Influence of the anti-androgen hydroxyflutamide on *in vitro* development of mouse embryos

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Hydroxyflutamide is a potent nonsteroidal anti-androgenic drug extensively used in laboratory investigations. Our present studies were aimed at determining whether this anti-androgen modulates development of embryos *in vitro*. One-cell and two-cell mouse embryos were collected by flushing the oviducts and cultured in defined culture media in the presence or absence of various doses of hydroxyflutamide. The development of embryos from one-cell and two-cell to blastocyst stage was assessed. Hydroxyflutamide caused a dose-dependent ($0\text{--}100\text{ }\mu\text{g ml}^{-1}$) inhibition of development of both one-cell and two-cell embryos; 100% inhibition was observed at $20\text{ }\mu\text{g ml}^{-1}$. The adverse effects of hydroxyflutamide on two-cell embryo development were completely reversed by testosterone in a dose-dependent manner, but not by oestradiol and progesterone. These results indicate that the anti-androgen hydroxyflutamide inhibits early embryo development suggesting that it may be useful during the preimplantation period for preventing conception.

Introduction

Hydroxyflutamide is a potent, nonsteroidal anti-androgen that has been reported to lack other agonistic or antagonistic hormonal properties (Neri *et al.*, 1972). It binds to androgen receptors and competitively inhibits the binding of testosterone and dihydrotestosterone. This compound has therefore been widely used as an anti-androgen in various studies (Peluso *et al.*, 1980; Clark and Nowell, 1980; Krey *et al.*, 1982; Chandrasekhar *et al.*, 1988; Armstrong *et al.*, 1989). Recent studies from our laboratories indicated that hydroxyflutamide blocks ovulation (Opavsky *et al.*, 1987) through interference with the LH surge at pro-oestrus and is reversed by the administration of LHRH (Chandrasekhar and Armstrong, 1988) or progesterone on the day of pro-oestrus (Chandrasekhar and Armstrong, 1989). In addition, hydroxyflutamide delays the initiation of implantation, fetal development and parturition in pregnant rats and suppresses decidualization after artificial stimulation of the sensitized uterus (Chandrasekhar *et al.*, 1990). The delay in the initiation of implantation by hydroxyflutamide may be due to its effects on embryo development in addition to its effects on uterine receptivity.

The role of androgens in intrafollicular oocyte and follicular maturation, ovulation and embryo development is not well understood. The aim of the present experiments was to determine the effects of hydroxyflutamide on development of mouse embryos *in vitro*. One- and two-cell embryos were cultured in defined culture media in the presence or absence of various doses of hydroxyflutamide, and the development of embryos was assessed. Our results indicate that hydroxyflutamide inhibits embryo development *in vitro*.

Materials and Methods

Materials

Ham's F-10 culture medium was obtained in powder form from Gibco Laboratories (Grand Island, NY). The media were prepared using Milli-Q-water, adjusted to 278–282 mOsm, filter sterilized and stored at 4°C for up to 1 month before use. Dulbecco's phosphate-buffered saline (PBS) was used for embryo collection and was prepared from powder (Gibco). Hydroxyflutamide was a gift from R. O. Neri (Schering Corp.; Bloomfield, NJ). Progesterone, oestradiol, testosterone, hCG and BSA were purchased from Sigma (St Louis, MO).

Embryo collection and culture

Four-week-old female and 12-week-old male mice ($B_6C_{3F_1}$) were purchased from Harlan-Sprague Dawley (Houston, TX). Animals were housed under a 12 h light:12 h dark regimen for at least 4 days before use. Female mice were superovulated by i.p. injection of 25 iu pregnant mares' serum gonadotrophin (Sigma) followed, after 48 h, by 25 iu hCG. After hCG injection, two females were caged with one male and allowed to mate. Mating was confirmed by the presence of a vaginal plug and females were killed between 10:00 and 14:00 h on day 1 of pregnancy (one-cell embryos) or between 08:00 and 10:00 h on day 2 (two-cell embryos) and embryos were flushed from the oviduct with sterile PBS with 0.3% BSA, using a 30-gauge needle on a 1 ml syringe. One-cell embryos (with two pronuclei) and two-cell embryos were pooled from several mice with a mouth-operated micropipette and washed through four 1 ml changes of equilibrated Ham's F-10 medium with 0.3% BSA. Embryos were allocated randomly to the experimental

