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行政院國家科學委員會補助專題研究計畫成果報告

過量攝食植物性雌激素 genistein 對小鼠卵泡發育及卵子品質之影響

The effect of phytoestrogen genistein on the mouse oocyte development

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結報：過量攝食植物性雌激素對小鼠卵泡發育及卵子品質之影響
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Introduction

Less incidence of breast cancer was reported in Asain women, and the reason for this was suspected as that Asain women consume large quantity of soybean, and reduce the incidence of breast cancer. In addition, several in vitro studies demonstrated that genistein, one major isoflavonoids presents in soybean (Barnes et al., 1996), can inhibit cancer cells (Yanagihara et al., 1993; Kuo, 1996; Brown et al., 1998; Shao et al., 2000), and prevent bone loss (Ishimi et al., 2000). However, although genistein may provide certain benefits, there were several detrimental effects ware reported. In ovarisectomised females, uterine hypertrophy was induced by the treatment of genistein to the mice (Burroughs et al., 1990; Ishimi et al., 2000). In ovary, two major morphological defects were observed in the genistein-treated females, one is ovary atrophy (Awoniyi et al., 1998), and the other abnormal follicals (Nagao et al., 2001). In addition, estradiol and progesterone were significantly reduced in the genistein-treated females (Awoniyi et al., 1998; Nagao et al., 2001).

Nowadays, genistein has been treated as "healthy food" and may be supplied as food supplement in the form of soybean extract. In order to achieve the anti-cancer or anti-osteoporesis, people may consume large quantity soybean or genistein tablets in a liberal manner. In Asia, vegeterians consume soybean as their major protein source. It is our great concern that when consumeers take soybean or genistein for their healthy reason, caution should be addressed as well. The aim of this study was to evaluate the effect of genistein on the growth of the offspings whose mothers receive genistein at various stages of gestation. Hopefully our result would provide some information to vegetarians, especially for the child-bearing aged women.

Materials and Methods

Animals

ICR females of 6-10 weeks were purchased from National Science Council, and kept in an enviroment with consistant temperature (25 ± 1℃), humidity (55 ± 5%) and regular dark light cycle (12-light and 12-dark).

Embryo culture, genistein treatment

Embryos at two-cell stage were retrieved from superovulated and mated females and cultured in M16 medium at 37℃ with 5% CO_2 atmosphere. Total 385 embryos were collected and divided into control and genistein-treated (experiemental) groups. In experimental groups, 2.5 and 5.0 μM of genistein were administrated to embryos immediately after being collected. Following 24 hours culture, both control and experimental embryos were transferred back to a pseudopregnant female, with 6-8 embryos to either left or right uterine horn randomly. On day 15 of gestation, females were autoposied and implanation rate was determined.

Feeding protocols

Animals were fed according to four feeding protocols in order to evaluate the effect of genistein on mouse development during gestation, lactation, and further developmental stages. The average content of genistein was 21.36 mg/kg body weight. [A] During early gestation stage [G10 group: Gen (-4~+5)], genistein was given from 4 days before till 5 days after gestation. [B] During early and late gestation stage [GP1 group: Gen (-7~+P1)], genistein was given to the mothers from 7 days before gestation till 1 day after birth. [C] Extending to early postnatal stage [GW3 group: Gen (-7~+ week 3)], genistein was provided from 7 days before gestation to the mothers till weaning. [D] Extending to pubertal stage [GW6 group: Gen (-7~+ week 6)], genistein was provided from 7 days before gestation to the mothers till weaning. In both Protocols [C] and [D], babies received genistein indirectly through milk, and after weaning, genistein were mixed to the food and fed directly to them.

Immunohistochemistry (IHC)

Immunohistochemistry methods were modified from Sar and Welsch (1999), and Bilinska (2001). Briefly, collected tissues were processed through ordinary histological methods, and serial sections at 5μm intervals were cut from wax blocks. Followed by dewax, rehydration procedures, 1st and 2nd antibodies to detect ERβ were applied to the sections and intensity determined.

Results

The effect of genistein on the implantation rate

Total 385 embryos were divided into control (0μM), and experimental (2.5μM, and 5.0μM) groups. Following 24 hours exposure to genistein, embryos in genistein-treated groups showed slightly lower survival rate. Furthermore, after embryo transfer, on day 15 of gestation, the implanatation rate significantly reduced (54.6% and 32.0% respectively). Individual umber of embryos in each step is shown in Table 1.

Table 1 Embryos numbers recorded at 2-cell, 4-cell stage, and the implantation rate was determined on day 15 gestation.

	genistein concentration	embryo number at 2-cell stage	embryo number at 4-cell stage	implants on day 15 gestation
control	0μM	127	126	101
experimental	2.5μM	130	117	71
experimental	5.0μM	125	99	40

The effect of genistein on mouse gross developmnet

Table 2 shows the development retardation of mouse following the treatment of genistein was observed in the experimental litters whose mothers received feeding protocol [B], ie, GP1 group.

Table 2 Reduced whole body length, body weight, and the brain weight of babies born from mothers received genistein treatment in GP1 groups.

	GP1 Contol (N=5)	GP1 Eexperimental (N=5)	p value
Body length (cm)	2.74 ± 0.09	2.52 ± 0.08	$p<0.005$
Body weight (g)	1.48 ±0.09	1.18 ± 0.06	$p<0.005$
Brain weight (g)	0.092 ± 0.045	0.089 ± 0.003	$p<0.005$

The effect of genistein on the developmental capacity of sex-related organs

For those offsprings from feeding protocols [C] and [D], ie who received genistein during their gestation, lactation, and further development, the weight of their sex-related organs were recorded (Table 3). The weight of ovary, uterus and testis from both groups were determined. In this experiment, all organs reduced in weight in the genistein-treated individuals in both GW3 and GW6 groups.

