THE JOURNAL OF EXPERIMENTAL ZOOLOGY 278:93–100 (1997)

17β-Estradiol Effects on Mast Cell Number and Spermatogonial Mitotic Index in the Testis of the Frog, *Rana esculenta*

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ABSTRACT Estrogen affects mast cell activity and cellular proliferation in several vertebrate tissues. Due to the presence of mast cells in the interstitial tissue of the testis and due to the annual changes of spermatogonial proliferation and estradiol level in the frog, *Rana esculenta,* we have studied the possible regulation of mast cell number (MCN) and primary spermatogonial mitosis exerted by 17β-estradiol (E_2). MCN changed in the testis during the annual reproductive cycle, showing peaks in December and in May. Administration of E_2 elicited an increase of MCN both in intact and hypophysectomized frogs, and this effect was counteracted by tamoxifen. In vitro experiments indicated that E_2 induced an increase of both MCN and the primary spermatogonial mitotic index. Moreover, a significant decrease of intratesticular androgen content was measured in E_2-treated testes. All the in vitro effects induced by E_2 were counteracted by tamoxifen. In conclusion, our data indicate that in *Rana esculenta* testis E_2 increases MCN and primary spermatogonial mitotic index via intratesticular mechanisms. A possible involvement of the decrease of androgen levels exerted by E_2 in the mechanism underlying the increase of MCN is also considered. *J. Exp. Zool. 278:93–100, 1997.*

Estrogens are produced by the vertebrate testis (Fasano and Pierantoni, '93), and estrogen receptors have been found in testes of rats (Sharpe, '82), reptiles (Mak et al., '83a), amphibians (Mak et al., '83b; Fasano et al., '89a), and elasmobranchs (Callard and Mak, '85; Callard et al., '85; Fasano and Pierantoni, '93). In addition, estradiol has been shown to exert a central role in the regulation of the hypothalamus-pituitary activity in vertebrates (Shanbacker, '84), acting both with central and local (intratesticular) mechanisms (Pierantoni and Fasano, '91).

Estrogens also affect mast cell activity in several vertebrate tissues. Estradiol treatment augments secretion of histamine and serotonin in rat peritoneal mast cells in a dose-dependent manner, whether stimulated by the segretagogue compound 48/80 or the substance P; these effects are counteracted by tamoxifen (for review see Vliagoftis et al., '92). Moreover, estradiol treatment increases mast cell number (MCN) in the uterus of the musk shrew (Mohanty and Chainy, '92) and in the testis of neonatally treated rats, where rare mast cells are normally observed (Gaytan et al., '86, '90a). Recently we have found that estradiol provokes proliferation of interstitial connective tissue and increases mast cell number in the Harderian gland of the frog, *Rana esculenta* (Di Matteo et al., '95).

Impairment of Leydig cell activity increases the MCN in rat (Gaytan et al., '90a,b), frog (Di Matteo et al., '92), and lizard (Minucci et al., '95) testis. Estradiol inhibits androgen production in the frog, *Rana esculenta,* and peaks in correspondence of both testosterone and 5α-dihydrotestosterone decrease during the annual reproductive cycle (Fasano et al., '89a). In this period of the year (February–March), the frog testis is also characterized by the highest number of primary spermatogonial mitosis (Rastogi et al., '85). Due to the well-known mitogenic activity of estrogens (Pierantoni and Fasano, '91) and to their effects on the impairment of the androgen production above described (Fasano and Pierantoni, '93), we attempt in the present paper to correlate estradiol treatment with both mast cell number and primary spermatogonial mitosis in the frog, *Rana esculenta,* testis.

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Received 5 July 1996; Revision accepted 6 November 1996

MATERIALS AND METHODS

Animals

Adult frogs, *Rana esculenta,* were collected from the surroundings of Naples by a local dealer. Frogs were maintained in plastic tanks (23 × 16 × 11 cm) with food (meal worms) and water available ad libitum.

Annual cycle

Eight animals per month were collected and immediately killed by decapitation. Testes were removed and quickly fixed in Bouin's fluid for histological observations. Serial paraffin sections (5 μm) were stained with 0.2% toluidine blue in Walpole buffer at pH 4.2 (Gabe, '68) for the recognition and evaluation of the mast cell number (MCN).

Small pieces (1 mm^3) of tissue for electron microscopy were fixed for 2 h at 4°C in Karnovsky's fluid in 0.1 M phosphate buffer at pH 7.4 and postfixed in 1% osmium tetroxide in the same buffer at 4°C. Samples were then dehydrated in a graded ethanol series and embedded in TAAB 812 resin. Ultrathin sections were counterstained with uranyl acetate and lead citrate and observed under a Zeiss transmission electron microscope (Zeiss, Germany).