Fig. 1. Development of embryos following culture of one-cell embryos for 96 h in Ham's F-10 medium with various doses of hydroxyflutamide. Bars indicate the mean percentage of embryos developed in each treatment group. Error bars represent the variation between replicate incubations. * $P < 0.01$ (χ^2 test) compared with control.

Fig. 3. Dose-dependent effects of testosterone on embryo development in the presence of 20 µg hydroxyflutamide ml⁻¹. Each bar represents the mean percentage of embryos developed; error bars indicate the variation between replicate incubations. Embryos were cultured without (□) or with 20 µg hydroxyflutamide ml⁻¹ (■) in the presence of various concentrations of testosterone. * $P < 0.01$ (χ^2 test) compared with control.

Fig. 2. Development of embryos following culture of two-cell embryos for 72 h in Ham's F-10 medium with various doses of hydroxyflutamide. Bars indicate the mean percentage of embryos developed in each treatment group. Error bars represent the variation between replicate incubations. * $P < 0.01$ (χ^2 test) compared with control.

treatment groups from the pooled one-cell or two-cell embryos. Stock solutions of hydroxyflutamide, prepared in ethanol, were added to culture medium to give various concentrations (0–100 µg ml⁻¹) of hydroxyflutamide but with a constant

Fig. 4. Modulation by steroid hormones of the effects of hydroxyflutamide on the development of two-cell embryos cultured for 72 h in Ham's F-10 medium. The medium contained testosterone, oestradiol or progesterone at 50 µg ml⁻¹ or vehicle (■) with or (□) without 20 µg hydroxyflutamide ml⁻¹. Bars indicate the mean percentage of embryos developed in each group with error bars representing the variation between replicate incubations. * $P < 0.01$ (χ^2 test) compared with the respective control.

ethanol concentration (0.05%), immediately before use. Controls received the same concentration of ethanol. In some experiments two-cell embryos were incubated with 2, 10 or 50 μg testosterone ml^{-1} , 50 μg oestradiol ml^{-1} , 50 μg progesterone ml^{-1} in the presence or absence of 20 μg hydroxyflutamide ml^{-1} . Embryos were cultured in 50 μl drops under autoclaved mineral oil (Sigma) that had been equilibrated in 5% CO_2 before use. Ten to twenty embryos per drop were maintained at 37°C under 5% CO_2 in air. Triplicate cultures were established within each individual experiment and each experiment was repeated three to seven times. Embryos were cultured for 72 or 96 h and scored, daily up to four days for progression to two-cell, four-cell, morula and blastocyst stages. Scoring of embryos was carried out in a blind fashion by a single observer for all the experiments.

Statistical analysis

The percentage of embryos that had developed to the blastocyst stage in control and various treatment groups were compared using χ^2 analysis.

Results

The presence of hydroxyflutamide in the culture media significantly ($P < 0.01$) reduced the percentage of embryos developing to blastocysts; the effects were dose dependent. Development of both one-cell and two-cell embryos were affected similarly by hydroxyflutamide (Figs 1 and 2). The percentage of embryos developing to blastocysts was reduced in the presence of 1–10 μg hydroxyflutamide ml^{-1} and the development was totally arrested at 20–100 μg hydroxyflutamide ml^{-1} . In the embryos exposed to 20 μg hydroxyflutamide ml^{-1} and higher doses, the development was arrested at the two-cell stage. The presence of ethanol did not alter the development of one-cell or two-cell embryos.

