days, but the level of DNA and protein contents were much higher in the experimental group than those in the control group.

Discussion:

The present study was designed to evaluate the effect of luminal perfusion with FBS on ischemia and reperfusion injury. In the control group without luminal perfusion of FBS, we observed considerable damage in the morphology (grading 5 by Chiu's grade) and decreased levels of DNA and protein content after 3 hours of blood supply occlusion. This change was induced by hypoxia, which would result in loss of villi and crypts and cell death. In general, if the cell damage by this kind of hypoxia is mild, the bowel mucosa will recover and the recovery

is rapid. But if the cell damage is extensive, it could cause intestinal necrosis (primary necrosis) [13]. In the experimental group, the mucosa morphological damage was grade 5 at day 0, similar to the morphological change of the control group at day 0. However, the levels of DNA and protein content were much higher in the experimental group. This might be due to the effects of the 3-day luminal perfusion of FBS prior to hypoxia and the continuous perfusion during 3-hour total occlusion of the blood supply. At day 3 the mucosal morphological grade was 3.1 in the control and 2.1 in the experimental group. DNA content was 3.46 μg and 4.54 μg, protein content was 54.85 μg and 74.69 μg per mg of intestinal tissues in the control and the experimental groups, respectively. At day 6, the morphological grade was 2.6 in the control and 1.5 in the experimental group. DNA content was 3.50 μg and 4.49 μg, protein content was 56.12 μg and 71.72 μg per mg of intestinal tissues in the control and the experimental groups, respectively. The recovery of morphological changes and elevation of DNA and protein content were rapid in the control and experimental groups, but the recovery from ischemia and reperfusion injury in experiment group was faster than in the control group. This response may be the result of homeostasis between proliferation activity of enterocytes and prevention of apoptosis. Adaptation following ischemia reperfusion injury is an important compensatory response of enterocytes. Apoptosis could occur immediately after ischemia reperfusion [13] and could be initiated by a variety of environmental stimulation and is probably responsible for crypt and villous loss and cell necrosis (secondary necrosis) [13].

Future studies will be necessary to elucidate the mechanism for beneficial effects of luminal perfusion of FBS following ischemia and reperfusion injury.

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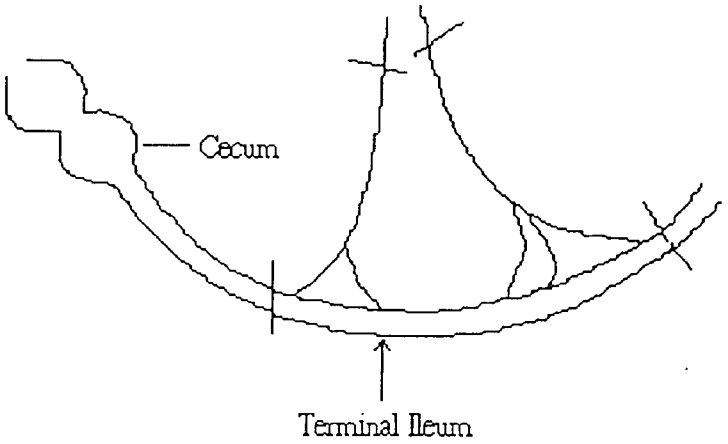

Fig.1 An experimental model to induce total vascular occlusion in a 15-cm long ileal segment. A 15 cm long loop of terminal ileum was subjected to 3-hour total ishemia by ligation of corresponding mesenteric arteries, veins, terminal ileum at both ends and the marginal vessels using no.3 silk sutures

