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Viability of α-momorcharin-treated mouse blastocysts in the pseudopregnant uterus

P. P. L. Tam, W. Y. Chan* and H. W. Yeung*

*Departments of Anatomy and *Biochemistry, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong*

Summary. Mouse morulae and early blastocysts developed normally to the late blastocyst stage in the presence of α-momorcharin in culture. When these embryos were transferred to a pseudopregnant uterus, they showed a poor ability to induce the decidual reaction and many failed to implant. Those that had implanted showed retarded embryonic development and many implantation sites contained only trophoblastic giant cells and extraembryonic membranes. Implantation of blastocysts was inhibited when the recipient animal was given α-momorcharin at the time of embryo transfer. We suggest that termination of early pregnancy by α-momorcharin is the result of the deleterious effect of the protein on the implanting embryos and the endometrium.

Introduction

A plant protein, α-trichosanthin, which is isolated from *Trichosanthes kirilowii* has been used clinically in China for the termination of pregnancy. Although this agent is very effective in inducing mid-term abortion, side effects such as induced hypersensitivity are often observed in the treated women (Anon, 1976; Zhong & Wang, 1983). Recent effort is directed towards the search for alternative abortifacients as well as the use of these agents in early pregnancy. In our laboratory, a glycoprotein named α-momorcharin was purified from *Momordica charantia* which is related botanically to *Trichosanthes*. When α-momorcharin was administered intraperitoneally to pregnant mice on Days 1–6 of gestation, the incidence of implantation was significantly reduced (Law, Tam & Yeung, 1983; Tam, Law & Yeung, 1984). In-vitro studies on the effects of α-momorcharin on the preimplantation embryo have shown that early cleavage-stage embryos developed normally to the compacting morula stage, but further development to the blastocyst stage was impaired (Tam *et al.*, 1984). When the embryo was treated at the morula stage, it developed into a blastocyst but subsequent in-vitro differentiation such as hatching, attachment and trophoblastic outgrowth was retarded (Law *et al.*, 1983). In the present study, we have tested the developmental potential of the α-momorcharin-treated embryos by transferring them to a pseudopregnant uterus. The viability of normal and treated embryos in the α-momorcharin-treated recipient was also studied.

Materials and Methods

Mice. Random bred ICR female mice were paired with males and the presence of vaginal plugs was checked the following morning (Day 1 of pregnancy). Pseudopregnant females were produced by mating the females to vasectomized males of proven sterility.

Culture and transfer of embryos. Morulae and early blastocysts were collected from Day-4 pregnant mice into complete PB1 medium (Whittingham & Wales, 1969). The embryos were washed once in warm culture medium (Dulbecco's modified Eagles medium + 20% fetal calf serum, both from Gibco, Grand Island, NY, U.S.A.) and were cultured for up to 24 h in the same medium under 5% CO_2 in air. The experimental embryos were exposed to α-momorcharin (1 or 5 μg/ml) for 1 to 24 h (Table 1). At the end of the culture period, the embryos were washed twice in PB1 medium and then were transferred in groups of 4 to 8 into the uterus of the pseudopregnant female anaesthetized with pentobarbitone sodium (Nembutal: Serva, West Germany). All the embryos were transferred irrespective of their developmental performance in culture. Embryos that were cultured for 1 or 6 h were transferred to Day-3 pseudopregnant females while those cultured for 24 h were transferred to Day-4 pseudopregnant females. Each recipient always received the control (untreated) embryos in the right uterine horn and the treated embryos in the left uterine horn. Some of the pseudopregnant females (N = 20) were given an intraperitoneal injection of 0·2 mg α-momorcharin/25 g body weight immediately after the embryos (treated with 5 μg α-momorcharin/ml for 24 h) were transferred. This dose of protein has been shown previously to inhibit implantation (Law *et al.*, 1983). Altogether 332 control (untreated) and 408 α-momorcharin-treated embryos were transferred to 66 pseudopregnant animals. In another batch of Day-4 pseudopregnant females (N = 24), the right uterine horn was mechanically stimulated by scraping with a 21-gauge syringe needle. The left horn was untouched and served as the control. Half of these females (N = 12) were given an injection of 0·2 mg α-momorcharin/25 g body weight after scraping. The animals were killed on Day 10 of pseudopregnancy and the wet weight of the uterine horns was determined. The value was expressed as a ratio of uterine wet weight to body weight of the animal.

