IN VITRO FERTILIZATION AND DEVELOPMENT OF OVA OBTAINED FROM FEMALE MICE TREATED WITH AMINOPTERIN AND MITOMYCIN C.

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Accepted July 11, 1986

Abstract.....We examined the effects of mitomycin C and aminopterin on ovulation *in vivo* and, fertilization, subsequent cleavage and implantation in slc-ICR female mice using methods of fertilization and culture *in vitro*.

Chemicals were injected into mice at the MI (meiosis I) stage or 3 hours before the MI stage in order to examine their toxicity. Mitomycin C did not affect ovulation, but decreased the rate of fertilization. A high dose of mitomycin C (4 mg/kg) inhibited fertilization and the development of ova to the 2-cell stage. Aminopterin affected neither ovulation nor fertilization.

At either the MII (meiosis II) stage or 3 hours before the MII stage, high doses (4 and 2 mg/kg) of mitomycin C arrested cleavage and implantation. The cleavage was blocked frequently between the 3-4 cell stage and the 5-8 cell stage. Aminopterin affected neither cleavage nor implantation.

Key words : *in vitro* fertilization, cultivation, mitomycin C, aminopterin

INTRODUCTION

The process of maturation of oocytes in the female animals involves a very long prophase of meiotic division followed by ovulation. It is therefore difficult to determine the developmental stage of oocytes accurately. Fertilization and development of ova *in vivo* are easily affected by secondary factors such as breeding environment. Recently, *in vitro* techniques of fertilization and germ cell culture have been developed by Bedford (1979) and Maudilian et al. (1978), respectively. These preparations devoid of environmental factors and the cultured medium can be easily manipulated and, therefore, it seemed that these preparations could be applied to the

study of the mechanism of chemicals-induced damage of germ cells.

In this paper we report the examination of the effects of mitomycin C (MMC) and aminopterin (APR) on ovulation *in vivo*, and capability of *in vitro* fertilization, cleavage and implantation of cultured ova.

MATERIALS AND METHODS

Chemicals : Mitomycin C (MMC, Sankyo K. K.) and aminopterin (APR, Sigma K. K.) obtained commercially were used.

Animals : slc-ICR mice were obtained from Shizuoka Laboratory Animal Center, Hamamatsu. All animals were purchased at the age of 6 weeks and used 3 weeks later. The animals were maintained in a controlled environment (room temperature ; $22 \pm 2^{\circ}\text{C}$, relative humidity of $55 \pm 5\%$. The lighting cycle was 12 hours of alternating light and dark).

A. Effects of MMC and APR treatment *in vivo* on ovulation and fertilization *in vitro*.

Spermatozoa were collected from the caudal epididymis of male mice and suspended in THY medium (Toyoda, 1979) containing 0.4% bovine serum albumin (BSA). Unfertilized ova were obtained as described by Watanabe et al. (1979). pregnant mare serum gonadotropin (PMSG, 5 I. U.) was injected intraperitoneally, and 48 hours later human chorionic gonadotropin (hCG, 5 I. U.) was also injected intraperitoneally.

According to Baker (1972) and Basler (1978) mouse ova enter the MI stage with bivalent chromosomes 3 hours after the hCG injection. Ovulation occurred within 6 hours after the injection, and 12 hours later the number of chromosomes decreased to half, which was associated with the appearance of the first polar body (MII stage). MMC or APR was injected subcutaneously at the same time of hCG injection, or 3 hours after the injection (MI stage). Fifteen hours after the hCG injection mice were sacrificed by cervical dislocation, and oocytes were flushed out from the ampulla tubae uterinae. Oocytes were separated from cumulus cells by pipetting the cumulus mass in a medium containing 0.1% trypsin. The condition of oocytes were examined under light microscope. Oocytes with polar body surrounded by cumulus cells were transferred into a fresh medium covered with paraffin oil. Capacitated spermatozoa were added to the oocytes suspension at a density of 1×10^7 spermatozoa/ml for insemination, and incubated at 37°C under carbogen gas (5% CO_2 , 95% air). After five hours of incubation the ova were fixed with formalin, stained with lacmoid, and examined by light microscopy. Whether the fertilization was successfully performed or not was judged by the penetration of spermatozoa and the presence of pronuclei. The formation of male pronucleus was confirmed by the presence of sperm tail, second polar body, and spermatozoa *in vitellus*.

In some cases fertilized ova were cultured for an additional 24 hours, and the ova

were observed at the 2-cell stage.

