Effects of Retinoic Acid on Morula-stage Embryo Development in Mice

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Background: In a previous study, we investigated the short-term effects of in utero retinoic acid (RA) exposure on early development at the cleavage stage before implantation to understand the possible roles of RA in blastocyst formation. We designed the present study to investigate the long-term effects of RA exposure on pre-implantation embryos *in vivo* and *in vitro*.

Method: To evaluate late pre-implantation exposure to RA, pregnant female mice were fed peanut oil with 50 mg/kg RA by oral gavage in the late afternoon of day 2 and early morning of day 3 of gestation. Mice were sacrificed on day 8. The number and morphology of embryos were recorded. The morula-stage embryos were treated with different doses of *t*-RA for 1 day and were cultured for the following 7 days *in vitro*. The post-implantation development *in vitro* was evaluated.

Results: The *in vivo* study showed that the rate of blastocyst implantation was not significantly different (9.2 vs 10.2 per mouse) and the rate of post-implantation embryo resorption was significantly higher in retinoic acid treated mice than in controls (35% vs 0%). RA was administered to morula-stage embryos at levels of 0, 0.001μ, 0.1μ, or 10μ M throughout *in vitro* culture. For embryos that continued to develop following implantation, the development stages were delayed when assessed in 7-day culture. The percentage of embryos in the later stages of development changed depending on the dose of retinoic acid used.

Conclusion: These findings document, for the first time, that RA exerts adverse effects on morula-stage embryos during the early stage of development.
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Key words: retinoic acid, mice, morula stage embryo, embryology

In a previous study, we investigated the short-term effects of in utero retinoic acid (RA) exposure on early development at the cleavage stage before implantation, to understand the possible roles of RA in blastocyst formation. In a prospective animal study, we plan to investigate the long-term effects of RA exposure on pre-implantation embryos *in vivo* and *in vitro*. It has been shown that *in vivo* and *in vitro* exposure of mice embryos at the morula stage to excess RA results in late adverse effects on subsequent embryo development. Retinoid treatment should be avoided at any stage of gestation.

RA, an active metabolite of vitamin A, has various biological functions. Retinoic acid plays an

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important role in normal embryogenesis and in the differentiation of normal and malignant cells.[1-8] Retinoic acid also participates in pattern formation and organ development during embryo growth,[9-13] but in excess, RA is teratogenic. Abnormalities induced by RA in laboratory animals include limb anomalies and dysmorphogenesis of both the vestibular and auditory portions of the inner ear, as well as craniofacial abnormalities.[14-18] RA is also a valuable compound in the therapy of cystic acne among other numerous dermatologic disorders.[19-21]

In 59 pregnant women exposed to isotretinoin during the first 28 days of gestation, 12 (20%) had spontaneous abortions and 20 out of the 47 (43%) newborns had congenital malformations.[22] Several studies have also demonstrated the potential adverse effects of RA when administered during mid and late pregnancy in mice. Most experiments have also shown that RA has the potential to affect embryo development in mice, with administration of RA on day 7 or day 8 of gestation causing retardation of general development, abnormal differentiation of the cranial neural plate and abnormal development of the hindbrain.[17] Administration of RA on day 9 of mouse gestation induced dysmorphogenensis of the inner ear.[14] Abnormalities of limb and neural plate development were induced when RA was administered between day 10 and day 16 of gestation.[15,16,18] In studying the effects of RA on peri-implantation embryos, we have found adverse effects on post-implantation development *in vivo* and *in vitro* in mouse blastocyts.[23-26] In a previous study, we also investigated the effect of in utero RA exposure on development at the cleavage stage before implantation to understand the possible roles of RA in embryogenesis.[27] The results showed that excess RA did not affect blastocyst formation. However, this only implies that RA may not have a short-term effect on pre-implantation embryos in early mammalian development. We designed the present study to investigate the long-term effects of RA exposure on pre-implantation embryos at the morula stage *in vivo* and *in vitro*.