Experiments in intact animals

Adult male frogs (n = 30) were collected in January and divided into three experimental groups. Animals were injected into the dorsal sac on alternate days for 2 weeks as follows: 1) Amphibians Krebs Ringer bicarbonate buffer (KRB) (Muller, '76) (100 μl); 2) KRB + estradiol (E_2) (2 μg/100 μl); 3) KRB + testosterone (T) (10 μg/100 μl). Ten additional animals were sacrificed at the beginning of the experiment as initial control.

Adult male frogs (n = 40) collected at the end of February were divided into four experimental groups (ten animals/group) and injected into the dorsal sac on alternate days for 2 weeks as follows: 1) KRB (100 μl); 2) KRB + E_2 (2 μg/100 μl); 3) KRB + E_2 (2 μg/100 μl) + tamoxifen (200 μg/100 μl).

At the end of experiments animals were anaesthetized with MS 222 (Sigma) and decapitated. Testis were removed and immediately fixed in Bouin's fluid for histological examination as above described.

Experimental in hypophysectomized animals

Adult animals (n = 100) collected at the end of February were hypophysectomized (HPX) and after 1 week were divided into three experimental groups and injected into the dorsal sac on alternate days for 5 days as follows: 1) KRB (100 μl); 2) KRB + E_2 (2 μg/100 μl); 3) KRB + E_2 (2 μg/100 μl) + tamoxifen (200 μg/100 μl). At the end of experiments animals were anaesthetized with MS 222 (Sigma) and decapitated. Testis sampling was carried out 24 h after the first, second, and third injection for the evaluation of MCN.

In vitro experiment

Adult frogs (n = 15) collected in February were killed by decapitation, and testes were excised. The right testes were incubated for 24 h in KRB alone (one testis/500 μl) or containing E_2 10^{-6} M or E_2 10^{-6} M + tamoxifen 10^{-4} M. After 8 h, the incubation medium was replaced with a fresh medium. Each tube contained 50 μg of colchicine for the evaluation of the primary spermatogonial mitotic index. MCN was also evaluated.

The left testes were incubated using the same conditions without colchicine. At the end of incubation, the left testes were stored at –80°C until analyzed for androgen content.

Hormone measurement

Intratesticular androgens were measured by radioimmunoassay as previously described (Pierantoni et al., '84). Since the antiserum was cospecific for testosterone and 5α-dihydrotestosterone, data are expressed as androgens. The intra- and interassay coefficients of variation were 5 and 8%, respectively. Sensitivity was 2 pg/tube.

Numerical and statistical analysis

For each experiment five randomly chosen sections from each animal/experimental group were viewed with the light microscope at a magnification of ×400. The MCN within the interstitial tissue and the tubule number of the testes were counted to give a value of MCN/100 tubules per animal.

The primary spermatogonial mitotic index was expressed as the number of metaphases per total primary spermatogonia counted multiplied by 100 in three randomly chosen sections/animal.

Significance of differences was evaluated using one-way analysis of variance followed by Duncan's test for multigroup comparisons.

RESULTS

Annual cycle

Mast cells of connective tissue type were observed in the interstitial tissue of the testes. At the ultrastructural level (Fig. 1), the testicular mast