Table 3 Weight of sex-related organs collected from offsprings of groups GW3 and GW6.

	GW3		**GW6**	
	Control (Mean ± S.D)	**Experimental** (Mean ± S.D)	**Control** (Mean ±S.D)	**Experimental** (Mean ± S.D)
Ovary	0.0039 ± 0.00065 (N=8)	0.0019 ± 0.00072 (N=12)	0.0092 ± 0.00070* (N=4)	0.0042 ± 0.00159* (N=6)
Uterus	0.0070 ± 0.00026 (N=4)	0.0062 ± 0.00039 (N=5)	0.1619 ± 0.07425 (N=3)	0.1454 ± 0.03890 (N=4)
Testis	0.0482 ± 0.00244* (N=6)	0.0262 ± 0.01135* (N=9)	0.0930 ± 0.01192 (N=4)	0.0548 ± 0.03344 (N=12)

*p<0.05

The effect of genistein on the developmental capacity of representative organs

Brain, kidney, and spleen were collected from the offsprings who received feeding protocols [C] and [D]. In both GW3 and GW6 groups, the weight of these organs demonstrated a reductin in the genistein-treated individuals, although some of them may not reach the level of statistic significance (Table 4).

Table 4 Weight of sex-related organs collected from offsprings of groups GW3 and

GW6.

	GW3		GW6	
	Control (Mean ± S.D)	**Experimental** (Mean ± S.D)	**Control** (Mean ±S.D)	**Experimental** (Mean ± S.D)
Brain	0.4524 ± 0.0130* (N=7)	0.3648 ± 0.0115* (N=11)	0.4712 ± 0.0168* (N=4)	0.4094 ± 0.0455* (N=9)
Kidney	0.0990 ± 0.0190 (N=7)	0.0600 ± 0.0142 (N=18)	0.1908 ± 0.0126* (N=8)	0.1202 ± 0.0653* (N=18)
Spleen	0.0482 ± 0.0024 (N=7)	0.0651 ± 0.0246 (N=11)	0.1402 ± 0.0342 (N=4)	0.1180 ± 0.0633 (N=9)

*p<0.05

The effect of genistein on the expression of ERβ in the ovary during mouse embryogenesis and further development

Figure 1 (A)-(E) demonstrates the expression pattern of ERβ examined on day 6, day 8, day 10, day 12, and day 14 mouse embryos. The major expression sites of ERβ were in the granulosa cells. Figure 1 (F)-(G) presents similar expression patterns in the ovaries in GW3 and GW6 mouse. A comparison list (Table 5) recorded the expression intensity of ERβ. During early embryogenesis, stronger ERβ signal in granulosa cells were observed in the genistein-treated embryos while opposite patterns were found during later developmental stages. Strongest ERβ signal were

noted in the GW6 ovaries.

Table 5 ERβ expression intensity in the mouse ovary during embryogenesis. ED stands for embryonic day.

	Granulosa cells		Thecal cells		Interstitial cells	
	Control	Experimental	Control	Experimental	Control	Experimental
ED6	+	++	-	-	-	-
ED8	+	++	-	-	-	-
ED10	++	++	-	-	-	-
ED12	++	+	-	-	-	-
ED14	++	+	-	-	-	-
GW3	++	+	-	-	-	-
GW6	+++	+	-	-	-	-

The effect of genistein on the mouse testis

Figure 2 (A)-(B) shows the expression pattern of ERβ was also evaluated in the testis in GW3 and GW6 groups. ERβ signal was limited in the sertoli cells while only very weak signal was found in the developing spermatocytes. In sertoli cells, ERβ signal reduced significantly in the genistein-treated groups in both GW3 and GW6 groups. This became obvious in the later stage, ie GW6 group.

Table 6 ERβ intensity in testis of control and genistein-treated embryos in GW3 and GW6 groups.

	Sertoli cells		Spermatocytes	
	Control	Experimental	Control	Experimental
GW3	+++	+	weak	weak
GW6	++++	+	Weak	weak

Discussions

In this study, we confirm that genistein may have certain detremental effects during mouse embryogenesis and later developmental stages. Reduced body length, body weight and brain weight were noted in the genistein-treated groups with various feeding protocols. The underlying mechanism may be that genistein acts as an inhibitor of protein tyrosine kinase (PTK), and subsequently hinders the binding of PTK with growth factors such as epidermal growth factor. Consequently, signal transduction pathways blocked, and gowth retarded (Chen et al., 1984). Furthermore, genistein has been reported to inhibit the function of topo isomerase II, and this may impede cell proliferation during embryogenesis and further developmental stages (Markovits et al., 1989).

Similar results were also observed in the sex-related organs. Significant reduction in weight were observed in the ovary (GW6 group), uterus (GW6 group), and testis (GW3). Several in vitro studies of testis cells have demonstrated that genistein may inhibit the activity of PTK and topo isomerase II and simultaneously induce the caspase-3 protease and consequently result in apotosis (Constantinou & Huberman, 1995; Constantinou et al., 1998; James et al., 1998; James et al., 2000). This may provide part of the reseon to explain the reduced weight in the sex-related organs in the genistein-treated offsprings.