METHODS

Animals Imperial-Cancer Research (ICR) albino virgin mice were kept under a 12-hour day, 12-hour night regimen, with food and water available *ad libitum*. All animals received humane animal care as outlined in the "Guidelines for care and use of Experimental Animals" (The Canadian Council on Animal Care, 1980). The morning after an overnight mating period, female mice with vaginal plugs were separated and used for the experiment. The day a vaginal plug was found was defined as day 0 of pregnancy. The pregnant mice were maintained on mouse breeder chow and water ad libitum.

Test compound RA was purchased from Sigma (St. Louis, MO, USA). Stock solutions of retinoids were prepared by dissolving RA into ethanol at a concentration of 100 mg/ml for *in-vivo* experiments. A stock solution of RA was then dissolved in peanut oil containing 5% ethanol before use. These solutions were kept in aliquots at –20°C in the dark. For *in vitro* experiments, RA was prepared in an aqueous solution of dimethyl sulfoxide (DMSO).

Collection of mouse morula-stage embryos

ICR mice, 6-8 weeks old, were induced to superovulate by injecting 5 IU pregnant mare serum gonadotropin (PMSG, Organon, 2000 Galloping Hill Road Kenilworth, N.J. 07033-0530) followed by injecting 5 IU human chorionic gonadotropin (HCG, Serono Pharma Schweiz Steinhauserstrasse 74 Postfach) 48 h later. The day a vaginal plug was found was defined as day 0 of pregnancy. The pregnant mice were maintained on mouse breeder chow and water *ad libitum*. Morula-stage embryos were obtained by flushing the uterine horn and fallopian tubes in the late afternoon of day 2 of gestation with Connaugh Medical Research Laboratories (CMRL-1066) culture medium (Gibco Laboratories, 1600 Fareday Avenue, Carisbad, California, 92008) containing 1 mM glutamine and 1 mM sodium pyruvate. The embryos were collected in uncoated plastic 35 mm Falcon culture dishes and washed a minimum of three times. Embryos from different females were pooled and selected randomly for the various experiments.

Embryo culture

For assessment of blastocyst formation, embryo implantation and further embryonic differentiation, morulas were cultured *in vitro*, using a method modified from that of Hsu (1979).[28] Incubation was carried out at 37°C in 5% CO_2 and 95% air. The embryos were cultured in 1 mL of culture medium in

four-well multidishes (Nunc, Roskilde, Denmark) at 37°C in 5% CO_2 in air. For group culture, 5 embryos were cultured per well. CMRL 1066 (Gibco) was used as the basic culture medium. It was supplemented with 1 mM glutamine and 1 mM sodium pyruvate plus 50 IU/ml penicillin and 50 mg/ml streptomycin. During the first 4 days the culture medium was supplemented with 20% fetal calf serum (FCS) (Gibco) and thereafter with 20% heated-inactivated human placental cord serum (HCS). After the first 3 days, when the embryos had attached, fresh medium was renewed daily until the eighth day of cultivation. Embryos were inspected daily under a dissecting microscope and classified according to the method of Witschi (1972).[29] In the following 5 days, developmental parameters, such as hatching through the zona pellucida, attachment to the culture dishes, trophoblastic outgrowth, and differentiation of the embryo proper into early or late egg cylinders (germ layer stage) were recorded daily. Embryonic development was observed through a phase-contrast microscope (Olympus, Shinjuku Monolith, 2-3-1 Nishi-Shinjuku Shinjuku-Ku, Tokyo 163-0914). To decrease observer bias, all data were analyzed using the following criteria.[23] Attachment was defined when a blastocyst was attached to the culture dish by day 3. An early egg cylinder (EEC) embryo was defined as an embryo that had reached stage 9 or 10 by day 5. A late egg cylinder (LEC) embryo was defined as an embryo that reached stage 11, 12 or 13 by day 7 of culture (Fig. 1).