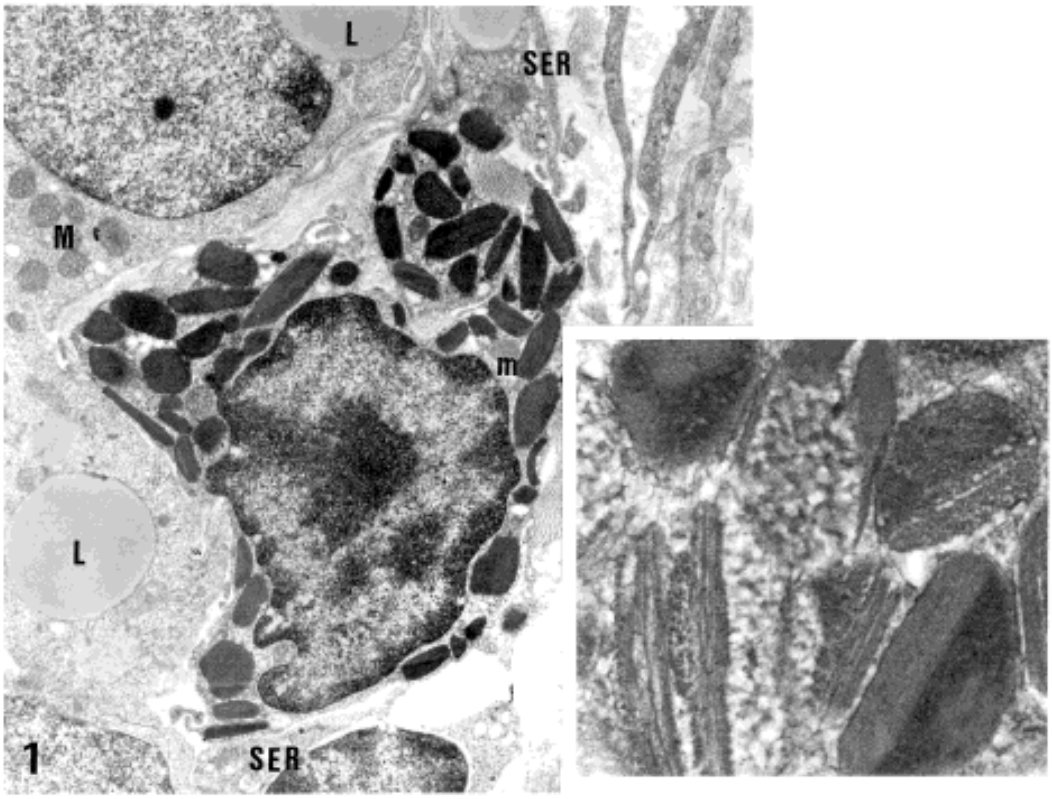


Fig. 1. Electron micrograph of a mast cell in the interstitial compartment of a February frog testis, showing mature and nonhomogeneous granules. Some Leydig cells are shown. L, lipid droplet; m, mitochondria; M, mitochondria with tubular cristae; SER, smooth endoplasmic reticulum. ×4,600. **Inset:** High magnification of mast cell granules with regularly arranged lamellae. ×45,000.

cells are similar to those found in the frog Harderian gland, with many mature and nonhomogeneous granules. The granules consist of regularly arranged lamellae which form parallel straight or curved figures (Fig. 1, inset) (Chieffi Baccari et al., '91). The irregular nucleus contained large masses of heterochromatin, particularly near the nuclear envelope. In the cytoplasm, the rough endoplasmic reticulum and the Golgi apparatus were not visible owing to the abundance of secretory granules. Some mitochondria were distinguishable among the secretory granules (Di Matteo et al., '92). With respect to the annual variation of MCN in the testicular interstitium, there were peaks in May and in December (Fig. 2). The highest values of MCN were found in May ($P < 0.01$ vs. the other months), while the lowest values were detected in April ($P < 0.01$ vs. Dec., Jan., May, June, July; $P < 0.05$ vs. Sept.) (Fig. 2).

Experiments in intact animals

Testes of frogs injected with E_2 during January showed a significant increase of MCN in the interstitial tissue as compared with control animals ($P < 0.01$), while no differences were found in testes of frogs injected with testosterone (Fig. 3).

The administration of E_2 provoked an increase of MCN ($P < 0.01$) also in the testes of animals injected during February. This effect was counteracted by tamoxifen. No differences as compared with control animals were evidenced when E_2 was given in combination with a tenfold or 100-fold in excess of the antiestrogenic compound (Fig. 4).

Experiment in hypophysectomized animals

No significant differences of MCN were found 24 h after the first injection of E_2 or E_2 + tamoxifen in the testes of HPX animals, while a small but significant increase ($P < 0.05$) of MCN was observed after the second injection only in the E_2-treated animals (Fig. 5). Interestingly, the MCN strongly increased ($P < 0.01$) after the third injection of E_2; tamoxifen injected with E_2 reduced the E_2 effect to control levels after the second as well as after the third injection (Fig. 5).