The expression pattern of estrogen receptor β in the ovary and testis was affected by genistein as well. During earlier embryogenesis, ie embryonic day of 6 and 8, ERβ signal in the ovary was up-regulated in the genistein-treated offsprings. However, this trend was reversed during later embryonic developmental stages, ie day 12 and 14, during which time ovary ERβ signal was inhibited in the experimental groups. This inhibitory effect was even more significant in the neonatal and pubertal development, ie in both GW3 and GW6 groups. In testis, inhibitory expression of ERβ was observed in the genistein-treated groups in GW3 and GW6 offsprings. In ER-positive breast carcinoma cell line, it has been reported that genistein may afftect ER downstream gene pS2 and TGF-β and decrease ERE-CAT level. In addition, in the presence of estrogen or 17-beta estrodiol, genistein significantly inhibit the expression of ER mRNA in breast carcinoma cell line (Shao et la., 2000), and in uterus (Michelle et al., 2000). This may explain the diverse expressin pattern of ERβ observed in the ovary during mouse embryogenesis. From day 10 onwards, endogenous estrogen may become evident and enhence the inhibitory effect of genistein on ERβ expression; in

day 6 and 8 day embryos, endogenous estrogen may not be sufficient and therefore no inhibitory effect of genistein was observed.

However, in addition to this suspection, there may be some other underlying mechanisms to be explored. For the male offsprings may not produce considerable quantity endogenous estrogen, and therefore should not be considered as one of the factors to enhence genistein's effect on the inhibition of ERβ expression in the testis in GW3 and GW6 mice.

Although in our study significant reduction of ERβ expression has been observed, the possible impact to the male offsprings remained unclear. Ngao and colleagues (2001) evaluated the reproduction ability in the genistein-treated males, and no significant harmful effect was noted. This result contrast to that recorded in the femele offsprings in the same study (Nago et al., 2001), profunding decrease in the female reproduction was found in the genistein-treated females.

Conclusions

In this study, we demonstrated that when mothers receive high dose of genistein, their offsprings may be affected in their growth in terms of whole body length, body weight, individual organ's weight, sex-related organs weight, and the expression pattern in the ovary and testis. Based on these findings, we would like to recommend that when genistein has been applied as anti-cancer, and/or anti-osteoporesis agents, caution should be make to its harmful effects, especially to women in child-bearing stages.

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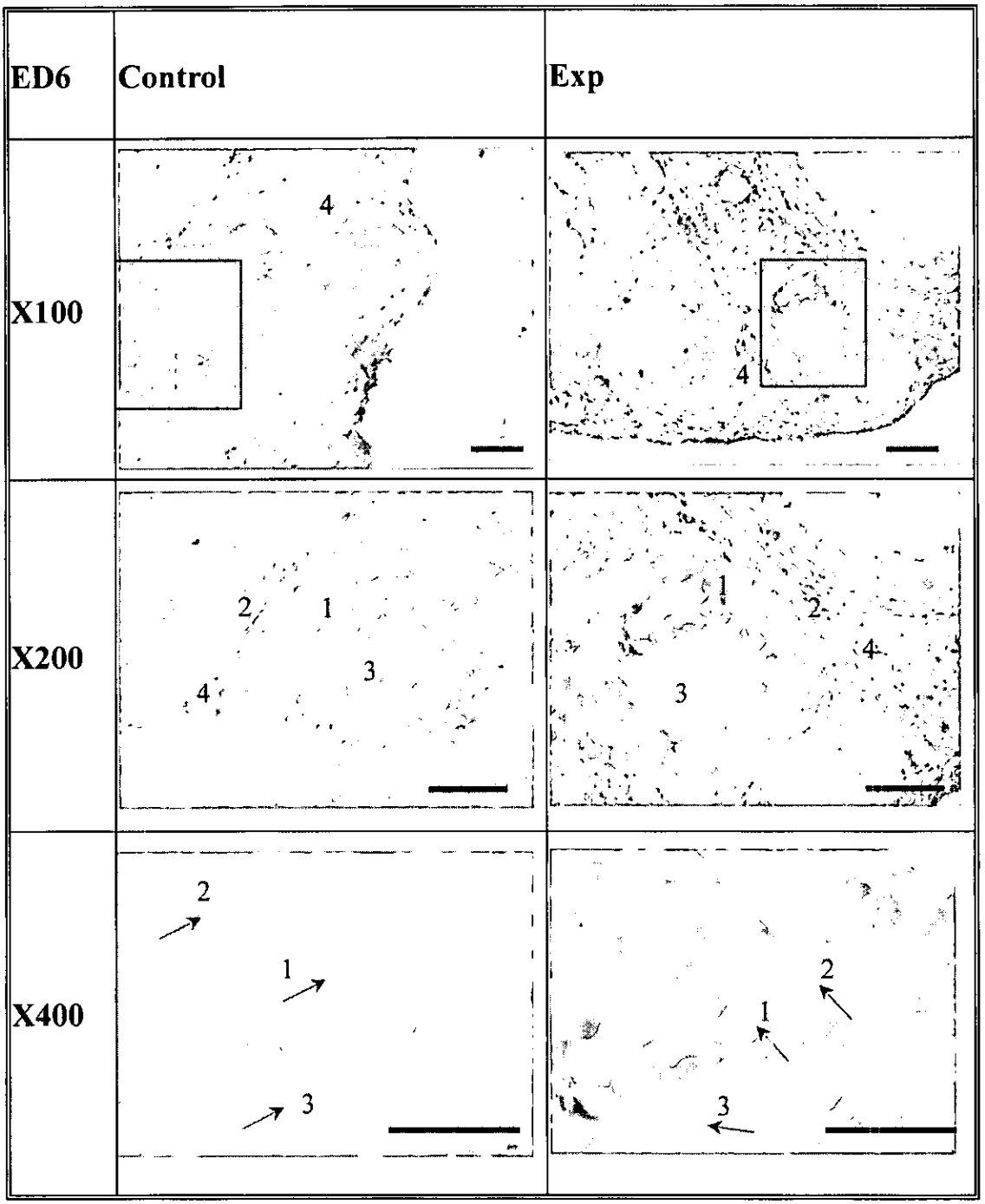


Figure 1 (A) Immunohistochemical detection of ERβ in the ovary in day 6 embryos whose mothers received feeding protocol [A], ie G10 group. Stronger ERβ signal was observed in the granulosa cells in the genistein-treated group.

