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Tamoxifen Inhibits the Migration of the Primordial Germ Cells of the Chick Embryos with Less Mortality Rate

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Abstract: The chick embryos, after 24 h of incubation were treated in front of the head area with three different doses of tamoxifen (100, 200 or 400 µg). There were no effects of the tamoxifen on the low dose group while it caused around 16% of the sterility of embryos in other treated groups. Embryos of higher treated groups showed more primordial germ cells (PGCs) remaining in the area of germinal crescent (GC) compared with zero cells in the control group. Also there was a significant reduction in the number of PGCs in the gonad area of the treated group as compared with the control group. So, tamoxifen prevented the migration of PGCs from GC possibly by occupying calcium-binding sites on calmodulin, or by inhibiting the gene expression of the microtubule formation in PGCs and caused the sterilization of the chick embryos. Tamoxifen had less side effects causing only~ 0.07% embryo abnormalities, thus can be used for producing transgenic chick embryo by PGCs transfer.

Key words: Primordial germ cells (PGCs), tamoxifen, chick embryo, germinal crescent, gonads

Introduction

The primordial germ cells (PGCs), that will produce the gamete later on, are formed outside the gonads. The first appearance of the PGCs is during the primitive streak formation, stage 4 (Hamburger and Hamilton, 1951). They arise from the germinal crescent (GC) in front of the head fold of the early chick embryo (Nieuwkoop and Sutasurya, 1979; Eyal-Giladi *et al*., 1981). From stage 12 at around 56 h post incubation (PI) the PGCs start their migration to the extra-embryonic blood vessels then they enter the vascular blood system and by stage 20-24 they migrate into the presumptive gonads ((Meyer, 1964; Fujimoto *et al*., 1976a; Eyal-Giladi *et al*., 1981; Al-Thani and Simkiss, 1991; Watanabe *et al*., 1994). The PGCs move by the pseudopodia which are formed by the translation of mRNA into the skeletal protein (micro tubules) inside the cell (Fujimoto *et al*., 1976b, 1985 ; Urven *et al*., 1989; Ukeshima *et al*., 1991; Wylie and Heasman, 1993; Kuwana, 1993). The PGCs attracted considerable attention since ablation of the cells in GC by microsurgery (McCarrey and Abbott, 1978, 1982; Aige-Gil and Simkiss, 1991a), by ultraviolet light (Aige-Gil and Simkiss, 1991a); also by laser irradiation (Mims and McKinnell, 1971) or by chemosterilant drug busulphan (Aige-Gil and Simkiss, 1991a). Unfortunately use of these techniques on a very young embryo produces a large number of malformations while in older embryos it gives a low efficiency of sterilization since the region containing the PGCs spreads over a large and more diffused area (Aige-Gil and Simkiss, 1991b).

Tamoxifen :{ (Z)-1-[4-[(dimethylamino)ethoxy]phenyl]-1,2-diphenyl -1-butene} is anti-oestrogen drug widely used for adjuvant therapy in the treatment of women with oestrogen receptor (ER) -positive breast tumours. Moreover it has a low incidence of serious side-effects (Legha and Carter, 1976; Mouridsen *et al*., 1978 ; Jordan, 1984, 1998; Jordan and MacGregor, 1998; Levenson and Jordan, 1998; White, 1999). The chronic tamoxifen treatment reduces the fertility of the male rat, weight of accessory sex glands, serum lutinizing hormone and testosterone levels without altering the potency or sperm counts (Gill-Sharma *et al*., 2001). It has been postulated that mammalian embryos produce oestrogen, required for their development (morula-blastocyst transformation) and implantation (Dikmann and Dey, 1974; Dikmann *et al*., 1976; Wu and Lin, 1982). The role of oestrogen in early embryonic development is reviewed by Niemann *et al*. (1989). Tamoxifen has been shown to function as a calcium- binding site on calmodulin (Lam, 1984; Lipton and Morris, 1986; Sato, 1990; Edwareds *et al*., 1992). Calmodulin is found in the pre-centriolar region of the mitotic spindle and it is needed for formation of spindle apparatus (Welsh *et al*., 1979; Geiser *et al*., 1993). Tamoxifen has also been shown to activate the specific kinases and phosphatases (Issanduo *et al*., 1990). Thus the ability of tamoxifen to interfere with the formation of calcium-calmodulin complex alters cell cycle kinetics in somatic cells (Osborne *et al*., 1983; Francavilla *et al*., 1989). Tamoxifen also induces alteration in meiotic maturation and cytogenetic abnormalities in mouse oocytes and one cell zygote (London and Mailhes, 2001).