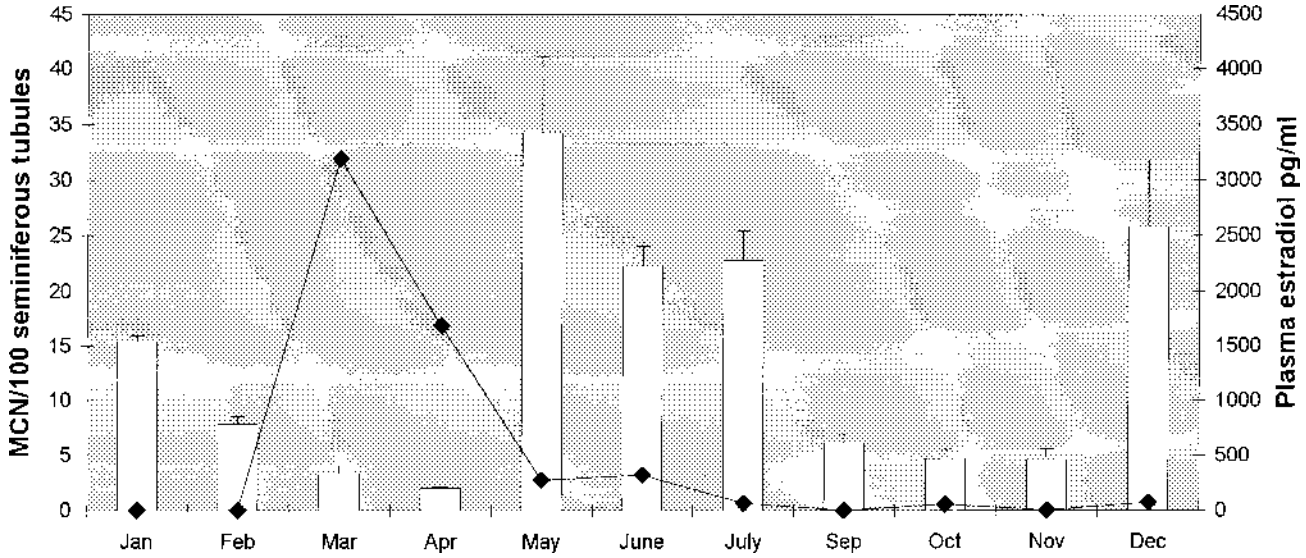


Fig. 2. Annual variation in the mast cell number (MCN) per 100 spermatogenetic tubules in the frog, *Rana esculenta*. Vertical bars indicate values ± SD. ■—■, E_2 profile. E_2-17β profile has been reported from Fasano et al. ('89).

In vitro experiment

February testes incubated with E_2 showed an increase of MCN ($P < 0.01$), while no differences were observed between the E_2 + tamoxifen incubated groups and controls (Fig. 6A).

The primary spermatogonial mitotic index of the E_2-incubated testes strongly increased ($P < 0.01$) as compared with controls (Fig. 6B). No differences were found between the primary spermatogonial mitotic index of control testes and that of the E_2 + tamoxifen incubated group (Fig. 6B).

February testes incubated with E_2 at 10^{-6} M showed a significant ($P < 0.01$) decrease of intratesticular androgen content as compared to control testes, and this effect was counteracted by the addition of tamoxifen (Fig. 6C).