Examination of implantation sites. The recipient animals were killed on Days 9–10 of pseudopregnancy. The numbers of decidual swellings were scored for each horn and then the whole uterus was fixed in Sanfelice fluid (10 parts 2% chromium trioxide, 6 parts 20% acetic acid, 10 parts 40% formaldehyde and 6 parts distilled water). Some of the conceptuses were dissected and examined under a dissecting microscope for an assessment of embryonic development and for the presence of ectoplacental tissues. The other conceptuses were processed for histology. Serial 7 μm sections of these specimens were stained with haematoxylin and eosin. A few specimens with an incomplete series of sections were not included in Table 2. The development attained by the embryo was determined according to the staging system based on external morphology and somite number (Theiler, 1972) and on the extent of cranial neurulation (Jacobson & Tam, 1982).

PLATE 1

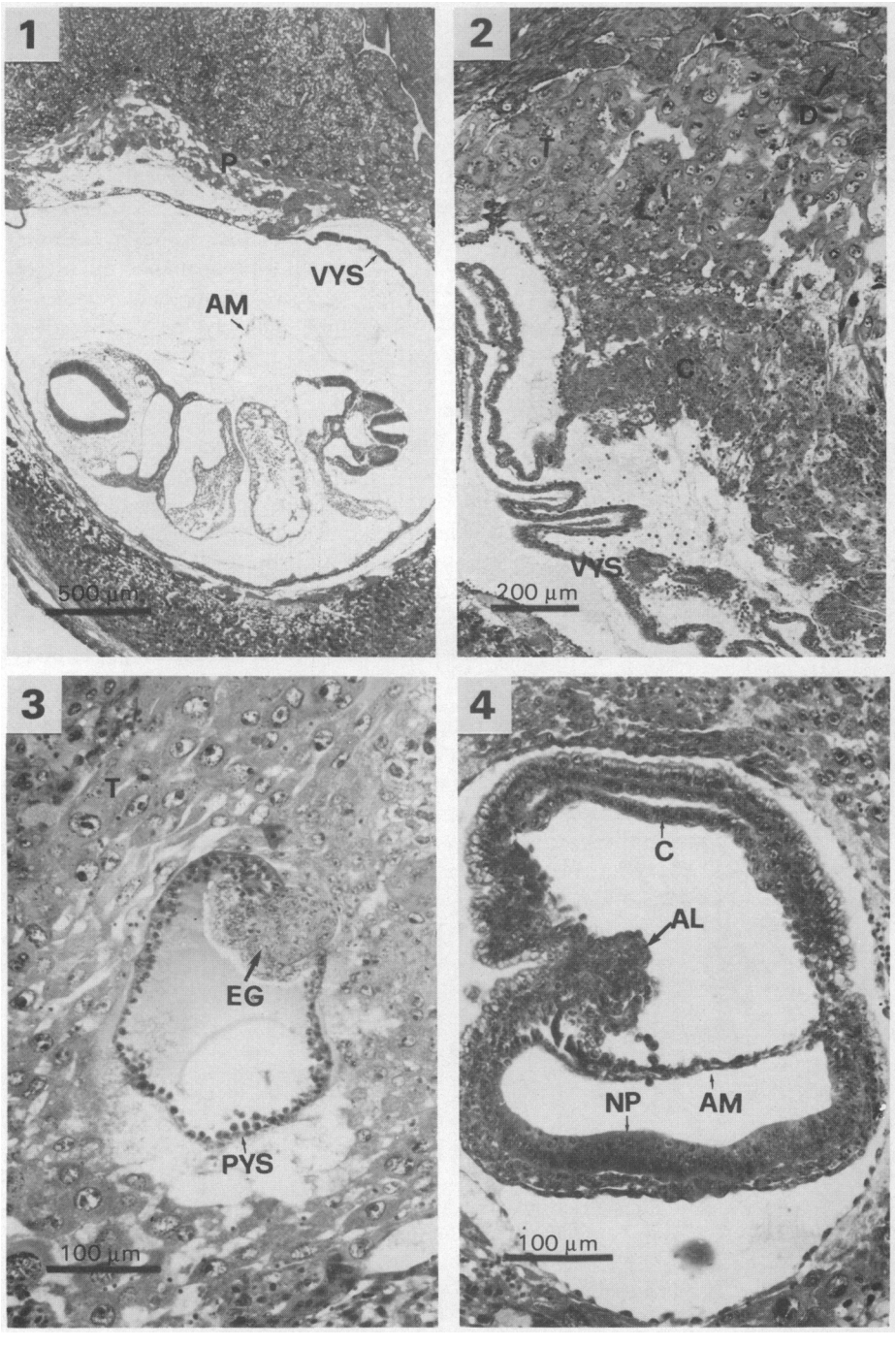
Fig. 1. A Day-10 24-somite embryo developed from an untreated blastocyst that was transferred to the pseudopregnant uterus. The chorioallantoic placenta (P), visceral yolk sac (VYS) and amnion (AM) were well formed.