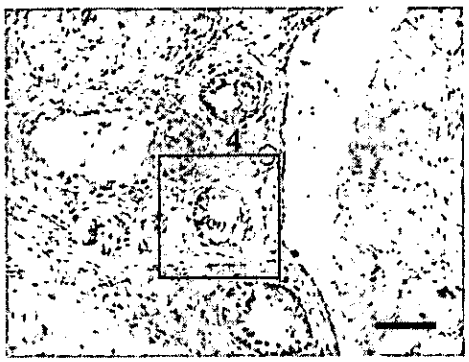
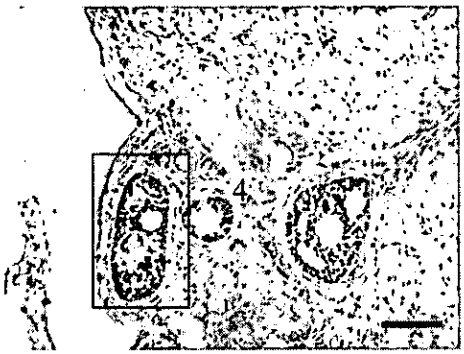
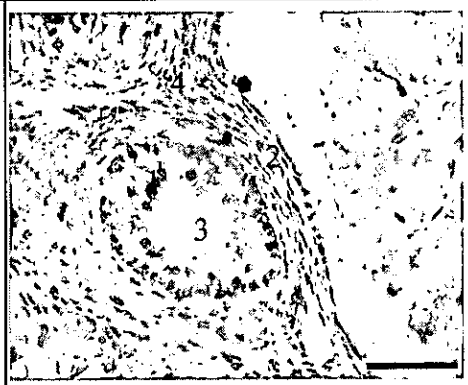
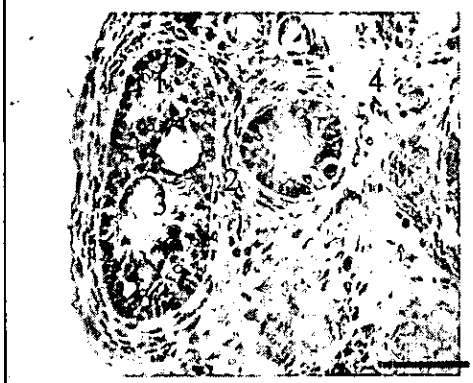
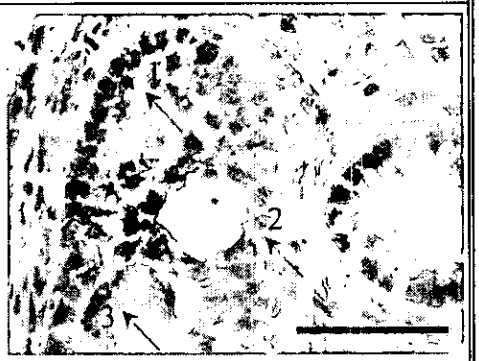
ED8	Control	Exp
X100		
X200		
X400		

Figure 1(B) Immunohistochemical detection of ERβ in the ovary in day 8 embryos whose mothers received feeding protocol [A], ie G10 group. Stronger ERβ signal was observed in the granulosa cells in the genistein-treated group.