B. Effects of MMC and APR treatment *in vivo* on subsequent cleavage and implantation of embryo *in vitro*.

Female mice were mated with males during the dark period (21:00-9:00), and the animals were checked next morning (9:00) for the presence of vaginal plugs. The sperm-positive females were injected subcutaneously with MMC or APR at the time of sperm-confirmation or 3 hours later (MII stage).

Mice were killed by cervical dislocation 36 hours after the confirmation of the vaginal plug. The oviducts were excised, put into BWW medium (Biggers et al. 1971) containing 0.3% BSA, and the ova were taken by the flush of the medium. The ova were washed several times and then placed in a drop of the medium under a layer of paraffin oil and cultured as described above.

The cultured ova were observed every 12 hours for 4 days. After compaction, some of the ova were surgically transplanted into uterine cavities of pseudopregnant females (mated with a vasectomized male). Thirteen days after transplantation, the mouse was killed by cervical dislocation in order to confirm the implantation.

RESULTS

A. Effects of MMC and APR treatment *in vivo* on ovulation and fertilization *in vitro*.

The average number of oocytes obtained from control mice was 23.3 (Table 1). There were no apparent differences in the number of oocytes obtained between the treated groups (MMC ; 1.0-4.0 mg/kg, APR 0.1-0.4 mg/kg injected at the same time of hCG injection or 3 hours later) and the control group. APR increased the number of ova without cumulus cells to 8.8% of the total number of oocytes. When the MMC or APR were injected at 3 hours before MI stage, the number of oocytes without polar body and surrounded by cumulus cells was markedly increased. However, when the chemicals were injected at MI stage, these cells increased only in MMC groups.

In vitro fertilization of mice oocytes obtained from mice treated with MMC or APR at the same time of hCG injection (Exp. 1) or 3 hours later (Exp. 2) are shown in Table 2. The fertilization rate in the control group was 78.3%. When the animals were treated with 1.0, 2.0 and 4.0 mg/kg MMC at 3 hours before MI stage, fertilization rate was decreased to 42.3%, 43.1% and 29.2% respectively. Effects of MMC on the fertilization rate at MI stage were almost the same as those at 3 hours before MI stage. In the case of APR, the fertilization rate of oocytes from APR treated mice did not apparently differ from that of the matched control group at both stages.

Approximately 40-90% of fertilized ova were cultured for an additional 24 hours in a fresh medium without MMC and APR. The results are shown in Table 3. In the control group, 97.5% of the ova grew to the 2-cell stage. On the other hand, the oocytes which were obtained from female mice treated with MMC at 3 hours after MI

Table 1 Ovulation in mice treated with MMC or APR at the time of hCG injection (Exp. 1) or 3 hours later (Exp. 2)

Dose (mg/kg)	MMC			APR			Control
	1.0	2.0	4.0	0.1	0.2	0.4	
Exp. 1							
Oocytes obtained (per mouse)	144 a) (24.0)	135 (22.4)	133 (22.2)	138 (23.0)	143 (23.8)	146 (24.3)	140 (23.3)
Oocytes examined	131	130	124	137	140	129	138
Oocytes with							
polar body +, cumulus cell + b)	59	51	72	55	45	42	124
polar body -, cumulus cell + c)	61	68	42	65	77	70	6
cumulus cell - d)	0	0	0	12	0	0	0
Degenerated oocytes	11	11	10	5	18	17	8
Exp. 2							
Oocytes obtained (per mouse)	132 (22.0)	129 (21.5)	141 (23.5)	138 (23.0)	131 (21.8)	139 (23.2)	—
Oocytes examined	132	129	139	138	130	133	—
Oocytes with							
polar body +, cumulus cell +	83	41	61	129	119	105	—
polar body -, cumulus cell +	36	78	66	0	0	18	—
cumulus cell -	0	0	0	0	0	0	—
Degenerated oocytes	13	10	12	9	11	10	—

a) Indicate the number of oocytes.

b) Oocytes with polar body and surrounded by cumulus cells.

c) Oocytes without polar body and surrounded by cumulus cells.

d) Oocytes which were not surrounded by cumulus cells.

Oocytes were obtained from 6 mice in each group.

In control group mice were treated with saline (10 ml/kg) at the same time of hCG injection.

