MATERNAL CONTRIBUTION IN REVEALING THE EFFECTS OF METHOXYACETIC ACID (MAA) ADMINISTERED BEFORE IMPLANTATION ON THE EMBRYONIC DEVELOPMENT OF SWISS WEBSTER MICE (*Mus musculus*)

by

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ABSTRACT

Maternal contribution to and direct action of methoxyacetic acid (MAA) on the embryonic development had been examined by conducting embryo transfer. To reveal the maternal contribution, compacted morulae and early blastocysts, which were collected from untreated Swiss Webster donor mice on day 3 of gestation, were transferred to day 2 pseudopregnant recipients, after having been treated with 2.0 mmol/kg body weight (b.w.) MAA by gavage on day 1 of pseudopregnancy. Direct effect of MAA on the embryonic development were observed by transferring compacted morulae and early blastocysts, similarly recovered from day 3 pregnant donor mice, after MAA treatment on day 2 of gestation with the same method and dosing, to untreated day 2 pseudopregnant recipients. Control donor mice and recipient were given distilled water only as the MAA solvent. Observations on fetuses resulting from embryo transfer were carried out on day 16 of gestation. Administration of MAA to the donors tended to decrease the implantation rate and the survival rate of the implanted embryos. When MAA was given to the recipients, the implantation rate and survival rate of embryos transferred decreased significantly ($p<0.05$) but the survival rate of implanted embryos were significantly higher ($p<0.05$) if compared to those of MAA treated donors. The intrauterine death tended to increase either in the treated donors or recipients. There was no effect of MAA on the fetal body weight and in producing fetal malformations. It is concluded that at the beginning of implantation, maternal contribution in revealing the effects of MAA on the embryonic development of Swiss Webster mice is predominant, whereas after implantation took place, the quality of the embryos become more important for their survival.

Keywords : methoxyacetic acid (MAA), preimplantation embryo, donor, recipient, embryo transfer, mice.

ABSTRAK

Telah dilakukan penelitian untuk mengetahui pengaruh asam metoksiasetat (MAA) yang diberikan pada induk mencit (*Mus musculus*) Swiss Webster umur kebuntingan tahap praimplantasi. Pengaruh tidak langsung melalui keadaan internal induk diteliti dengan cara mentransfer embrio tahap morula kompak dan blastokista awal, yang dikoleksi dari donor tanpa perlakuan pada umur kebuntingan 3 hari, ke dalam uterus induk resipien pada umur kebuntingan semu 2 hari dan telah diperlakukan dengan MAA secara "gavage" dengan dosis 2 mmol/kg berat badan (bb) pada umur kebuntingan 1 hari. Pengaruh langsung MAA terhadap embrio tahap praimplantasi diteliti dengan cara melakukan transfer embrio yang dikoleksi dari induk donor yang telah diperlakukan dengan MAA dengan dosis dan cara yang sama pada umur kebuntingan 2 hari, ke dalam uterus induk resipien bunting semu 2 hari yang tidak diperlakukan dengan MAA. Induk donor dan resipien kontrol hanya diperlakukan dengan akuabides steril. Pengamatan terhadap fetus hasil transfer dilakukan pada umur kebuntingan 16 hari. Hasil yang diperoleh dari penelitian transfer embrio menunjukkan, bahwa pemberian MAA terhadap induk donor

menyebabkan persentase keberhasilan implantasi, keberhasilan hidup embrio yang ditransfer dan keberhasilan hidup embrio yang terimplantasi cenderung menurun. Jika MAA diberikan pada induk resipien, persentase keberhasilan implantasi dan keberhasilan hidup embrio yang ditransfer menurun secara nyata ($p<0,05$) dari kontrolnya, tetapi persentase keberhasilan hidup embrio terimplantasi meningkat secara nyata ($p<0,05$) dibandingkan dengan induk donor yang diperlakukan dengan MAA. Pemberian MAA terhadap induk donor maupun resipien menyebabkan persentase embrio yang mengalami kematian intrauterus cenderung meningkat, tetapi tidak mempengaruhi berat badan fetus dan kemunculan kelainan perkembangan embrio mencit Swiss Webster, sedangkan sesudah terimplantasi terlihat kualitas embrio lebih menentukan keberhasilan hidup embrio tersebut.