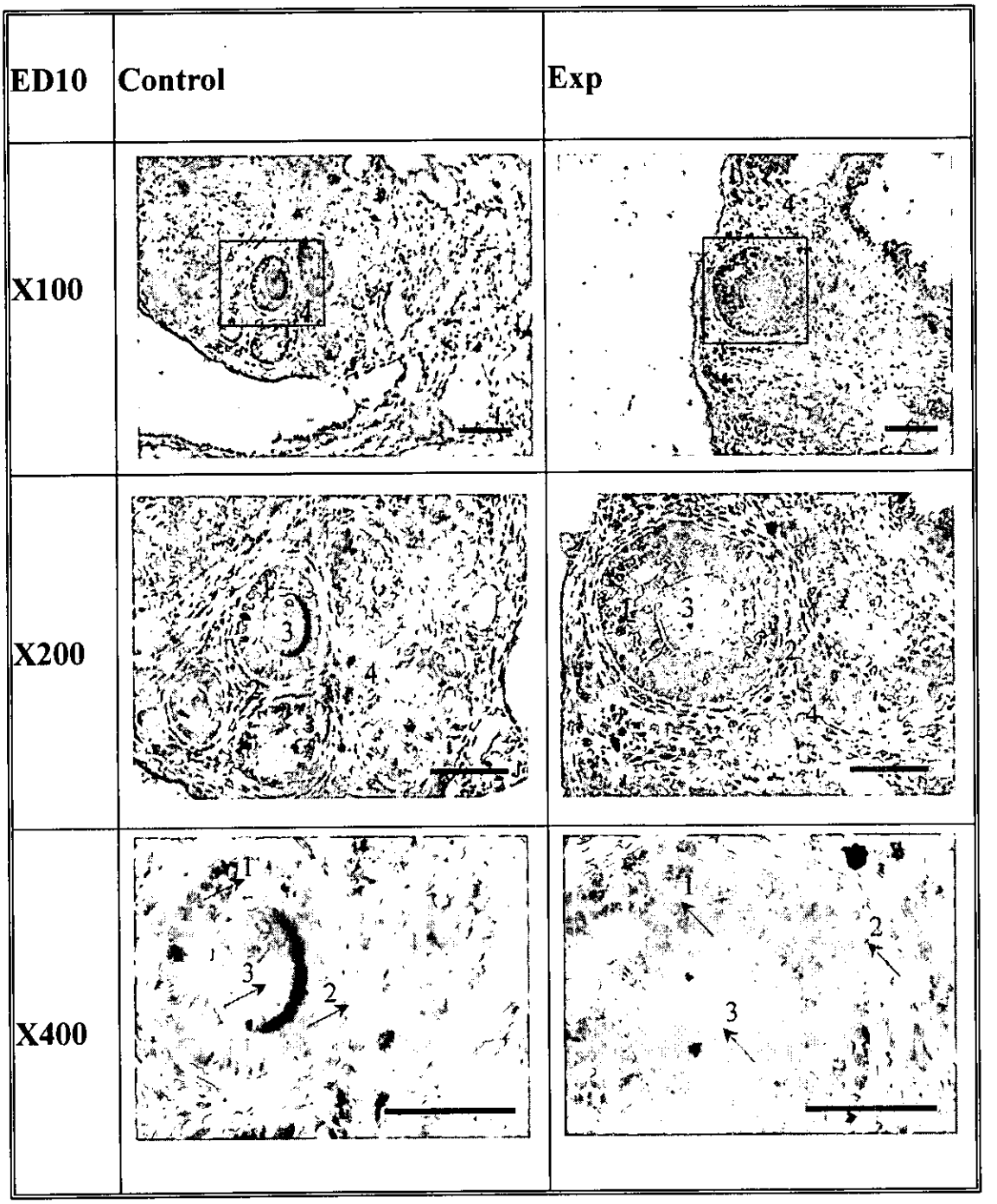


Figure 1(C) Immunohistochemical detection of ERβ in the ovary in day 10 embryos whose mothers received feeding protocol [A], ie G10 group. Stronger ERβ signal was observed in the granulosa cells in the genistein-treated group.

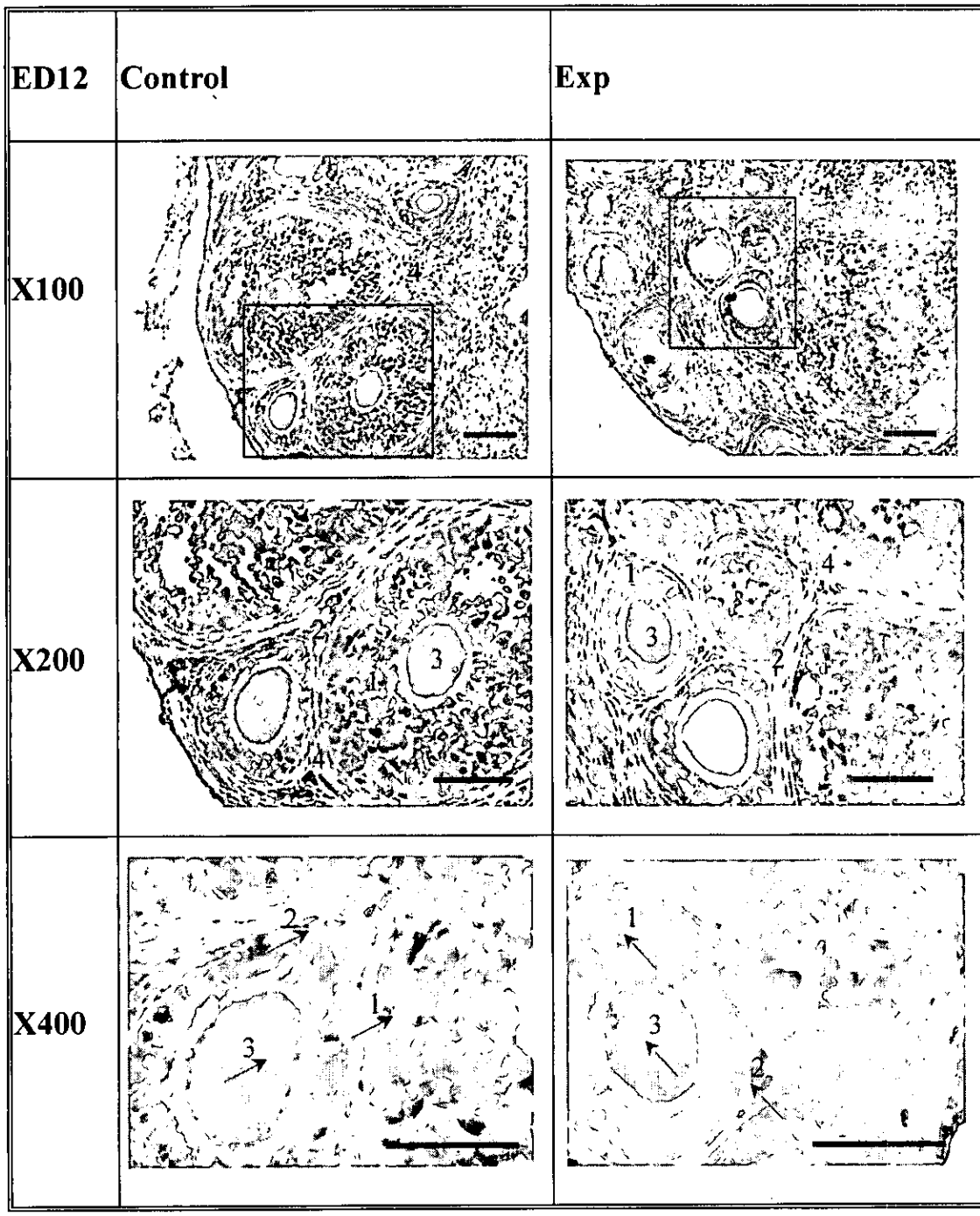


Figure 1(D) Immunohistochemical detection of ERβ in the ovary in day 12 embryos whose mothers received feeding protocol [A], ie G10 group. Forom this stage onwards, reduced ERβ signal was observed in the granulosa cells in the genistein-treated group.