The aim of this study was to test the hypothesis that the tamoxifen might prevent the migration of PGCs with less side effects on the mortality of chick embryos. Present work will be helpful in future studies involving the transfer of PGCs to produce transgenic chick embryo.

Materials and Methods

Specimens: Eggs of white leghorn were obtained from the College of Agriculture, King Saud University. They were incubated at 39°C and 60% relative humidity for 24 h. before treatment so that they have reached about the end of primitive streak stage by that time.

Experimental treatments: Tamoxifen: {(Z)-1-[4-[(dimethylamino) ethoxy]phenyl]-1,2-diphenyl - 1-butene} was obtained from Sigma Chem. Co. USA, suspended in olive oil (10-20 µg/µl). Doses of 100, 200 or 400 µg were given to each egg in volume of 10 or 20 µl. Control groups were treated with the equal volume of vehicle, olive oil only. The injections were administrated by applying the solution directly to the surface of the embryo after making a hole in eggshells. After that the holes were sealed by paraffin wax and the eggs were returned to incubator.

Embryo studies: To count the number of PGCs in area of GC and the mid-gut, the embryos were examined at 3rd day (72 h PI). Embryos were fixed in Rossmans fixative and stained by Periodic acid Schiff reagent method. Whole mounts or sections were prepared. To count the PGCs in the gonad area the embryo were dissected at day 10 of incubation. Then gonads were fixed in Rossmans fixative, sectioned and stained with eosin and haematoxylin.

The index of sterility (IS) as proposed by Aige-Gil and Simkiss (1991a) were used according to the following equation:

$$IS = N - X / N$$

Where N, is the number of PGCs in the control and X, is the PGCs of treated embryos.

Statistical analysis: The statistical analysis of the data was done by simple t-test and Chi-square analysis (X^2) to compare each two

Table 1: The effect of tamoxifen on the mortality and the migration of primordial germ cells of the chick embryos injected with a single dose of 100, 200 and 400 µg

Dose injected (µg)	No. of eggs	Mortality %	PGCs in Germinal Crescent	PGCs in mid gut	PGCs in gonads	PGCs in male#	PGCs in female !	Sterility %
100	Treated (142)	24.46	0.4± 0.14	1.4± 0.26	159.9± 10.4	227.4± 41.5	123.4± 6.7	zero
	Control (125)	20.80	0.2± 0.2	2.6± 0.5	128.4± 12.3	153.2± 21.5	103.6± 10.0	
200	Treated (115)	28.69**	1.0± 0.29	2.2± 0.24*	171.85± 6.7**	183.7± 13.4	159± 8	16.08
	Control (123)	22.76	0± 0	4.8± 0.66	204.8± 9.73	227.8± 20.3	181.8± 3.3	
400	Treated (141)	39.01**	2.4± 0.26*	2.9± 0.37*	154.55± 7.2**	180.5± 14.9	128.4± 6.0**	16.36
	Control (149)	20.13	0± 0	8.6± 1.2	184.8± 9.8	198.2± 17.3	171.4± 10.0	

PGCs, mean number of primordial germ cells; #, both testes; !, left ovary only, *P< 0.001; ** P < 0.05 as compared with the same control group

means of every treatment group. All calculations were done by computer using program INSTAT.