Kata kunci : asam metoksiasetat (MAA), embrio praimplantasi, donor, resipien, transfer embrio, mencit.

INTRODUCTION

Methoxyacetic acid (MAA) is a derivative of di(2-methoxyethyl) phthalate or DMEP, which is one of the phthalic acid esters (PAEs). Phthalic acid esters are industrial chemicals, used mainly as plasticizers in the manufacture of flexible plastics.

PAEs is embryotoxic and teratogenic to mammals (Campbell *et al.*, 1984). Out of eight different phthalate esters investigated, it was found that DMEP was a potent teratogen (Ritter *et al.*, 1985). DMEP is activated *in vivo* and hydrolized to 2-methoxy ethanol (2-ME) followed by oxidation to MAA, which is cosidered as the proximate teratogen (Ritter *et al.*, 1984).

MAA has a relatively long biological half life (Scott *et al.*, 1987, Sleet *et al.*, 1988). The elimination half life of MAA in the cynomolgus monkey is 18-25 hours (Wittfoht *et al.*, 1988), whereas in human is about 77 hours (Groeseneken *et al.*, 1989 in Welsch *et al.*, 1996). The long elimination half life of 2-MAA in people will cause accumulation of the agent upon continuous exposure to MAA even at low level. MAA was found to cross the placenta and may produce malformation. This finding may be important with regard to human exposure, particularly pregnant woman (Wittfoht *et al.*, 1988).

MAA also is found to be toxic and teratogenic to rats (Brown *et al.*, 1984) and mice (Clarke *et al.*, 1991, Darmanto *et al.*, 1994, Sudarwati *et al.*, 1996). Most studies on the effects of MAA have been conducted at the period of organogenesis, whereas relevant studies on preimplantation stage are still rare.

When toxic agents act on fertilized ovum, blastocyst or early embryos prior to organogenesis, it has been stated that such embryos will die or grow normally, and this known as "all or none" law (Tuchmann-Duplessis, 1975; Russel & Russel, 1956 in Nagao *et al.*, 1986). However, some agents such as trypan blue, cyclophosphamide, and methylnitrosourea given to pregnant animals before implantation have been shown to yield malformed fetuses (Nagao *et al*, 1986).

Spielmann *et al.* (1977) reported that the interference with embryonic development and the incidence of malformations in fetuses from teratogen treated dams may be due to the effects of the drug directly to the embryos or indirectly throught the maternal environment or the changes in the internal uterine environment. The effect responsible in producing malformations could be elucidated by conducting embryo transfer experiments (treated donor x untreated recipient and untreated donor x treated recipient).

Spielmann *et al.* (1977) carried out the embryo transfer experiments to see whether cyclophosphamide (CPA) of 60 mg/kg given to pregnant Wistar rat on gestation day 3, predominantly affects the embryos or internal environment of the dam. The results showed that CPA interferes either with the development of the embryos before implantation (the quality of the embryos) or with the decidual reaction of the uterus. The same technique was used by

Nagao *et al.* (1986) to investigate the effects of 3 or 5 mg/kg of mitomycin C given to day 2 pregnant donor mice, and to day 1 pseudopregnant recipient. It was noted that the internal maternal environment has more significant effect on the embryonic development than the direct action of the drug on the embryos.

Maternal contribution to and the direct action of MAA given at the preimplantation stage, on the embryonic development of Swiss Webster mice will be clarified by embryo transfer. The objectives of this study is to determine maternal contribution in revealing the effects of MAA given before preimplantation on the embryonic development.