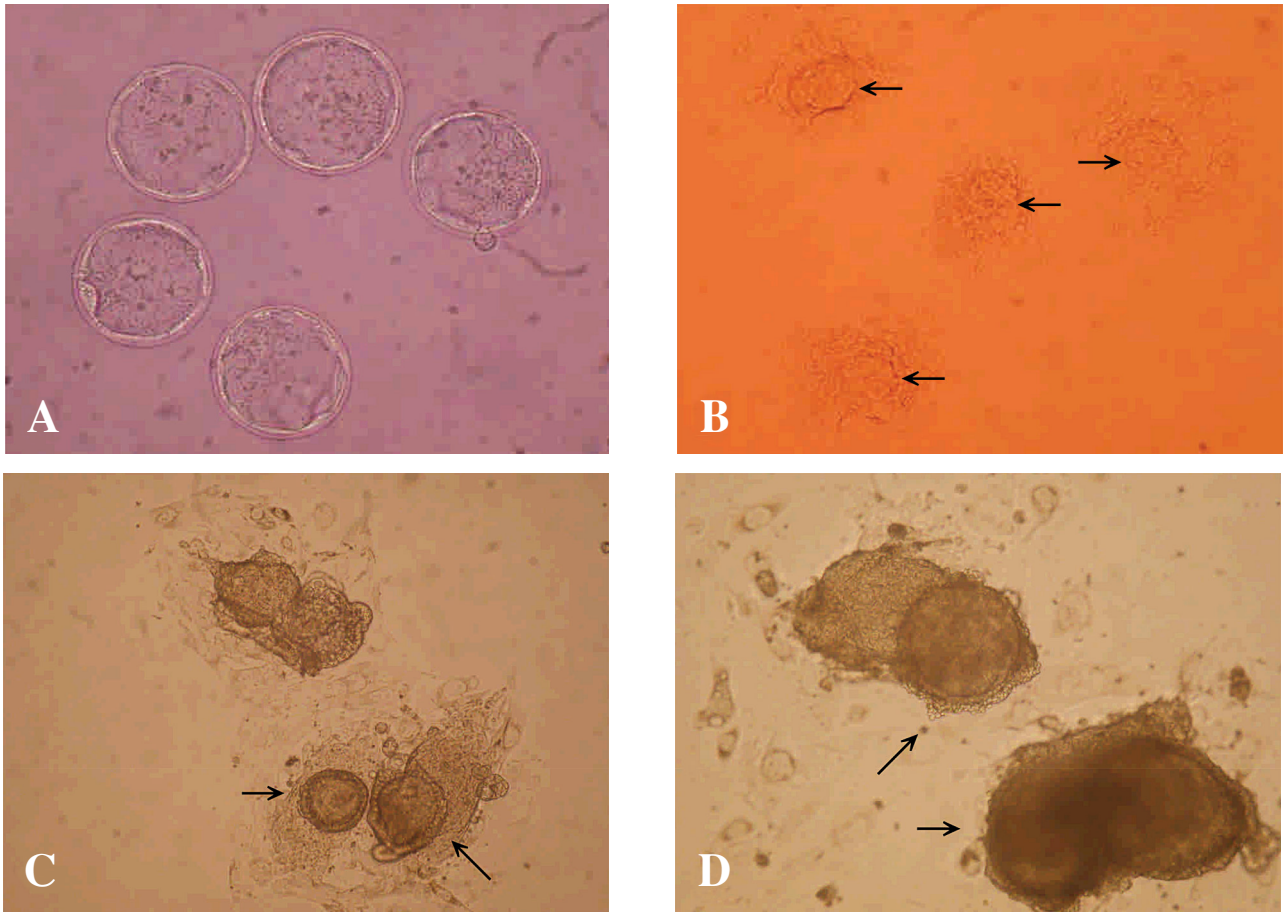

Fig. 1 The sequence of mouse embryo development *in vitro* from blastocyst to the advanced stage. (A) Blasotcysts with zona pellucida. (B) Implanted blastocysts left an inner cell mass (Arrow) on the outgrowth trophoblasts. (C) Early egg cylinder with the inner cell mass protruding from the outgrowth trophoblasts which formed a small cavity in the center (gastrula) (Arrow). (D) Late egg cylinder with primitive streak or neurula (Arrow). Original magnification: 100×.