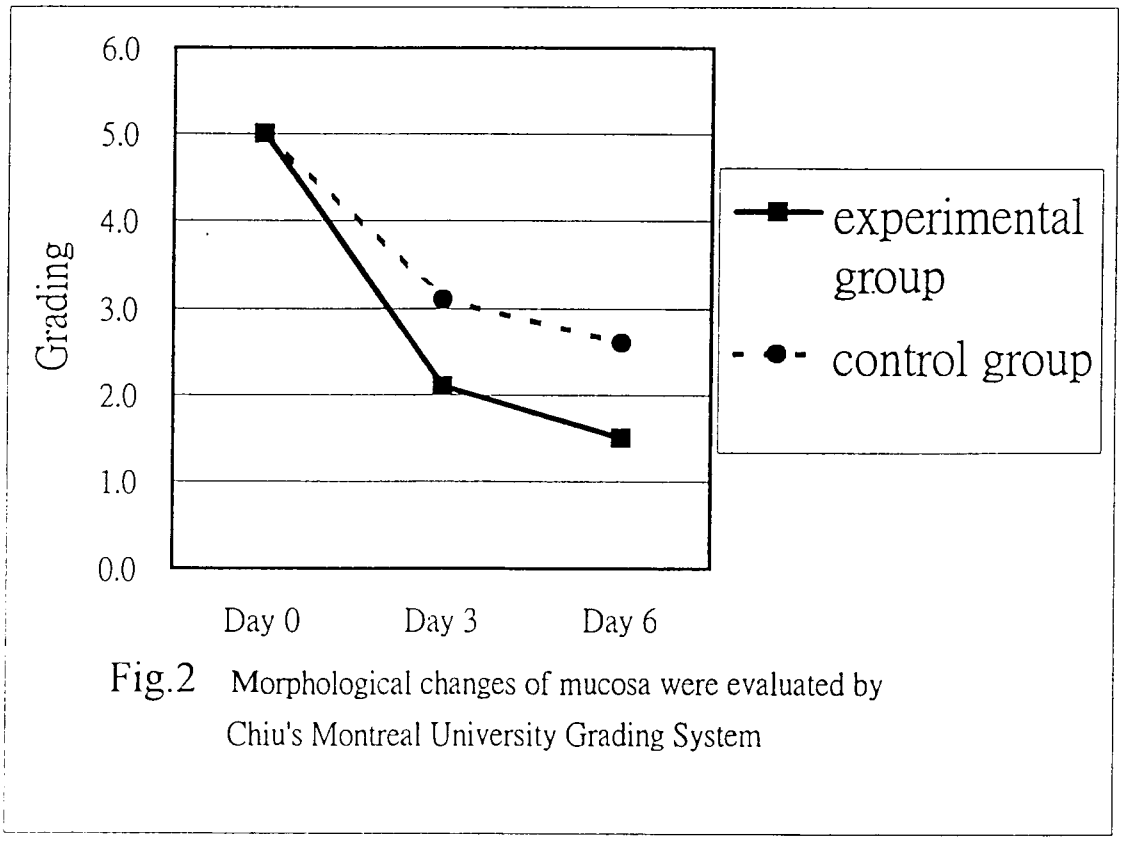

Fig.2 Morphological changes of mucosa were evaluated by Chiu's Montreal University Grading System

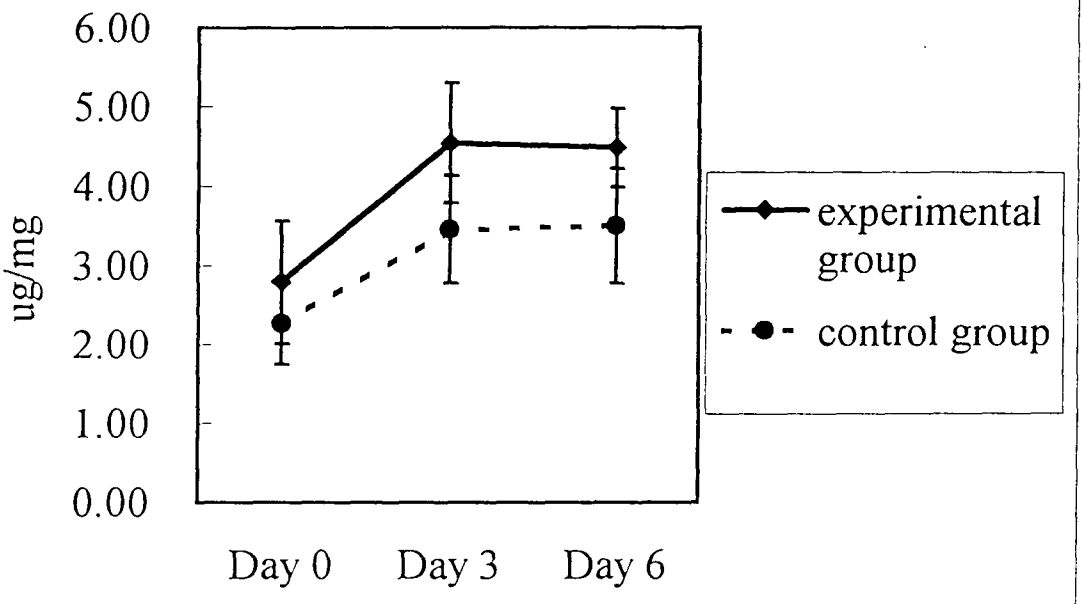

Fig.3 Mean Intestinal DNA content for experimental group and control group

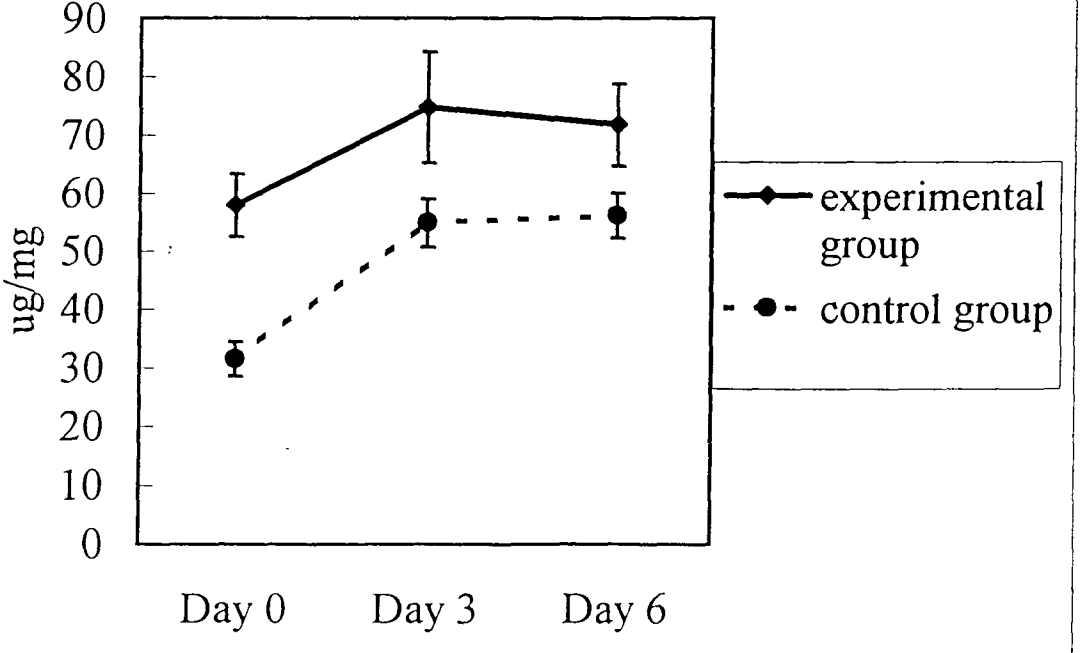

Fig.4 Mean Intestinal protein content for experimental group and control group