Table 2 *In vitro* fertilization of mice oocytes obtained from mice treated with MMC or APR at the same time of hCG injection (Exp. 1) or 3 hours later (Exp. 2)

Dose (mg/kg)	MMC			APR			Control
	1.0	2.0	4.0	0.1	0.2	0.4	
Exp. 1							
Oocytes inseminated	59 a)	51	72	55	45	42	60
Fertilized ova b)	25	22	21	35	29	27	47
Unpenetrated ova	25	20	38	9	7	7	8
Polyspermy	9	9	11	5	5	8	5
Degenerated ova	0	0	2	6	4	0	0
Fertilization rate (%) c)	42.3	43.1	29.2	63.6	64.4	64.2	78.3
Exp. 2							
Oocytes inseminated	83	41	61	60	60	60	—
Fertilized ova	40	19	13	48	45	32	—
Unpenetrated ova	25	4	30	8	4	17	—
Polyspermy	18	6	5	4	11	11	—
Degenerated ova	0	12	13	0	0	0	—
Fertilization rate (%)	48.1	46.3	21.3	80.0	75.0	53.3	—

a) These figures except for fertilization rate indicate the number of ova.

b) Fertilized ova were confirmed by both the observation of sperm *in vitellus* and the formation of pronuclei.

c) Fertilization rate: number of fertilized ova/number of oocytes inseminated.

Oocytes were obtained from 6 mice in each group.

In control group mice were treated with saline (10 ml/kg) at the same time of hCG injection.

Table 3 Growth of *in vitro* fertilized ova to two cell stage
(Oocytes were obtained from mice treated with MMC or APR)

Dose (mg/kg)	MMC			APR		Control
	1.0	2.0	4.0	0.1	0.2	0.4
	Growth rate % ^{a)}					
Exp. 1	69.2	85.0	45.0	76.9	100.0	85.0
	(13) ^{b)}	(20)	(15)	(13)	(16)	(20)
Exp. 2	74.3	46.2	ND	100.0	94.4	66.7
	(35)	(13)	ND	(11)	(18)	(12)

a) Growth rate: number of ova which reached 2 cell stage/number of examined ova.

b) Figures in parenthesis indicate the number of ova examined.

In Exp. 1 oocytes were obtained from female mice treated with MMC or APR (s. c.) at 3 hours before MI stage, and in Exp. 2 oocytes were obtained from female mice treated with these agents at MI stage.

ND: not determind.

stage (1.0, 2.0 and 4.0 mg/kg) grew to the 2-cell stage only by 69.2%, 85.0% and 45.0%, respectively. When the mice were treated with MMC at MI stage, the number of cells grew to the 2-cell stage were 46.2% in 2.0 mg/kg and 74.3% in 1.0 mg/kg. When the mice were treated with 4.0 mg/kg of MMC, the number of fertilized ova with radiated second polar bodies was so few that we could not continue the culture any further. In the case of APR (0.4 mg/kg, treated at MI stage), 66.7% of the cell grew to the 2-cell stage. When the dose of APR was decreased to 0.2 or 0.1 mg/kg, the value was almost the same as that in the control group.

B. Effects of MMC and APR treatment *in vivo* on subsequent cleavage and implantation of embryo *in vitro*.

The number of ova obtained from female mice was 14.0 per mouse on the average. The effects of MMC and APR treatment at the time of sperm confirmation are shown in Table 4. Almost all the cells, taken at 36 hours after sperm-confirmation in the control group and APR groups, were at 2-cell stage. On the other hand, MMC increased the number of embryo at 1-cell stage dose dependently. The ratio of the 1-cell stage embryo was 8.6, 12.8 and 51.7% in 1.0, 2.0 and 4.0 mg/kg group, respectively.

In the control group, the ova grew up to the 3-4 cell stage after about 15 hours of incubation (63.8%) and to 5-8 cell stage after about 39 hours (85.5%). After 51 hours of incubation 68.6% of the ova were at morula stage, and after 63 hours 62% of the ova were at early blastocyst stage. Finally, 30.1% of the cultured ova reached the zona shedding stage after 75 hours. The embryo before and after the compaction at the 8-cell stage are shown in Photo 1, and those at zona shedding stage, in Photo 2.