Fig. 2. A Day-10 conceptus derived from a treated (1·0 μg α-momorcharin/ml for 24 h) blastocyst. Only a collapsed visceral yolk sac (VYS) together with chorion (C) and trophoblastic giant cells (T) were found. D = decidual cells.

Fig. 3. A Day-9 abnormal egg cylinder (EG) formed by a treated (1·0 μg α-momorcharin/ml for 24 h) blastocyst. PYS = parietal yolk sac, T = trophoblastic giant cells.

Fig. 4. A Day-9 presomite neural plate (NP) stage embryo developed from a treated (1·0 μg α-momorcharin/ml for 24 h) blastocyst. This embryo was retarded when compared to control embryos which were already at the neurulation stage and had about 10 somites. C = chorion, AL = allantois, AM = amnion.

PLATE 1



(*Facing p.* 568)

Results

The embryos obtained from Day-4 pregnant mice were at the morula (37–47%) and early blastocyst (53–63%) stages. Most embryos developed normally to the expanded blastocyst stage (control, 96·1%; 1 μg α-momorcharin/ml, 96·3%; $P > 0.05$ χ^2 test), but about 30% of the Day-4 embryos failed to develop in the presence of 5·0 μg α-momorcharin/ml. An exposure of 1·0 μg α-momorcharin/ml for 1 or 6 h *in vitro* did not affect the ability of these embryos to implant in the pseudopregnant uterus (Table 1). When the embryos were exposed to 1–5 μg α-momorcharin for 24 h, a significant reduction in the number of implantations was observed (Table 1). A within-recipient comparison of the implantation rates between the untreated and the treated embryos in the two horns showed that untreated embryos thrived significantly better than those embryos exposed to 5 μg α-momorcharin/ml for 24 h (Table 1). Implantations were found in 28 horns (out of 46) receiving untreated embryos. An average of 4·7 untreated embryos were transferred to each horn, and a mean of 3·8 implantations were recovered. Implantations were found in 24 horns (out of 46) receiving the treated embryos. An average of 5·9 treated embryos were transferred to each horns which resulted in 3·2 implantations ($P < 0.025$, compared to untreated embryos; χ^2 test). In 5 horns that received treated embryos, only necrotic decidual masses and haemorrhagic clots were found in the uterine lumen.

Table 1. Effects of α-momorcharin on the incidence of implantation in mice at Day 9–10 of pseudopregnancy

	Untreated embryos		Treatment		Treated embryos	
No. of recipients	No. transferred	No. (%) of implantation sites	Dose (μg/ml)	Time (h)	No. transferred	No. (%) of implantation sites
Untreated pseudopregnant recipients						
5	22	11 (50)	1·0	1	22	11 (50)
14	65	19 (30)	1·0	6	86	23 (27)
10	58	27 (47)	1·0	24	77	23 (30)*
17	72	48 (67)	5·0	24	90	20 (22)**[b]
Overall	217	105 (48)			275	77 (28)**[a]
Treated pseudopregnant recipients†						
20	115	12 (10)	5·0	24	133	5 (4)*

† 0·2 mg α-momorcharin/25 g body weight i.p.

* $P < 0.05$, ** $P < 0.001$ compared with values for untreated embryos (χ^2 test).