Experimental design 1

To assess the *in-vivo* effect of RA administered during the pre-implantation period, one treatment group of pregnant female mice received 0.2 ml of peanut oil containing 50 mg/kg RA by oral gavage on the late afternoon of day 2 and early morning of day 3 of gestation. Control pregnant female mice received an equivalent dose of alcohol alone in peanut oil on the same days of gestation. Mice were sacrificed by cervical dislocation on day 8 of gestation. The numbers of implantation sites and resorptions (early and late) in each uterine horn were recorded. Then, the uterine horns were opened and each embryo was explanted to assess the anomalies and developmental stages according to Witschi's classification under a dissecting microscope.[29] Morphologically normal head-fold embryos at Witschi's stage 15 were considered appropriate for day 8 of gestation.

Experimental design 2

In order to investigate the *in-vitro* effects of retinoic acid on implantation and post-implantation development, morula-stage embryos were randomly assigned into four dose groups, and cultured with 20% fetal bovine serum in CMRL-1066 media for the first 4 days and 20% human cord serum for the next 4 days in the presence of 0, 0.001μ, 0.1μ, or 10 μM RA.

Statistical analysis: All statistical comparisons were made between treatment and control groups. Differences in the numbers of mice with developing deciduas, with complete resorption of deciduas or without any deciduas and differences in the number of embryos at different stages of development were analyzed using Fisher's exact test. The data were presented as the percentage of mice or embryos. Implantation sites and developing decidua per mouse were analyzed using Welch's t-test. The data were presented as the mean ± standard error of the mean. Fisher's exact test was also used to compare the developmental rates to attachment, EEC, and LEC stages according to culture day. Differences of $p < 0.05$ were considered to be significant.

RESULTS

Effect of RA administered to morula-stage embryos *in vivo*

Twenty mice embryos were administered RA during the morula stage (day 2-3 of gestation) and fifteen control mice were treated with vehicle alone. All mice were sacrificed on day 8 of gestation (Table 1). There was no difference in the number of implantation sites per mouse (9.2 vs 10.2) or in the percentage of mice with no implantation sites between the RA treated and the control groups. The number of mice with visible implantation sites but no decidua formed was significantly higher in the RA treated group than the controls (7/20 vs 0/15). As a consequence of increased resorption, the percentage of mice with developing decidua was significantly lower in the RA treated group (55 % vs 93 %).

The effects of RA administered on day 2-3 of gestation on subsequent embryo development in embryos recovered from the decidua on day 8 of gestation are summarized in Table 2. All stages of embryo development *in vitro* were significantly affected by RA ($p < 0.001$). In the RA-treated mice, only 28 embryos were found to be at Witschi stage 15 compared to 82 embryos in 15 control animals. Among the embryos recovered from the deciduas, the percentage of embryos reaching stage 15 in RA treated mice was also significantly lower than in the controls (19.8% vs 57.7%).

Dose effect of RA throughout *in-vitro* culture

Embryos were cultured in regular medium with 0, 0.001μ, 0.1μ, or 10 μM RA. A total of 200 morula-stage embryos were evaluated for their development with different doses of retinoic acid. As indicated in Fig. 2, the percentage of embryos that reached

Table 1. Effects of Retinoic Acid Administered on Day 2 and 3 of Gestation in Mice

	Treated mice	Control mice	*p* value
No. of mice with vaginal plug	20	15	
No. of mice with developing decidua (%)	11 (55)	14 (93)	0.956
No. of mice with complete resorption of decidua (%)	7 (35)	0 (0)	0.012
No. of mice without decidua (%)	2 (10)	1 (7)	0.727
Implantation sites per mouse	9.2 ±2.4	10.2 ±1.8	0.234