By the treatment of MMC (4.0 mg/kg s.c.) the number of oocytes in 1-cell stage at 3 hours of culture increased. Most of these cells were unfertilized oocytes. In the

Table 4 *In vitro* development of ova obtained from mice treated with MMC or APR at the time of sperm-confirmation

Stage of development	Duration of culture (hours)														Control (N=83)
	3	15	27	39	51	63	75	3	15	27	39	51	63	75	
	MMC 1.0 mg/kg (N=81)														
1 cell	9	4	4					13	10	8					8 8 8
2 cell	91	4	4	4				87	52	10	11	11			92 24 1
3-4 cell	89	22						38	33	41	41				64 34
5-8 cell	4	70						48							4 45 86
morula			93	78					36	36					12 5 69
early blastocyst				15	69						16	1			13 63
late blastocyst						60						14			8 23 47
zona shedding						4									30
degeneration			4	7	31	36			11	11	84	85			10 10 14 23
	APR 0.1 mg/kg (N=90)														
1 cell	10	10	10					2	2						
2 cell	90	1	1	1				95	9			100	1	1	
3-4 cell		1	1	1	1			2	82	20	2		58	6	
5-8 cell	83	64						6	72	2			41	36	
morula	4	21	81	28					2	93	44		57	99	33
early blastocyst			3	48	29	1				2	29	60			13 30
late blastocyst					44	41					9	14	32		52 54 38
zona shedding						31							7	41	42
degeneration			2	13	23	27	27			6	2	9	19	27	1 1 16 20
	APR 0.4 mg/kg (N=69)														

The figures in the table indicate percent of total number of ova in each determination.
 Figures in parenthesis indicate the number of ova obtained from 6 mice in each group.

Photo. 1. Developed from two-cell mouse ova cultured for 27 hr.
a : eight-cell stage before compaction.
b : eight-cell stage after compaction.

Photo. 2. Developed from two-cell mouse ova cultured for 75hr.
a : late blastocyst escaping from zona pellucida.
b : late blastocyst completely free of zona.

Table 5 Development *in vitro* of embryos of mice treated with chemicals *in vivo* (treated with chemicals at the MII stage)

Stage of development	Duration of culture (hours)																	
	MMC 1.0 mg/kg (N=81)						MMC 2.0 mg/kg (N=94)						MMC 4.0 mg/kg (N=84)					
1 cell	10	4	4				28	26	26				81	81			4	4
2 cell	90	6	4	4			72	35	9	7	6		15	2	2		96	16
3-4 cell	85	22					39	55	33	33			13				76	23
5-8 cell	5	70	12				11				2		13				5	41
morula			80	78				34	29	2						29	90	59
early blastocyst				15	52				20	1							2	33
late blastocyst					49					17							1	38
zona shedding					15													37
degeneration			4	7	36	36		26	32	78	80		4	4	4	87	87	99
Control (N=82)																		
																	3	15
																	27	39
																	51	63
																	75	75
APR 0.1 mg/kg (N=79)																		
1 cell	100	19	3															
2 cell							100	26	1				10	9	9			
3-4 cell	76	24						70	37				89	2	2	2		
5-8 cell	5	43	1					4	49	93			1	1	1	1	1	
morula			30	94	61								83	66	3			
early blastocyst							13	5	75				4	20	78	28		
late blastocyst			3	34	48					14	67					3	48	29
zona shedding				1	38	43				9	26	50					44	41
degeneration					33							34					31	
			3	4	14	24				1	1	7	16			2	13	23
																	27	27

The figures in the table indicate percent of total number of ova in each determination.

Figures in parenthesis indicate the number of ova obtained from 6 mice in each group.

case of MMC (2.0 mg/kg) treatment at the time of sperm-confirmation, the growth of the ova was arrested, and only 14% of the ova reached late blastocyst stage at 75 hours while 84% of the ova were degenerated at 63 hours. At higher dose (4.0 mg/kg) the toxicity was more pronounced. It seems that MMC affects embryos and arrests growth at the 3-4 cell stage. Ova developed normally in the doses 1.0 mg/kg MMC or 0.1-0.4 mg/kg APR (Table 5).

In the case of ova obtained from mice treated at 3 hours after sperm-confirmation (MII stage), the effects of MMC (2.0 and 4.0 mg/kg) appeared to be a little stronger (Table 5) than those in the case of ova obtained from mice treated with MMC at the time of sperm-confirmation (Table 4). At either stage APR did not have about significant effects.

Some cultured ova which had been compacted at the 8-cell stage were transplanted to pseudopregnant mice, which were then killed on the 13th day of pregnancy in order to count the number of implantations, and to check whether the embryos were viable or had died early or late. The results are shown in Table 6. Injection of MMC (2.0 and 4.0 mg/kg) was shown to cause a considerable decrease in the rate of implantation. When 4.0 mg/kg MMC was injected into mice 3 hours after the confirmation of the vaginal plug, almost all ova appeared to be abnormal making them impossible to transplant. Even when the dose was reduced to 2.0 mg/kg, only 12.5% of the ova achieved implantation. When MMC was injected upon confirmation of vaginal plug, the rate of implantation (Exp. 1) was better than those in the Exp. 2, but it was still much lower than that of the control group (85.9-90.0%).