For between-horn comparison, [a] $P < 0.01$ (30 non-zero values), [b] $P < 0.001$ (10 non-zero values), Wilcoxon test.

The implantation of untreated and treated embryos was markedly (both at $P < 0.001$, compared to those in the untreated pseudopregnant recipients, χ^2 test) inhibited when the pseudopregnant female was given α-momorcharin at the time of embryo transfer. In many α-momorcharin-treated recipients there was a fluid-distended uterus which showed poor differentiation of stroma and glands in the endometrium. Only 4 treated recipients carried implantations, the others either had aborted (with the necrotic decidua remaining in the uterus) or had no implantation sites. However, the untreated embryos implanted more successfully than did the treated embryos in the treated recipients. The decidual reaction of the endometrium as indicated by the increase in wet weight of the uterus upon stimulation was significantly diminished by α-momorcharin treatment (treated: scraped horn = 0·47 ± 0·15 (n = 12), control horn = 0·36 ±

0·04 ($n = 12$), $P > 0·05$, paired *t* test; untreated: scraped horn = 0·92 ± 0·18 ($n = 12$), control horn = 0·31 ± 0·09 ($n = 12$), $P < 0·01$, paired *t* test).

About 70% of the implantations derived from untreated blastocysts that were transferred to normal recipients contained an embryo and of these >95% were morphologically normal (Pl. 1, Fig. 1; Table 2). The ectoplacental cone was well developed on Day 9 and a chorioallantoic placenta was formed on Day 10. However, the transferred embryos showed a wide variation in developmental stages (Table 2). The embryos that were treated with α-momorcharin (1 μg/ml) for 1 h developed at the same rate as did untreated embryos. However, the embryos that were exposed to the same dose of α-momorcharin for 6 h implanted normally and seemed to develop normally up to Day 9, but by Day 10 they had begun to show developmental retardation (Table 2). Other embryos that were treated with α-momorcharin (1 or 5 μg/ml) for 24 h failed to form a proper embryo and the implantation sites contained either no identifiable embryonic derivatives or a meshwork of trophoblast cells and extraembryonic membranes (Pl. 1, Fig. 2; Table 2). The few embryos that developed from these two groups of treated blastocysts were mostly retarded (Pl. 1, Figs 3 & 4). Only 12 untreated blastocysts had implanted in the α-momorcharin-treated females, 8 of them formed an embryo. These embryos were, however, significantly retarded in development (Table 2). No viable embryos were found in the implantation sites derived from the treated blastocysts (Table 2).

Table 2. Development of untreated and α-momorcharin-treated embryos in Day 9–10 pseudopregnant uterus

Treatment					No. of embryos at stages*								Somite no.§	
					Presomite		Somitic							
Dose (μg/ml)	Time (h)	No. of decidua analysed	No. (%) with embryos	Day	10	11	12	13	14	15	16	Test *vs* control‡	Mean ± s.e.m. (*n*)	Test *vs* control
Untreated pseudopregnant recipient														
Control		105	73 (70)	9	4	4	13	13	14	0	0	—	10·3 ± 0·8 (40)	—
(untreated)				10	1	0	0	1	3	17	3	—	25·5 ± 1·0 (24)	—
1·0	1	11	8 (78)	9	0	0	1	4	3	0	0	n.s.	12·1 ± 1·4 (8)	n.s.
1·0	6	21	19 (90)	9	0	2	3	2	3	0	0	n.s.	9·4 ± 1·9 (8)	n.s.
				10	0	0	1	0	5	3	0	$P < 0·01$	17·6 ± 1·7 (9)	$P < 0·001$
1·0	24	23	4 (17)[b]	9	1	1	1	0	0	0	0	$P < 0·01$	6 (1)	n.t.
				10	0	0	0	0	0	1	0	n.t.	22 (1)	n.t.
5·0	24	20	1 (5)[b]	9 & 10	One embryo at stage 13							n.t.	10 (1)	n.t.
Treated pseudopregnant recipient (0·2 mg α-momorcharin/25 g body weight)														
Untreated		12	8 (67)	9	0	0	2	2	0	0	0	n.s.	7·5 ± 0·9 (4)	$P < 0·01$
				10	0	0	0	0	1	3	0	n.s.	23·0 ± 1·7 (4)	n.s.
5·0	24	5	0	9 & 10	No viable embryo							n.t.	—	—

* Stages of development: 10 = Amnion formation, egg cylinder; 11 = presomite neural plate; 12 = early-somite (1–7), cranial neurulation; 13 = turning, 8–12 somites; 14 = formation and closure of cranial neuropore, 13–20 somites; 15 = forelimb bud, 21–29 somites; 16 = closure of caudal neuropore, hindlimb bud and tail bud, 30–34 somites. Six malformed embryos (three each from control and experimental groups) were excluded.

