韓國家畜繁殖學會誌 18(4) : 265~274(1994)
Korean J. Animal Reprod.

Rabbit Platelet Activating Factor and Its Relationship with Embryo Development

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토끼 혈소판촉진인자와 배발달과의 관계

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요 약

가토 20마리의 혈액중 혈소판의 수를 수정 전후로 조사한 결과, 수정전과 비교하여 수정후 1일부터 유의하게 감소하였으며 (26.84%, $P<0.01$), 이러한 감소는 수정후 5일까지 계속되었다. 수정후 5일동안 혈소판의 감소율과 혈소판의 최저치를 수정란의 수와 비교한 바, 이들간의 유의한 상관관계($r=0.5032$)가 있었다. 가토수정란 (2세포기) 각각 30~40개를 5마리의 비장적출수란가토의 난관에 이식한 다음, 혈소판수의 변화를 조사한 결과, 이식전에 비해 이식후 90분 ($P<0.025$)과 135~270분 ($P<0.025$)에 유의한 감소를 확인하였다. 특히 수정란 이식후 180분경에 가장 크게 감소하였다 ($P<0.001$). 60~70개의 2세포기 가토수정란을 24시간 배양한 다음, 배양액을 3마리의 비장적출가토에 주사한 후 혈소판의 변화를 조사하였을 때, 주사후 120경부터 혈소판의 수가 유의하게 감소하기 시작하였다. 이들 배양액을 동결, 해동한 다음 주사한 실험에서도 유사한 결과를 얻었다. 또한 이 배양액을 비장적출생쥐에 주사한 경우에도 가토에서와 마찬가지의 혈소판 촉진현상이 나타났다. 혈소판 촉진인자(PAF)의 길항제인 kadsurenone이 첨가된 배양액에서 24시간동안 가토수정란을 배양한 다음, 이 배양액을 4마리의 비장적출생쥐에 주사한 결과, 혈소판수의 변화가 일어나지 않았다. 또한 kadsurenone이 첨가된 배양액에서는 2세포기 가토수정란의 상실기와 배반포기까지 발달율은 각각 4와 7%로 대조구의 7과 49%보다 유의하게 낮았다. 따라서 본 연구에서 가토의 초기배는 가토나 생쥐의 혈소판수를 감소시키고, 특히 길항제 처리는 이러한 혈소판 감소현상을 억제시킬 뿐만 아니라 가토 초기배발달을 억제한다는 것을 확인하였다. 결론적으로 가토초기배에서는 PAF 또는 수정란 유래의 PAF가 분비된다는 것을 알 수 있었으며, 이러한 인자는 동결처리에서도 그 기능은 전혀 변하지 않는다고 본다.

I. INTRODUCTION

It has been demonstrated that the drastic decrease in platelet number in peripheral blood during the early stage of pregnancy in mice was considered as an earliest body reaction of the pregnant female (O'Neill, 1985). This reaction is depending on the presence of fertilized egg and the degree of decrease of platelet number is also directly related to the number of embryo present in the genital tract, there was a negative relationship between them (O'Brien, 1976). Studies in human, sheep, cattle, etc. revealed that platelet activating factor (PAF) was released from the early stage embryo (O'Neill, 1992). Injection of 8-16-cell embryo-cultured medium into the abdominal cavity of splene-

ctomized female mice, caused as drastic decrease in platelet number in 5 min after injection, the decrease continued to 120 min (O'Neill, 1985). It has been discovered that the amount of PAF secretion was the highest at morula stage (Angle, 1988). and continuous culture of mouse blastocysts also secreted PAF. It has been discovered that embryo-derived platelet activating factor (EDPAF) was also found in the embryo-cultured medium (O'Neill, 1987). It has been considered that EDPAF plays an important role in recognition of pregnancy. The aim of present studies is to find out the evidence; (1) whether there is also a drastic decrease in platelet number at the early stage of pregnancy in rabbit, (2) is there any relationship between platelet number and number of embryo, (3) will the rabbit embryos transferred into splenectomized rabbit genital tracts cause a decrease of platelet number, (4) will the injection of rabbit embryo-cultured medium to the splenectomized mice also cause a decrease of platelet number, (5) will PAF produce any effects to the early development of rabbit embryo, so as to convince whether PAF is released from the rabbit embryo.