MATERIALS AND METHODS

1. Animals

Swiss Webster mice (*Mus musculus*) from the Department of Pharmacy, ITB were housed in the animal room of Department of Biology, which was maintained at relative humidity 84.78 ± 2.07 %, temperatures 22.86 ± 0.25° C to 26.83 ± 0.25° C, under an altering 12 hour light/dark schedule (6 AM-6 PM). Males were placed separately from females. Diet pellets (CP 551, P.T. Charoen Pokphand Indonesia) and tap water allowed *ad libitum*.

2. Preparation of embryos and recipients

Donor mice were prepared by mating 8-12 week old females with normal males overnight. To obtain large number of embryos to be transferred, donors were superovulated prior to mating, by intraperitoneal injection of PMSG (Folligon, from Intervet) 5 IU/ mouse, followed 48 hours later by the same dose of hCG (Chorulon, from Intervet). The present of vaginal plugs was designated as day 0 of gestation.

Donor embryos were collected by flushing the uterus and the caudal part of the oviduct which was excised from the donor dams with 1 ml syringe+ 26 G neddle, containing M2 medium, sacrified on gestation day 3, by cervical dislocation. Five to eight compacted morulae and early blastocysts were transferred to each uteri horn (right and left) to day 2 pseudopregnant recipients. Pseudopregnant recipients were prepared by mating female mice with provensterile vasectomized males.

3. Embryo transfer to recipients

MAA (Wako Pure Chemical Industries, Japan) was dissolved in sterilized distilled water. Embryo transfer to evaluate the quality of MAA-treated embryo (Group I): Donors were treated with MAA 2.0 mmol/ kg b.w. by gavage on gestation day 2. On gestation day 3, donor embryos were recovered and transferred into untreated day 2 pseudopregnant recipients. Donor administered with sterilized distilled water as MAA solvent were served as controls.

Recipient mice were killed on gestation day 16, and the reproductive organs were removed intact from the animal. The uterine horn was cut opened and the number of implantations, live fetuses, malformed or dead fetuses, resorbed embryos and the body weight of live fetuses were examined. The parameters evaluated were implantation rate, survival rate of embryo transferred, survival rate of implanted embryos, mortality of implanted embryos, malformation rate of fetuses and fetal body weight.

Embryo transfer to evaluate the MAA-treated internal uterine environment to the ability and viability of the embryos (Group II) : Donors were untreated and on gestation day 3, embryos were transferred into day 2 pseudopregnant recipients which had been treated with MAA 2.0 mmol/kg b.w. by gavage on day 1 of pseudopregnancy. Control recipients were administered with solvent only. Recipient mice were killed on gestation day 16. The same parameter as Group I were evaluated.

All the data were statistically analysed using Student's t-test or Wilcoxon's rank sum test (Wilcoxon & Wilcox, 1965).

RESULTS AND DISCUSSION

The result of this experiment is presented in Table 1. Embryo transfer to evaluate the quality of MAA-treated embryo (Group I) was shown that MAA 2.0 mmol/kg b.w. given to donor mice on gestation day 2, tended to decreased the implantation rate (41.85%), survival rate of transferred embryos (31.31%) and the survival rate of implanted embryos (58.68%) compared to controls (62.14%, 52.42%, 83,81%). In this MAA treatment, the intrauterine death (21.32%) and fetuses with malformations or hemorrhage (4.5%) tended to be higher than those in the control (16.19% and 2.0%), whereas the mean fetal weight was similar to that of the control (0.69 g) and the malformation rate of fetuses with malformation or hemorrhage (1.25%) showed a tendency to increase from controls (8.40% and 0%).

Statistical analysis between treatment groups of the MAA treated donors and treated recipients did not show a significant difference in the implantation rate, survival rate of transferred embryos, mortality rate of implanted embryos and in the incidence of malformation or hemorrhage, as well as in the mean fetal body weight. There were two fetuses with hemorrhage (4.5%) was found in the MAA treated donors (group I) and one fetus with the cleft palate in its control. In the MAA treated recipients (group II) one fetuses with brachydactyly of digit IV (1.25%) was obtained.