Table 2. Effects of Retinoic Acid Administered on Day 2 and 3 of Gestation on Embryo Development

	Treated mice	Control mice	*p* value
Total no. of developing decidua	101	142	
Mean no. of developing decidua per mouse	9.2 ±1.4	10.1 ±1.6	0.86
Stage of development			
Stage 15 embryos (%)	20 (19.8)	82 (57.7)	< 0.001
Stage 14 embryos (%)	67 (66.3)	51 (35.9)	< 0.001
≤ Stage 13 embryos (%)	14 (13.9)	9 (6.4)	0.073

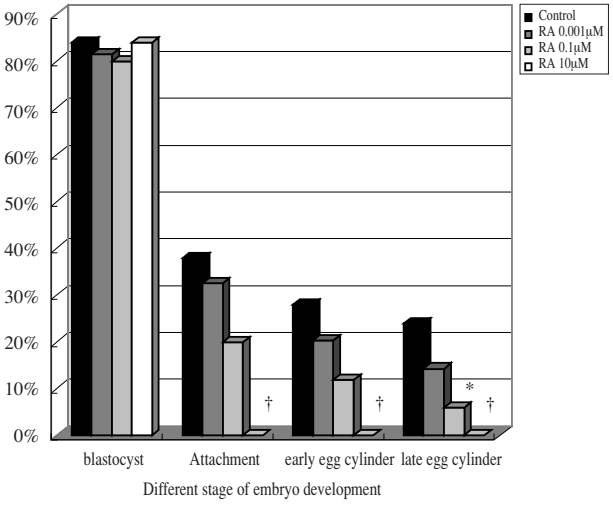

Fig. 2 Impact of retinoic acid on the embryo development of mice morula exposed for 24 h to increasing concentrations of retinoic acid and subsequent 7-day culture. Data are given as percentages of embryos reaching different stages and are based on at least 50 values in the control group, 49 values in the 0.001μ group, 50 values in the 0.1μ group and 51 values in the 10μ group. * and † represent statistical differences between treated embryos and control embryos at $p < 0.05$ and $p < 0.001$ levels, respectively.

the late stages of development decreased with increased doses of retinoic acid. Furthermore, treatment with 10μM RA resulted in failure in implantation and post-implantation development so that by the end of 3rd day of culture, no embryos had developed to the early or late egg cylinder stage.

DISCUSSION

The present study showed that RA exposure *in vivo* at the morula stage of gestation did not affect the implantation rate, but affected post-implantation embryo development. The immediate effect of RA on blastocyst formation was not evident because the implantation rate was similar in the treatment and control groups. However, the late effects of RA were significant at the post-implantation stage because smaller numbers of mice developed deciduas and a lower percentage of embryos reached Witschi stage 14 to 15.

The dose effects of retinoic acid were assessed throughout *in-vitro* culture in order to determine the actual effects of retinoic acid on blastocyst formation, implantation and subsequent embryo development. The most striking findings were that RA exposure at the morula stage affects implantation and post-implantation embryo development in a dose dependent manner. The rate of blastocyst formation from the RA-treated morula did not change, but the subsequent implantation was significantly affected at doses of 0.1 μM or 10 μM. However, the implantation of embryos was not affected by RA exposure *in vivo*. The findings *in vitro* seem to be different from the data *in vivo*. The differences in the implantation rate were possibly due to a more direct impact of RA on morula *in vitro* than *in vivo*. The *in vivo* impact was possibly compensated for by the maternal environment. However, adverse effects of RA on subsequent embryo development after implantation were shown by both the in vivo and in vitro experiments. These findings apparently demonstrate that late effects of RA on morula-stage embryos occur significantly at the subsequent post-implantation stage. Immediate effects of RA were not shown in the study because of normal blastocyst formation in RA-treated morula.