Ⅱ. MATERIALS AND METHODS

1. Experimental animals

Rabbits(Giant Blanc) – healthy, non-pregnant females, above 6 months old.

Mice (Kunming Strain) – healthy, non-pregnant females, 2 months old.

2. Platelet number counting

10 μl blood was collected from the ear vein of rabbits or by cutting tails of mice, diluted with 2.5% NaCl solution, platelet number counted by using haemocytometer under microscope (× 160)

3. Mating

Ovulation was induced by injecting 10 μg LLH into the ear vein of female rabbit, immediately followed by artificial insemination (AI)

4. Embryo transfer

Six subcutaneous injections of FSH (0.05mg each) were given to donor rabbits at the neck, 12 h between each injection, 12 h after the last injection of FSH, one injection of LH (10 IU) was given into the ear vein, and AI done at the same time.

The recipient rabbits were also received one injection of LH (10 IU), at the same time as did in the donor rabbits, for the purpose of sychronization.

Twenty-four h after the injection of LH, embryos were flushed from the oviducts of donors by using Medium 199. Embryos were then transferred into the oviducts of recipients.

5. Embryo culture *in vitro*

Embryos were cultured in Medium 199 containing 15% Albumin Bovine V. For the experimental group, embryos were cultured in 1 ml medium in each pit of the plate. For the Antagonist experimental group, 20 μg kadsurenone was dissolved in 0.3 μl DMSO, then added into the culture medium as mentioned above, and the embryos were introduced at last.

6. Data processing

Compare the data of platelet number before and after experimental treatments, calculate the dropping percentages and examine the significance of difference by applying statistical methods.

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Ⅲ. RESULTS

1. Experiment 1

The platelet number of 20 rabbits were decreased in 5 days after mating (AI) (day 1~5) as compared with that before mating (day 0), as follows ; 69.3($\times 10^4/mm^3$, day 0), 50.7, 59.0, 3 and 68.6($\times 10^4/mm^3$, day 1-5), showing that more decrease in day 1 ($50.7 \times 10^4/mm^3$), or with a decrease of 26.84%($p<0.001$), as compared with that before mating(day 0, $69.3 \times 10^4/mm^3$).

Taking the lowest decrease value of each of the 20 rabbits in 5 day after mating compared with that before mating, the dropping percentages ranged form 18.97% to 61.40% ($p<0.001$), as shown in Table 1.

During the interval from mating to implantation, there was a significant decrease in platelet number, a significant positive correlation ($r=0.5032$) was found between dropping percentages of platelet number and number of embryos in uterus as determined by laparotomy at the 15th day after mating.

2. Experiement 2

Thirty 2-cell embryos were transferred into the oviducts of three splenectomized rabbits (No. 1, 2, 3), and forty 2-cell embryos were transferred into the oviducts of two splenectomized rabbits (No. 4, 5), Platelet number were determined in every 45 min interval before (0) and after (45~270 min) embryo transfer, as shown

Table 1. Platelet number before and after mating (AI) and number of embryos

Rabbit No.	Platelet number at day 0 ($\times 10^4/mm^3$)	Lowest platelet number after AI ($\times 10^4/mm^3$)	Dropping percentage (%)	Number of embryo at 15 th day in uterus
1	70	30	57.14	15
2	74	39	47.30	11
3	106	55	48.11	9
4	37	26	29.73	12
5	49	38	22.45	7
6	57	45	21.05	9
7	57	22	61.40	13
8	61	31	49.18	11
9	80	52	35.00	11
10	98	63	35.71	4
11	55	38	30.91	13
12	64	46	28.13	13
13	53	33	37.74	15
14	65	48	26.15	12
15	58	47	18.97	8
16	92	44	52.17	12
17	74	43	41.89	12
18	72	57	20.83	2
19	96	56	41.67	11
20	68	49	27.94	7