We next investigated whether the hydroxyflutamide-induced arrest of embryo development could be reversed by steroid hormones. Testosterone reversed the inhibition by 20 μg hydroxyflutamide ml^{-1} in a dose-dependent manner. Complete reversal of the inhibition was observed in the presence of 50 μg testosterone ml^{-1} (Fig. 3). In addition, the ability of oestradiol and progesterone, at 50 μg ml^{-1} , to rescue mouse embryos from development arrest caused by 20 μg hydroxyflutamide ml^{-1} was also tested. Oestradiol was ineffective in restoring the embryo development (Fig. 4). However, progesterone had a positive effect; about 35% of the embryos developed to blastocysts (Fig. 4). The embryo development, in the absence of hydroxyflutamide (Fig. 4), was unaffected in the presence of testosterone, oestradiol or progesterone; the percentage of embryos developing from the two-cell to blastocyst stage was similar to that without steroids.

Discussion

This report is the first direct evidence of inhibition of embryo development by the anti-androgen hydroxyflutamide. Hydroxyflutamide arrested the embryo development of both one-cell and two-cell embryos. The inhibition was dose dependent,

and developmental arrest was obtained at a dose of 20 μg ml^{-1} . The adverse effects of hydroxyflutamide were reversed by testosterone in a dose-dependent manner, but not by oestradiol and progesterone, suggesting a role for androgens in embryo development.

Our previous studies demonstrated that hydroxyflutamide when injected daily on day 4, 5 and 6 of pregnancy in rats causes a delay in implantation of embryos (Chandrasekhar *et al.*, 1990; Chandrasekhar, 1991). The delay in implantation of embryos in these studies (Chandrasekhar *et al.*, 1990) appeared to be due to the effects of hydroxyflutamide on the uterus, as unimplanted embryos, flushed from the uterus on day 6 of pregnancy, were at the blastocyst stage and when hydroxyflutamide treatment was terminated on day 6, these blastocysts went on to implant. In these studies (Chandrasekhar *et al.*, 1990), hydroxyflutamide treatment was started on day 4 of pregnancy, when embryos would be at the blastocyst stage. The fact that these blastocyst-stage embryos were viable during hydroxyflutamide treatment (Chandrasekhar *et al.*, 1990) suggests that this anti-androgen does not affect the ability of the embryo to implant. However, in the study reported here the early stages of embryo development were significantly arrested by hydroxyflutamide. Data from this study together with our previous studies (Chandrasekhar *et al.*, 1990; Chandrasekhar, 1991) raise the possibility that hydroxyflutamide may be used as an antigestational drug.

From these earlier studies *in vivo* (Chandrasekhar *et al.*, 1990) it was unclear whether hydroxyflutamide had direct effects on embryo viability and its development. Results from the present *in vitro* study support the contention that hydroxyflutamide inhibits development of embryos, possibly through its direct actions on the embryo. The ability of testosterone, but not oestradiol and progesterone, to completely reverse the developmental arrest of the embryos induced by this compound indicates that these effects are specific to anti-androgenic properties of hydroxyflutamide. Although progesterone and oestradiol are required *in vivo* for complete embryo development *in utero* (Roblero, 1973; Roblero and Garavagno, 1979), there is no convincing evidence for direct effects of oestradiol and progesterone on preimplantation embryo development. Several workers (Paria and Dey, 1990; Mattson *et al.*, 1988; Colver *et al.*, 1991) have suggested roles for various growth factors in the development of preimplantation embryos. Our present studies provide evidence for a direct influence of an anti-androgen on embryo development.

In summary, we demonstrated that hydroxyflutamide inhibits preimplantation embryo development. The effects of this anti-androgen were reversed by testosterone in a dose-dependent fashion, but not by oestradiol and progesterone. These findings may have important clinical implications in the development of antigestational drugs for early embryo development.

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