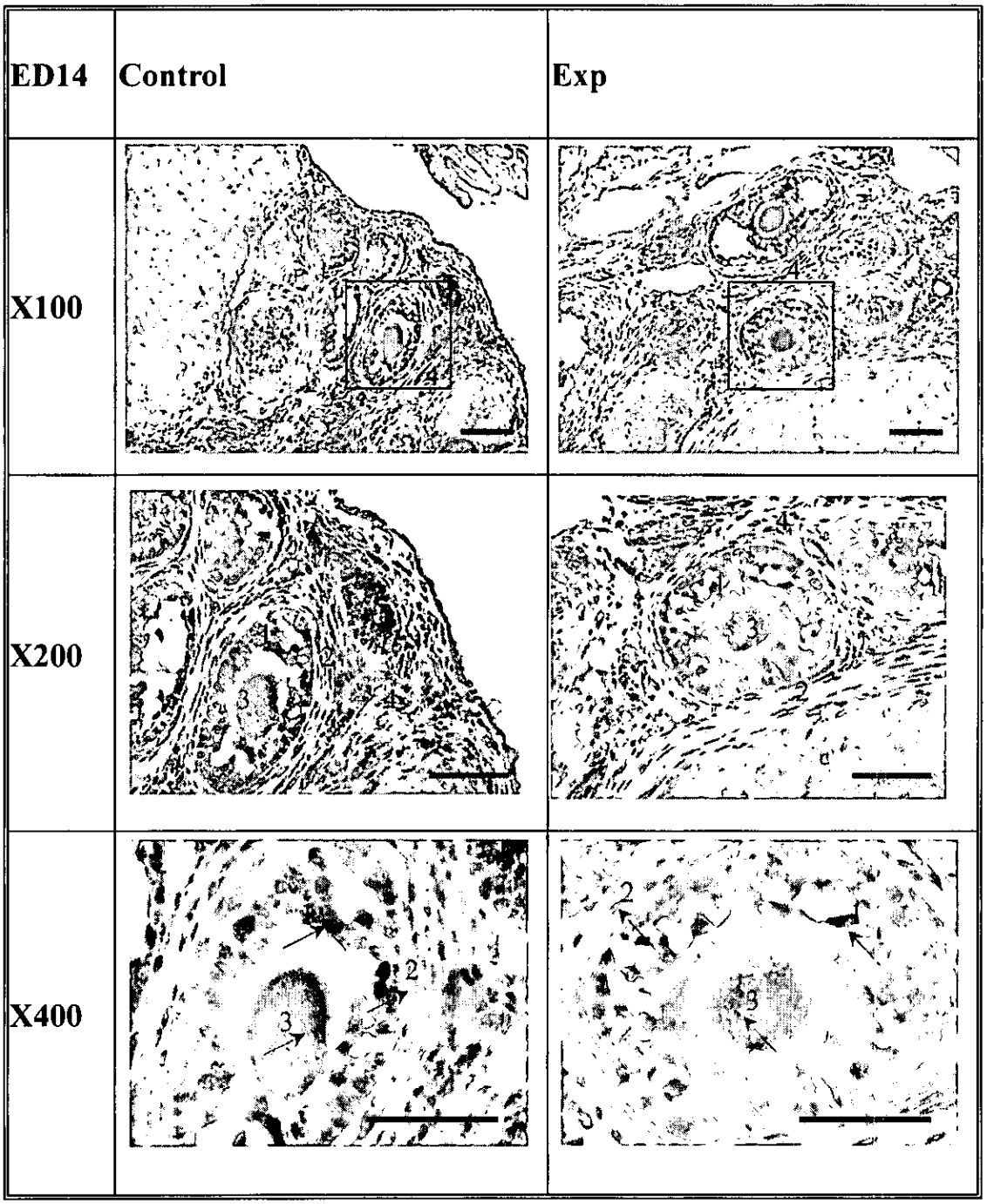


Figure 1(E) Immunohistochemical detection of ERβ in the ovary in day 14 embryos whose mothers received feeding protocol [A], ie G10 group. Reduced ERβ signal was observed in the granulosa cells in the genistein-treated group.