Table 1. Influence of maternal MAA treatment on the development of embryos transferred to pseudopregnant recipient

	GROUP I		GROUP II	
	Untreated recipient		Untreated donor	
	Donor+solvent	Donor+MAA	Recipient+ solvent	Recipient+MAA
Dose (mmol/kg b.w.)	0 (control)	2	0 (control)	2
Number of recipients	10	10	10	10
Number of embryos transferred	80	72	130	131
Implantation rate (Number of implanted embryos)[1]	62.14 (51)	41.85 n.s. (31)	53.83 (68)	34.41* (42)
Survival rate of embryos transferred (Number of live fetuses)[1]	52.42 (43)	31.31 n.s. (23)	48.42 (62)	30.23* (38)
Survival rate of implanted embryos (Number of live fetuses)[1]	83.81 (43)	58.68 n.s. (23)	91.60 (620	90.17 n.s # (38)
Mortality rate of implanted embryos (Number of intrauterine death)[1]	16.19 (8)	21.32 n.s. (8)	8.40 (6)	9.83 n.s. (4)
Malformation rate of fetuses (Number of malformed fetuses)[2]	2.00 (1)a	4.50 n.s (2) b	0 (0)	1.25 n.s (1)c
Mean fetal body weight (g)[1]	0.69	0.69 n.s.	0.66	0.64 n.s.

*Significantly different from control ($p<0.05$)) by Student's t test[1]
n.s = non significantly different from control)
# Significantly different between treatment groups ($p<0.05$) by Wilcoxon's rank sum test[2]
a = cleft palate, b= haemorrhage, c= brachydactyly IV in the fore limb

Embryo transfer to evaluate the MAA-treated internal uterine environment to the ability and viability of the embryos (Group II) in which MAA 2.0 mmol/kg b.w. was administered to the recipients on day 1 of pseudopregnancy, it was revealed that the implantation rate (34.41%) and the survival rate of transferred embryos (30.23%) were significantly lower ($p<0.05$) than controls (53.83%, 48.42%), while the survival rate of implanted embryos (90.17%) though it tended to decrease from its control (91.16%), but it was significantly higher ($p<0.05$) compared to that in the MAA treated donors (58.68%). The mortality rate of implanted embryos (9.83%)

The result of the experiment showed that MAA treatments either to the donors or to the recipients decreased the implantation rate of the embryos transferred compared to that of the control (Table 1). The low implantation rate occurred in the embryo transfer of MAA treated donor to the untreated recipients (normal internal uterine environment), was caused by the decrease in the quality of MAA treated donor embryos. The same result which was revealed in the MAA treated recipients, indicated that the internal maternal environment contributed in lowering the implantation rate of untreated donor embryos transferred.

The transfer of MAA treated donor embryos to untreated recipients showed a tendency in decreasing the implantation rate and survival rate of the embryos transferred, which was due to the decrease of the quality of the donor embryos, exposed to the MAA since they were in the oviduct. Sumarsono *et al.* (2002) also found that the ability of the blastocyst to implant into uterus tended to decrease from 58% in control 42% in treated embryos colleted from donor treated with 2.0 mmol/kg b.w. MAA, while the survival rate of implanted embryos were significantly decreased ($p<0.05$) compared to control (52% in control to 34% to treated embryos). This data support our result that the quality of embryos decreased as the effect of MAA and also decreased the quality of embryo to implant in the uterus of recipient. The lower implantation rate and survival rate from the controls, in this type of embryo transfer experiment, were significantly found by Nagao *et al.* (1986) using 3 or 5 mg/kg b.w. of mitomycin C in pregnant CD-11 donor mice and by Lou *et al.* (1996) who treated pregnant Wistar rat with aspirin at the dose of 0.25, 0.5 or 1.0 mg/kg b.w., but Spielmann *et al.* (1977) obtained an increased implantation rate with the decreased survival rate in the treatment of cyclophosphamide 60 mg/kg b.w. to Wistar rat donors.