Table 6 Implantation of mice ova of morula stage (Ova were obtained from mice treated with MMC or APR and developed to morula stage in vitro)

Dose (mg/kg)	MMC			APR			Control
	1.0	2.0	4.0	0.1	0.2	0.4	
Implantation rate %							
Exp. 1	70.0	80.0	10.0	80.0	66.6	66.6	90.0
	(10)	(10)	(10)	(15)	(15)	(15)	(20)
Exp. 2	70.0	12.5	ND	80.0	60.0	66.6	85.0
	(10)	(8)	ND	(15)	(15)	(15)	(20)

In Exp. 1 ova were obtained from female mice treated with MMC or APR (s. c.) at 3 hours before MII stage, and in Exp. 2 ova were obtained from female mice treated with these agents at MII stage.

Implantation rate: number of implanted embryos/number of examined embryos.

Figures in parenthesis indicate the number of embryos examined.

See "Materials and Methods" for the method of implantation.

ND: not determined.

DISCUSSION

MMC and APR showed adverse effects on ovulation in mice. Typical feature of the toxicity was the appearance of oocytes without polar body. The degree of toxicity of MMC at 3 hours after MI stage did not differ significantly from its effects at MI stage. On the other hand, effects of APR on ovulation appeared more pronounced when the oocytes were treated at the same time of hCG injection (3 hours before MI stage).

When the mice were treated with MMC at MI stage or at 3 hours before the stage, the rate of fertilization of the oocytes by *in vitro* insemination was decreased markedly, and the growth of the ova up to the 2-cell stage was also inhibited. The difference in the timing of MMC treatment did not cause a difference in its effect on the fertilization rate. But the growth of the ova to 2-cell stage was inhibited more severely by the treatment of MMC (2.0 mg/kg) at MI stage. MI stage seemed to be more sensitive also to the toxicity of APR (0.4 mg/kg). Since the disappearance of the follicle and granulosa cells was associated with the loss of resistance of ova against adverse effect of MMC, it is likely that these cells protected the oocytes from the invasion of chemicals.

We found approximately 10% of polyspermy in both control groups. The rate of polyspermy seemed not to be affected by MMC and APR. As Miyamoto et al. (1972), Fraser et al. (1978) and Fukuda et al. (1978) reported, the increase in polyspermy at fertilization *in vitro* reflects a relationship between sperm density and polyspermy, since the number of spermatozoa present around the ova was greater *in vitro* than *in vivo*.

Tanaka (1981) reported that the injection of MMC into female mice just after mating induced remarkable chromatid type abnormalities in ova. Hitotsumachi (1981) reported that injection of MMC into male mice (3.0-5.0 mg/kg) induced chromosomal aberrations in spermatogonia 24 hours after the injection, resulting in a decrease in the number of sperm. In the present experiment, the 4.0 mg/kg MMC decreased the fertilization rate and suppressed ova growth to the 2-cell stage in ova treated at the MI stage. When the female mice were injected with this dose of MMC at the time of sperm-confirmation, it also suppressed the maturation of oocytes and decreased the fertilization rate. The treatment with 2.0 mg/kg of MMC at the MII stage inhibited the growth of oocytes and decreased the fertilization rate in the same way. It was confirmed that MMC blocked the growth of ova from the 3-4 stage to the 5-8 cell stage. From these results we can conclude that MMC inhibited the maturation of oocytes and the growth of fertilized ova as well as the maturation of sperm (Hansmann, 1974).

Tanimura (1965) examined the effect of APR on mice and demonstrated that 0.3-2.5 mg/kg of APR was lethal to dams and to fetuses and had a growth-inhibiting effect. Thiersch et al. (1959) reported that injection of 0.15 mg/kg APR on CFW mice

every day for a week caused complete resorption of embryos. In this paper, we showed that APR (0.4 mg/kg) inhibited the growth of ova from 1-cell stage to 2-cell stage.

Female germ cells have been thought to be highly resistant to mutagens. It is assumed that oocytes with a long dictyoten stage are capable of recovering effectively from DNA injuries, and that some injured ova degenerate. In the present experiment, however, we have shown that oocytes before ovulation were sensitive to MMC and APR.

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