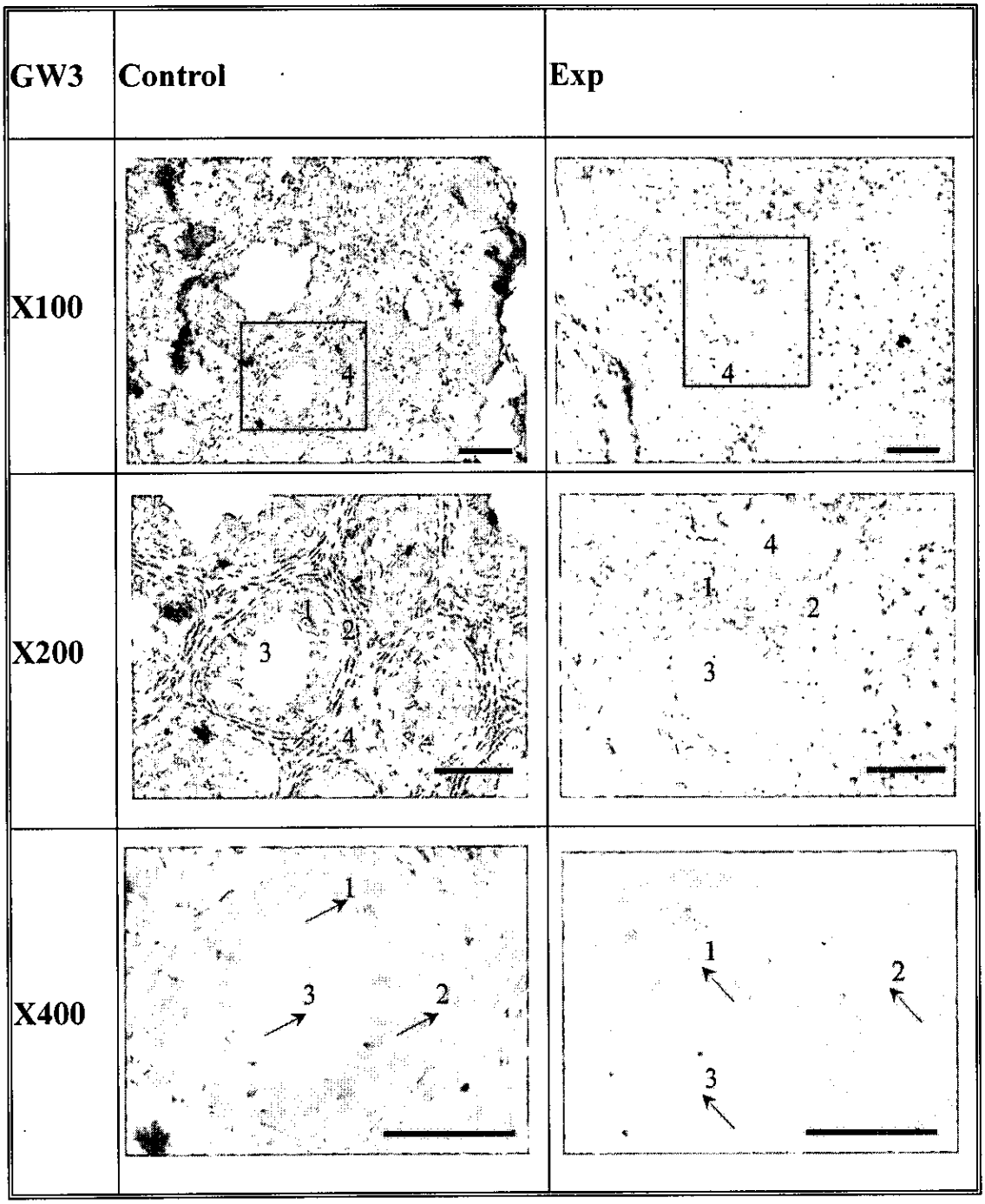


Figure 1(F) Immunohistochemical detection of ERβ in the ovary in 3-week old offsprings who and their mothers received feeding protocol [C], ie GW3 group. Reduced ERβ signal was observed in the granulosa cells in the genistein-treated group.