Table 2. Platelet number ($\times 10^4/mm^3$) before (0) and after transferring embryos into oviduct

Rabbit No.	Determining time(min)							Number of embryos transferred
	0	45	90	135	180	225	270	
1	60	27	56	49	19	26	42	30
2	54	23	41	22	15	24	35	30
3	80	20	22	30	17	23	27	30
4	53	41	44	26	33	35	33	40
5	61	61	42	33	39	46	46	40
Mean	61.6	34.4	41.0	32.0	24.6	30.8	36.6	
Dropping percentage(%)		44.16	33.44	48.05	60.07	50.00	40.58	
P		<0.025	<0.025	<0.005	<0.001	<0.005	<0.005	

in Table 2.

It is demonstrated that the platelet number of these five rabbits were constantly decreased in 270 min after embryo transfer, The means of dropping percentages of platelet number of the five rabbits were ranged from 33.44% to 60.07%, showing a significant decrease at 45~90 min ($P<0.025$), a highly significant decrease at 135~270 min ($P<0.005$), and a most significant decrease at 180 min ($P<0.001$), after embryo transfer.

While the two rabbits in control group, receving same surgical operation as the in five rabbits of Group I, except the rabbits were transferred the equal volume of non-embryo flushing medium, the platelet number of these two rabbits were also determined at the same time as in five rabbits of experimental group, showing a highest dropping percentage only 17.02 % at 45 minutes, but with no significance ($P>0.500$).

It is therefore concluded, that the remarkable decrease of platelet number is due to the presence of embryos, in splenectomized rabbits.

3. Experiment 3

Sixty 8-16-cell rabbit embryos were cultured for 24 h, 2 ml of the embryo-cultured medium was used for injecting each of the two splenectomized rabbits (No. 1, 2), 2 ml of the seventy 8-16-cell embryo-cultured medium, also after 24 h, was used for injecting one splenectomized rabbit (No. 3). The platelet number were determined before (0) and after injection (5~120 min). The results were presented in Table 3, showing that the platelet number decreased significantly at 5 min ($P<0.050$) and constantly decreased from 5 to 120 min after injecting embryo-cultured medium, the dropping percentages ranged from 3.35% to 65.13%, with the highest decrease at 10 min (65.13%, $P<0.010$), the and also significantly high at 15 min ($P<0.050$). While in the five rabbits in controlled group, after injecting non-embryo cultured midium, also 2 ml, the highest dropping percentage of platelet number at 60 min was 17.05%, but with no significance ($P>0.050$). It may be concluded that the manipulation of non-embryo-cultured medium had no effect on the decrease of platelet number, it is certain substance that derived from the embryo in the cultured medium caused the decrease of platelet number.

4. Experiment 4

Three groups were used in this experiment.

Table 3. Platelet number ($\times 10^4/mm^3$) before (0) and after injecting 8-16-cell embryo-cultured medium

Rabbit No.	Deatermining time (min)									
	0	5	10	15	20	30	45	60	90	120
1	82	35	36	45	66	66	59	71	95	41
2	58	41	16	18	24	45	30	37	43	54
3	75	56	23	21	34	49	74	72	70	73
Mean	71.7	44.0	25.0	28.0	41.3	53.3	54.3	60.0	69.3	56.0
Dropping percentage(%)		38.63	65.13	60.95	42.40	25.66	24.27	16.32	3.35	21.90

Group Ⅰ. Ten 2-cell embryos were cultured in 1 ml medium, 24 h after, the embryo-cultured medium was taken out, preserved in deep-frozen cabinet, at −25°C, for one month, then thawed at room temperature, 0.4 ml of this freezing-thawing cultured medium was injected into the abdominal cavity of splenectomized mouse. Four mice were used in this group.

Group Ⅱ. Thirty 2-cell rabbit embryos were cultured in 1 ml medium containing 20 μg antagonist kadsurenone for 24 h, 0.4 ml of this cultured medium was injected into the abdominal cavity of splenectomized mouse, Three mice were used in this group.