† $P < 0·01$ compared with the control value (χ^2 test).

‡ Groups with <3 normal embryos were not tested (n.t.); n.s. = not significantly different from control values by Mann–Whitney test.

§ Groups with <4 embryos were not tested (n.t.); n.s. = not significantly different by Student's *t* test.

Discussion

Our previous study (Law *et al.*, 1983) on the development of α-momorcharin-treated blastocysts showed that the inner cell mass and the trophoblast differentiated poorly in culture. The present

study showed that when these treated blastocysts were transferred back to the uterus, many were incapable of implanting. About 27% of the blastocysts treated with 1 μg α-momorcharin/ml for 6 h had implanted and nearly all formed an embryo. The post-implantation development of these embryos seemed normal up to Day 9 but obvious developmental retardation began by Day 10. The poor development of the α-momorcharin-treated embryo in an untreated uterus could be due to (1) a retardation effect that was secondary to the impaired differentiation of the trophoblast and the ensuing abnormal events of implantation, (2) the toxic effect of α-momorcharin released by the embryo which created a localised unfavourable uterine environment or (3) damage of the inner cell mass leading to abnormal development or failure to develop. When embryos were treated with a higher dose of α-momorcharin for 24 h, only a few embryos were recovered on Days 9 and 10. Most of the implantation sites contained trophoblastic giant cells, fibrotic tissues and extraembryonic membranes. The absence of embryo-derived tissues suggested that cell degeneration may have occurred in the inner cell mass shortly after implantation. Law *et al.* (1983) showed that although over 90% of α-momorcharin-treated blastocysts showed inner cell mass development, there was some histological evidence of cellular damage. A study on the effect of another abortifacient protein, α-trichosanthin, on the rabbit blastocyst showed that ultrastructural changes were found in the inner cell mass cells after incubation with this protein for 24 h (Tseng, Teng, Wu & Chou, 1979). However, the subsequent development of these blastocysts was not followed.

In the present study, the untreated and treated embryos failed to establish themselves in the α-momorcharin-treated uterus. The affected uterus also showed a poor decidual reaction to mechanical stimulation. There was a diminished proliferation of the stromal tissue and the glands in the endometrium. These results therefore suggest that the in-vivo inhibition of early pregnancy could be a direct action of α-momorcharin on the uterine environment making it unfavourable for embryonic implantation. The study has not ruled out the possibility that the poor implantation of untreated embryos in the treated uterus resulted from the embryotoxic effect of α-momorcharin released by the uterus in the immediate vicinity of the embryo. Law *et al.* (1983) found that α-trichosanthin inhibits implantation in a similar fashion to α-momorcharin during early pregnancy. Several studies have shown that the effects of α-trichosanthin on the uterus may be crucial to its abortifacient activity. In the mouse, the protein inhibited the decidual reaction of the pseudopregnant uterus to a mechanical stimulus (Zhou, Li, Shu, Bao & Chu, 1982). The suppression in decidualization was correlated with a decrease in the number and the binding affinity of cytoplasmic receptors for progesterone and oestradiol in the decidual cells (Chu, Zhou & Li, 1982). Circulating concentration of progesterone remained unchanged after protein treatment, suggesting that the insensitivity of the uterine tissue is not a secondary response to luteolysis. In the pseudopregnant rabbit uterus, a decrease in the number and binding affinity of progesterone receptors coincided with an enhanced spontaneous contraction of the uterus (Chu, Zhou & Zhao, 1982). It is therefore likely that the interaction of an unfavourable uterine environment with embryos of impaired developmental potential is the major factor leading to the termination of early pregnancy by α-momorcharin in the mouse.

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