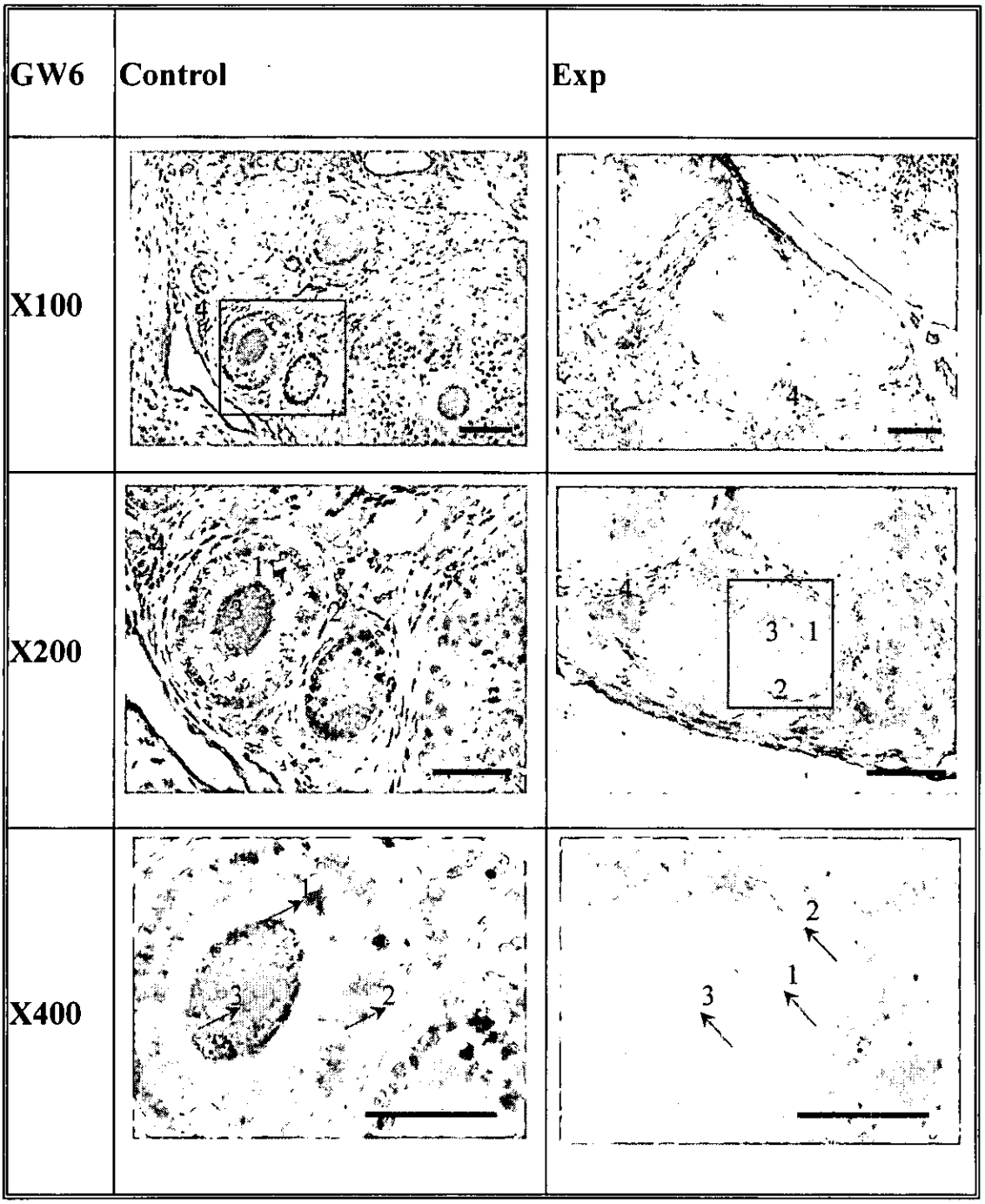


Figure 1(G) Immunohistochemical detection of ERβ in the ovary in 6-week old offsprings who and their mothers received feeding protocol [D], ie GW6 group. Reduced ERβ signal was observed in the granulosa cells in the genistein-treated group.

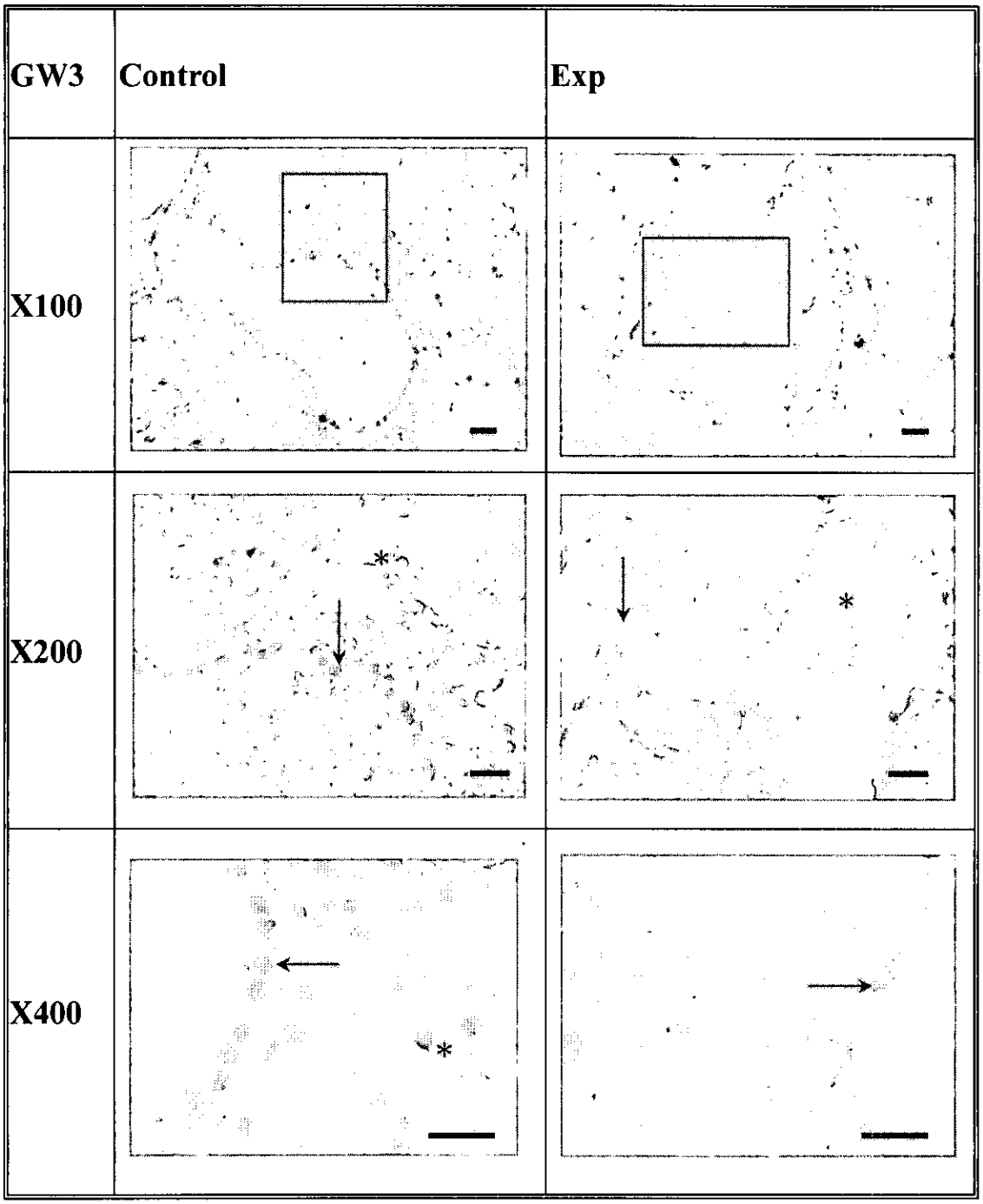


Figure 2(A) Immunohistochemical detection of ERβ in the testis in 3-week old offsprings who and their mothers received feeding protocol [C], ie GW3 group. Significantly stronger ERβ signal was observed in the sertoli cells in the control group.