Group Ⅲ. 1 ml medium with no embryo was treated as the same procedure was used as in Group Ⅰ. 0.4 ml of this non-embryo-cultured medium was injected into the abdominal cavity of splenectomized mouse. Four mice were used in this Group.

A total of eleven mice were used in the above three groups, blood samples were collected and platelet number was determined before (0) and after (5, 10, 15, 20, 30, 45, 60, 90 & 120 min) injections. The results are shown in Table 4.

The result of Group Ⅰ showing that platelet number were decreased immediately at 5 min after injecting the rabbit embryo-cultured medium, the average of four mice was 85.7 $\times 10^4/mm^3$, that of before injection was $117.0 \times 10^4/mm^3$, with a dropping percentage of 26.75%, with a high significant difference ($P<0.005$); the average dropping percentages ranged from 26.75% to 62.14%, showing a gradually decrease in platelet number when the determining time after injection was prolonged.

Comparing the platelet number before and after injection, showing a significant difference at 5~15 min, and a greater difference at and after 20 min ($p<0.001$).

The average platelet number of four mice in Group Ⅲ, as compared with that of Group Ⅰ, there was little, or insignificant change before and after injection, the lowest platelet number was found at 45 and 90 min after injection($92.0 \times 10^4/mm^3$), that of before injection was $108.8 \times 10^4/mm^3$, with a dropping percentage only 15.44%, there was no significant difference ($p>0.050$).

It has been clearly illustrated that, the decrease in platelet number was due to the effect of certain substance released form the embryo, and also that, it was this substance released from rabbit embryo causing the decrease of platelet number even in the heterogeneous mice, with the same effect as in rabbit.

The present experiment also reveals that, the frozen-preservation of rabbit embryo-cultured

Table 4. Platlet mumber ($\times 10^4/mm^3$) of splenectomized mice before (0) and after injecting 3 kinds of culturd medium

Group No.	Mouse No.	Determining time (min)									
		0	5	10	15	20	30	45	60	90	120
I	1	112	85	69	78	51	76	69	28	36	39
	2	104	92	69	54	55	59	47	63	36	41
	3	123	77	76	89	64	69	51	55	42	40
	4	129	87	91	85	82	66	71	50	63	70
	Mean	117.0	85.7	76.3	76.5	63.0	67.5	59.5	49.0	44.3	47.5
	Dropping percentage(%)		26.75	34.79	34.62	46.15	42.31	49.15	58.12	62.14	59.40
	P		<0.005	<0.005	<0.010	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
II	1	124	109	118	121	122	114	110	116	119	108
	2	83	85	76	73	74	80	78	72	69	70
	3	110	102	116	106	103	101	110	113	97	97
	Mean	105.7	98.7	103.3	100.0	99.7	98.3	99.3	100.3	95.0	91.7
III	1	97	104	95	135	105	95	94	84	86	84
	2	96	112	94	88	90	86	65	91	89	93
	3	132	127	117	115	90	122	105	117	95	107
	4	110	122	129	111	87	105	104	89	98	96
	Mean	108.8	116.3	108.8	112.3	93.0	102.0	92.0	95.3	92.0	95.0

Group I : Injecting frozen 10 2-cell rabbit embryos-cultured medium

Group II : Injecting frozen medium of 30 20-cell rabbit embryos-cultured medium containing kadsurenone

Group III : Injecting frozen non-embryo-cultured medium

medium, even for a period as long as one month, the substance released from the embryo still maintained the biological activity causing the decrease of platelet number.

The result of Group II also reveals there was little change in platelet number before and after injection, the mean of platelet number at 120 min after injection, as determined in three mice ($91.7\times10^4/mm^3$), was the highest decrease as compared with that before injection ($105.7\times10^4/mm^3$), with a dropping percentage of 13.25%, and no significant difference was shown ($p<0.400$). As compared with Group I, the culture midium of Group II contained PAF antagonist kadsurenone, which inhibited the rabbit embryo-cultured medium to produce an effect of decreasing the platelet number in mice. It is therefore, a conclusion could be reached, indirectly, that the PAF is produced from the rabbit embryos.