When MAA treatment was given to recipients, the implantation rate and the survival rate of untreated donor embryos transferred, became significantly lower. These low rates were assumed to be caused by the decreased quality of the internal maternal environment, changed by the MAA given one day prior to the embryo transfer. The same result were reported by Spielmann *et al.* (1977), Nagao *et al.* (1986), Lou *et al.* (1996) and Sumarsono *et al.* (2002) in their experiments as previously described.

The result of the above embryo transfer experiments (group II), indicated that at the preimplantation and early implantation stages, the internal maternal environment is more pronounced than the quality of the embryos in affecting the development of preimplantation embryos.

MAA given to pregnant mice was distributed in the various tissues and organs including the uterus (Terry *et al.*, 1995). MAA may be incorporated with the secretes of the uterine glands, which was taken up by the preimplantation embryos, as shown by caffeine exposed to the rabbit blastocysts (Fabro & Sieber, 1969 in Spindle & Wu, 1985). At this stage of development, the nourishment of the embryos still depend on the uterine secretions (Tuchmann-Duplessis, 1975), and therefore, MAA in the uterine secretes was absorbed by the embryos and interfered with the development of preimplantation embryos.

The survival rate of postimplantation embryos, derived from MAA treated donor embryos, which were transferred into untreated recipients (group I), tended to decrease as the consequence of the higher incidence of the embryonic intrauterine death. This incidence may be due to the decreased quality of the MAA treated donor embryos, that caused them unable to survive. However, treatment of MAA to the recipients, yield a significant higher survival rate of postimplantation embryos compared to that in the MAA treated donor. This higher survival rate of the implanted embryos obtained, was due to the preselection of good quality embryos at the pre- and early implantation stages, whereas those with lower quality have been deleted so that they were failed to implant and to survive. This result elicits that to the postimplantation development, the quality of the embryos is more important for their survival. The higher survival rate of postimplantation and lower incidence of intrauterine death in the treated recipients were also reported by Nagao *et al.* (1986) in the treatment of mitomycin C to CD-1 mice.

Ryanto (2000) found that 2.5 mmol/kg b.w. MAA given to day 2 pregnant donor mice decreased significantly the mitotic index and total cell numbers of blastocyst. And also tended to increased the chromosome aberration.

The administration of MAA either to the dams or recipients, tended to raise the incidence

of malformed fetuses. This malformation seemed to be spontaneously occurred, since the incidence was low, i.e. 4.5% in the group I and 1.25% in group II (Table 1.). From all the result of the experiments reviewed, it was shown that some agents given at the preimplantation stage, may either produced significant incidence of fetal malformations or did not. MAA belonged to the group of agents which did not produce fetal malformation significantly, but only decreased the quality of the embryos and the quality of internal maternal environment. MAA is an agent, which goes along with the "all or nothing" law.

CONCLUSION

Methoxyacetic acid (MAA) at the dose of 2.0 mmol/kg b.w. given to day 1 pseudopregnant recipient Swiss Webster mice (*Mus musculus*), which received embryo transfer of 3 day old untreated donor embryos on gestation day 2 of pseudopregnancy, decreased the quality of the internal maternal environment. But the same treatment of MAA to donor mice on gestation day 2, lowered the quality of the embryos, which was indicated by the increased intrauterine death of the embryos transferred to the untreated recipients.

The influence of the internal maternal uterine environment of the recipient is more pronounce in the early implantation stage of the transferred embryos, whereas at the late and postimplantation stage, the quality of the embryos is more important for its survival. There was no significant incidence of malformations in the fetuses resulting from embryo transfer either in the MAA treated donor embryos or in the treated recipients.

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