Results

The mortality rate increased with increase in the dose: 24.64, 28.69 and 39.09% embryos were died after the injection of 100, 200 and 400 µg tamoxifen respectively which are significantly higher (P< 0.05) than control groups (20.13%) (Table 1). Although the high dose of tamoxifen caused more mortality rates, but it resulted into very low abnormal effects in the development of embryos (0.07%), which included the finger less or finger fusion or excencephaly in embryos treated with 400 µg drug.

The migration of PGCs is also affected by the treatment with tamoxifen. Treatments with 400 µg tamoxifen prevented the migration of PGCs and they remained in the GC area with a mean of 2.4± 0.26 cells/embryo, which is highly significant (P < 0.001) as compared with zero cells in control embryos (Table 1). Moreover, in the area of mid gut at day 3, the control embryos showed higher cell numbers 4.8± 0.66 and 8.6± 1.2 as compared with 2.2± 0.24 and 2.4± 0.26 in treated embryos injected with the doses of 200 and 400 µg respectively. In gonad area, at the age of ten days also the control embryos showed higher PGCs number (204.8± 9.73; 184.8± 9.8) than the high doses (200 and 400 µg) treated embryos (171.85± 6.73; 154.55± 7.25 PGCs respectively) which are statistically significant (P< 0.05). The comparison of the PGCs number in the male and female gonads (Table 1) indicated that there were no side effects on distribution of PGCs between the two sexes. However the number of PGCs varies in the control and treated ovaries (P< 0.05) as ovaries in control had 171.4± 10.4 whereas 400 µg treated ones had 128.04± 6.02 PGCs. The over all mortality rate of the treated embryos was 30.9% (127/398) while that of total control groups, was 21.15% (84/397). The sterility rate caused by 200 and 400 µg tamoxifen was around 16% of the PGCs number arrived in gonads.

Discussion

The control over the development of reproductive system and the migration of PGCs to gonads have got considerable interest because of its implication for both manipulation (Gordon, 1990) and sexual differentiation (McLaren, 1988). Such studies have explained some of the reasons of sterility and reduction of the PGCs in gonads (Mims and Mckinnell, 1971; Lee *et al.*, 1978; Aige-Gil and Simkiss, 1991a and Tajima *et al.*, 1998). However, the chemosterilization and radiation techniques caused very high mortality and many abnormalities in the early developed embryos. The increase of the tamoxifen dose (200 and 400 µg) increased the mortality rate of chick embryos with less side effects and produced about the same sterility (16%). The implications of these results will enhance the possibility to avoid certain diseases by gene therapy, or for the production of transgenic embryo by transfer of PGCs.

The results of the present study supported hypotheses that tamoxifen prevents the migration of PGCs by acting as a calcium and calmodulin antagonist and occupying the calcium-binding sites on calmodulin (Lam, 1984; Lipton and Morris, 1986; Sato, 1990; Edwareds *et al.*, 1992) which is utilized in mitotic spindle of PGCs. Alternatively acting as anti oestrogen receptors (Wade and Powers, 1993) it could inhibit the gene expression of the microtubule formation in PGCs and might prevent the migration of PGCs from the GC and ultimately causes the sterilization of chick embryos. Similarly, Pareia *et al.* (1984) reported the inhibition of morulae to blastocyst transformation in mammalian embryos. So, it is inferred that tamoxifen prevented the migration of PGCs from the (GC) possibly by occupying the calcium-binding sites on calmodulin, or by inhibiting the gene expression of the microtubule formation in the PGCs and caused the sterilization of the chick embryos. Further, it is concluded that tamoxifen with a single dose does interfere with the migration of the PGCs with less side effects in the development of the avian embryos.

The results of this study indicated that tamoxifen could be used for chemosterilization of the chick embryo (200 to 400 µg doses) with less embryo abnormality and mortality. Therefore, it is suggested that tamoxifen treatment can be used for manipulating the chick embryo to produce transgenic experimental birds in future.

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