5. Experiment 5

The present experiment contains 4 groups : Group 1, using culture medium only ; Groups 2 and 3, 20 μg of kadsurenone and 0.3 μl DMSO were added into the culture medium. Group 4, 0.3 μl DMSO was added into the culture medium. Twenty seven 2-cell embryos were cultured in each of the four groups with three different culture medium. Observations were made on the development of cultured embryos at different times, i. e., at 6.5, 25, 46, 77, 99.5 and 124 h after culturing. The results are shown in Table 5.

Table 5. Comparison of rabbit embryo developing in 3 kinds of culture medium

Cultured time (h)	Group No.	Embryo developing stage								
		2-cell	4-cell	8-cell	16-cell	32-cell	64-cell	morula	blastocyst	hatched blastocyst
0	1	27								
	2	27								
	3	27								
	4	27								
6.5	1	8	19							
	2	9	18							
	3	10	17							
	4	7	20							
25	1			26	1					
	2		5	22						
	3		5	22						
	4			23	4					
46	1				5	22				
	2		5	5	7	10				
	3		1	9	10	7				
	4			2	8	17				
77	1				2	7	7	7	9	
	2			7	4	9	6	1		
	3		1	5	5	8	5	3		
	4				2	4	5	10	6	
99.5	1				2	7	2	4	11	1
	2			6	5	9	5	2		
	3		1	5	5	8	4	4		
	4				2	4	5	5	11	
124	1				2	6	3	2	11	3
	2			6	5	9	5	1	1	
	3		1	5	5	8	4	1	3	
	4				2	4	5	2	14	

Group No. 1 : Culture medium

Group No. 2, No. 3 : Culture medium containing 20 μg kadsurenone & 0.3 μl DMSO

Group No. 4 : Culture medium containing 0.3 μl DMSO

The results obtained from the experimental studies showing that the embryo development in all the four groups seems to be approximately the same at 6.5 h after culturing. At 25 h after cultivation, the embryos of Group 1 and 4 developed to 16-cell stage : while in Groups 2 and 3, 19% of the embryos were still stay at 4-cell stage, none of them reached 16-cell stage.

After 46 h culturing, the embryos in Group 1, 100% developed to 16~32-cell stage, among them, 81% (22/27) reached 32-cell satge ; in Group 4, 93% (25/27) reached 16~32-cell stage, among them, 63% (17/27) reached 32-cell stage. However, in both Groups 2 and 3, 63%

(17/27) of embryos developed to 16-32-cell stage, respectively, showing the speed of embryos development is rather slow, much behind that in Groups 1 and 4.

After culturing 77 h, in both Groups 1 and 4, all the embryos were developed to, or above, 16-cell stage, among them, 26% (7/27) and 37% (10/27) reached morula stage, and 33% (9/27) and 22% (6/27) reached blastocyst stage, repectively. While that in Group 2, 26% (7/27) were still stay at 8-cell stage, only one embryo (4%) developed to morula stage; in Group 3, one embryo even still stop at 4-cell stage, five at 8-cell stage, only three embryos (11%) reached morula stage. And none of them reached blastocyst stage. The above observations evidently showing that, in Group 2 and 3, the embryo development is rather slow, as compared with that in Groups 1 and 4.

After culturing 99.5 and 124 h, showing that the embryo development in both Groups 1 and 4 was rather fast, 11 blastocysts (41%) were found, and in Group 1, one embryo (4%) reached hatched blastocyst stage. When the embryo cultured after 124 h, in Group 1, 11 embryos (41%) developed to blastocysts and 3 (11%) to hatched blastocyst stage, and in Group 4, 14 embryo (52%) found at blastocyst stage. While in Group 2, only one (4%) blastocyst was found, and 6 embryos (22%) were still stay at 8-cell stage ; in Group 3, only 3 blastocysts (11%) were found, and one embryo (4%) was still at 4-cell stage, and five (19%) at 8-cell stage.