Since RA is an important morphogen inducing pattern formation, it is reasonable to conclude that excess RA may disrupt normal development and lead to serious retardation, as demonstrated in the present study. In previous studies the adverse effects of RA were found to be dose-dependent during the different stages of embryo development. Mouse embryos exposed to excess RA (12 mg/kg) on day 7 of development showed retardation of general development,

abnormal differentiation of the cranial neural plate and abnormal development of the hindbrain.[17] The morphological features of embryos from treated mice were characterized by reduced somite numbers, reductions in pharyngeal arch size and number, a rostrally displaced otocyst, and delayed closure of the anterior neuropore, as well as retardation of heart development. Maternal exposure to RA (20 mg/kg) on day 9 of gestation has been found to induce dysmorphogenensis of the inner ear in mouse embryos.[14] Similarly, a congenital limb anomaly was induced following exposure to a non-physiological level of RA (120 mg/kg) on day 10 and day 11 of gestation in pregnant Swiss-Webster mice.[15,16] In our previous study we showed that RA affected the germ layer and subsequent neurula development from day 3 to day 8 of gestation.[23-26] These findings suggest that embryos at different stages have different tolerances for increases in RA concentration. RA suppressed mesodermal gene expression in mouse embryonic stem cells[8] and induced endodermal specific gene expression in F9 embryonal carcinoma cells.[6] These findings suggest that the teratogenic effects of RA on early post-implantation embryos may be mediated by disrupting germ layer specific gene activities. However, RA also induced apoptosis in the inner cell mass of mouse blastocysts.[25]

Retinoids have been used in the treatment of a variety of hyperproliferative diseases in humans, including acute promyelocytic leukemia, squamous cell carcinoma of the skin, cervical intraepithelial neoplasia, bladder papilloma and leukoplakia of the oral cavity and larynx.[30-33] Isotretinoin (13-cis-retinoic acid) is an effective therapy for cystic acne and other dermatologic disorders.[19-21] Unfortunately, retinoids are highly teratogenic in humans even in the therapeutic dose range of 0.5 to 1.5 mg/kg/day. The major malformations found among isotretinoin-exposed infants involved the cranium and face, heart, thymus, and brain.[22]

Although 50 mg/kg of RA and even higher doses of RA (100 mg/kg) did not adversely affect blastocyst formation from early or late pre-implantation embryos, the late adverse effects of 50 mg/kg of RA in late pre-implantation embryos had not been investigated in previous study.[27] In this study, we showed that *in vivo* and *in vitro* exposure of mice embryos at the morula stage to excess RA results in late adverse effects on subsequent embryo development. Retinoid treatment should be avoided at any stage of gestation.

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維他命 A 衍生物對老鼠桑椹期胚胎的影響

黃富仁

背 景： 在我們的前期研究中，已顯示過量的維他命 A 衍生物作用在小鼠的桑椹期胚胎，並不會造成著床前囊胚期胚胎發育的負面影響。此研究將進一步探討維他命 A 衍生物，對桑椹期胚胎著床後胚胎發育的影響。

方 法： 在此前瞻性的小鼠體內研究，維他命A 衍生物的給予劑量爲 0 毫克／公斤與 50 毫克／公斤兩組；在第八天下午收集胚胎觀察並記錄結果。在小鼠的體外研究，收集桑椹期胚胎分別在不同維他命A 衍生物濃度下 (0, 0.001μ, 0.1μ, 10μM) 作用一天後再培養七天，觀察胚胎發育並記錄結果。

結 果： 不同劑量的維他命 A 衍生物作用在桑椹期胚胎，並不影響著床前囊胚期胚胎的形成。但它會影響囊胚期胚胎著床後的發育。

結 論： 此研究發現過量維他命 A 衍生物作用在小鼠桑椹期胚胎，雖然沒有造成胚胎短期傷害，卻造成了囊胚期胚胎著床後的長期傷害。

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關鍵詞：維他命A 衍生物，小白鼠，桑椹期胚胎，胚胎發育學

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