The results of above experimental studies clearly illustrating that ; in Group 1, the embryo developed much better in culture medium, in Group 4, little difference has been found in embryo development as compared with that in Group I, The result also revealing that ; DMSO with a concentration of 0.03%, has little effect on the embryo development. While in Groups 2 and 3, the embryo development was much slow, illustrating that : the PAF antagonist kadsurenone present in the culture medium produces an inhibiting effect on the embryo development.

Ⅳ. DISCUSSION

The decrease of platelet number in early pregnancy has been studies by O'Neill in mice (O'Neill, 1985). The phenomenon of early pregnancy-associated thrombocytopenia has been demonstrated in early pregnant rabbits as shown in Experiment 1 of the present studies, and also showing that there is a negative corelation between platelet number and number of embryo. In Experiment 2, it has been demonstrated that transfer of 2-cell embryo into the oviduct of splenectomized rabbit will cause a drastic decrease in the number of platelet. The results obtained from both Experiments 1 and 2 illustrating that the presence of the embryo has been considered as the direct reason causing the decrease in the number of platelet.

The spleen has been considered having a blood storing function. The spleen of mouse at 4~5 days of pregnancy will produce splenic contractions, and causing a drastic reduction of splenic platelet pool at the same time, and liberating the platelets into peripheral blood (O'Neill, 1985). This is the reason why splenectomized animals were used as the most suitable experimental animal patten related to number of platelet in researches.

In Experiments 3 and 4, injecting rabbit embryo-cultured medium into splenectomized rabbits and mice caused a drastic decrease in platelet number demonstrating that, the embryo could release certain substance into the culture medium, which produce and effect to decrease the number of platelet, and not only in rabbits,

but also in heterogeneous mice ; not only produced from 2-cell embryo, but also from 8-16-cell embryo; not only in fresh embryo-cultured medium, but also in preserved deep-frozen embryo-cultured medium, they can produce the same effect causing the decrease of platelet number. The above findings illustrating that certain substance released not only from 2-cell embryo, and also from 8-16-cell embryo ; and the biological activity of the substance present in the cultured medium will be still maintained after deep-freezing ; it is therefore, the embryo-cultured medium could be preserved in deep-freezing.

The technique used for determining the peripheral platelet number could be also used for determining the persence of PAF (Collier, 1990 ; Stock, 1992).

It is therefore, the decrease of platelet number could be indirectly applied to determine the presence of certain substance released from the rabbit embryos. This subtance is probably the PAF.

Kadsurenone is a competitive antagonist of PAF receptor. It inhibits PAF inducing aggregation of platelet (Shen, 1985). In Experiment 4, it has been demonstrated that, when the embryos were cultured in the medium contining kadsurenone, there will be no influence on the number of platelet. This result may be illustrated PAF is probably derived from the embryos, and kadsurenone has an antagonist effect on PAF. In Experiment 5, it has been demonstrated that kadsurenone will cause a delayed development of early stage rabbit embryos, and this may illustrate PAF possibly released from the rabbit embryos, and has also an effect to promote the development of embryos. The receptor which kadsurenone is competitive to PAF, may be located in rabbit embryo. While the exact location and the number of PAF receptor will be submitted to the examination in future experimantal investigations. In Experiment 4, since kadsurenone is competitive to PAF receptors while rabbit embryos were cultured, the embryo-cultured medium could not produce any effect on the decrease of platelet number on the splenectomized mice. When the kadsurenone containing embryo-cultured medium was injected into the splenectomized mice, PAF effect was inhibited in aggregation of platelet. These results should be submitted to the examination of future experimental studies.

The results of the five experiments carried out in the present studies may be summarized as follows : it has been illustrated the PAF may be released from the rabbit embryos, and has an effect on the number of platelet either in rabbits or in mice, and it is benificial to the early embryo development. However, further investigations should be carried out in order to prove accurately on : (1) the presence of EDPAF, (2) quantitative bioassay of EDPAF, and (3) the presence of PAF receptors.

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