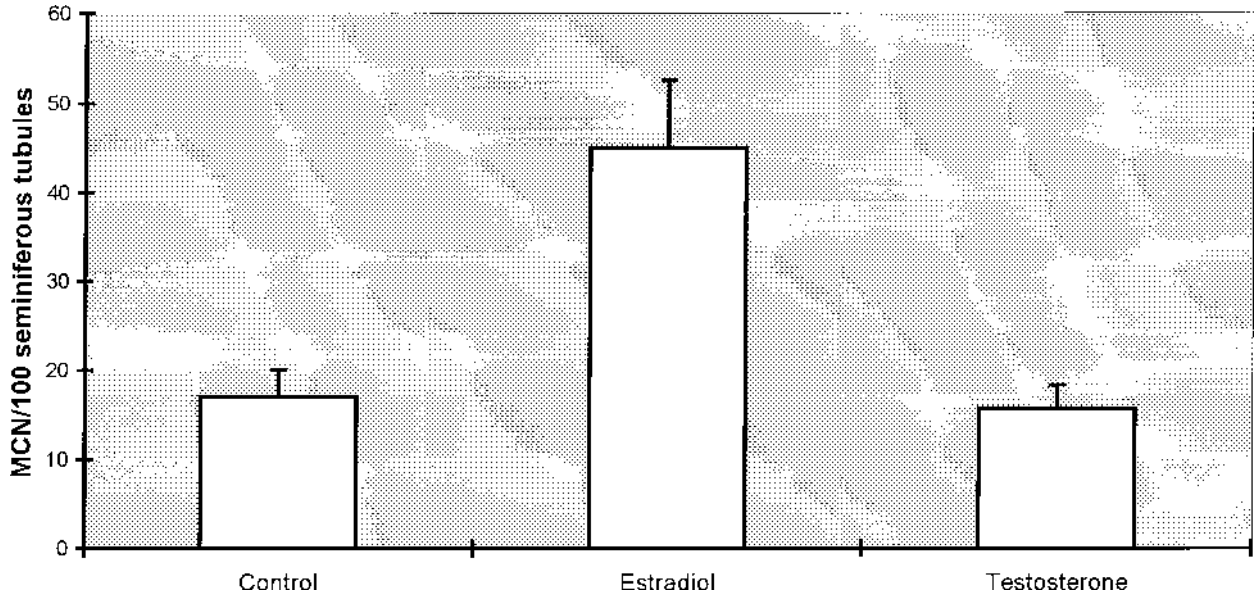


Fig. 3. Effect of sex hormones (estradiol-17β, 2 μg/100 μl, and testosterone, 10 μg/100 μl) on MCN in January frog testis. Animals were injected on alternate days into the dorsal sac for 2 weeks. Vertical bars indicate values ± SD.

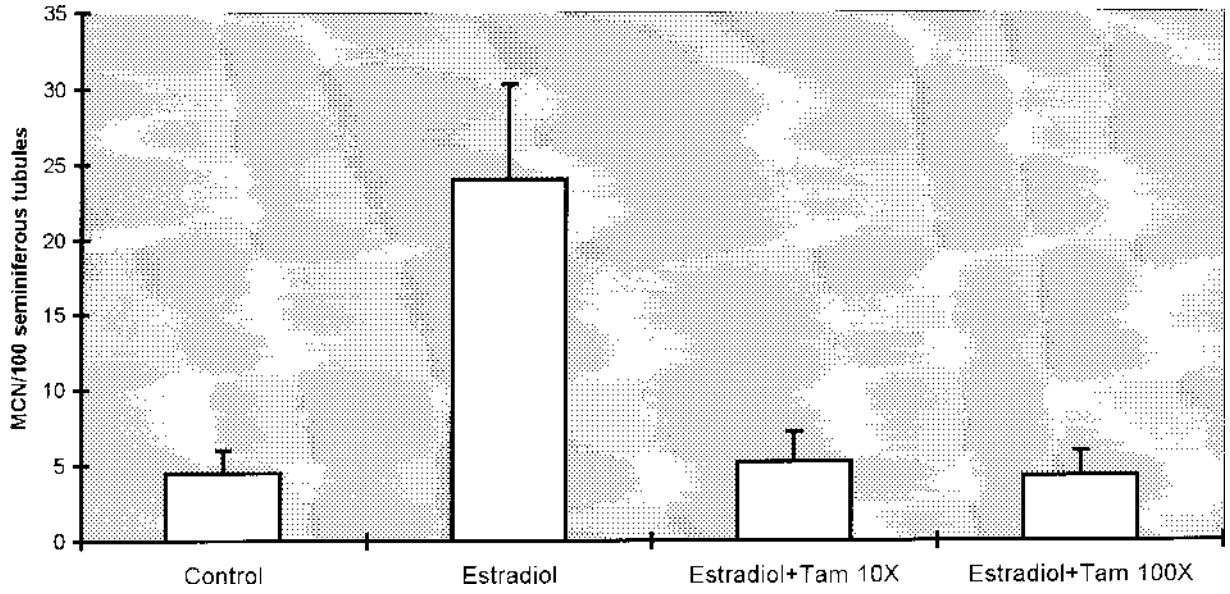


Fig. 4. Effect of estradiol-17β (2 μg/100 μl) and estradiol-17β given in combination with a tenfold or 100-fold in excess of its synthetic antagonist on MCN in February frog testis. Tam, tamoxifen. Animals were injected on alternate days into the dorsal sac for 2 weeks. Vertical bars indicate values ± SD.

DISCUSSION

The present study confirms that mast cells of connective type are present in the interstitial tissue of the frog, *Rana esculenta,* testis. In addition we show that their number varies during the year, with peaks in May and December. Previously we demonstrated in frogs that MCN is influenced by thermal manipulations. Low temperature increased MCN while high temperature did not in both testis (Di Matteo et al., '92) and Harderian gland (Chieffi Baccari et al., '91). The increased MCN at low temperature has been interpreted as an adaptation for preventing thrombus formation under the condition of slow blood flow characteristic of hibernation.

Our results show that in January, when MCN is at high value, estradiol treatment provokes a significant increase of MCN. The increase is also detected in February and is counteracted by tamoxifen treatment. The responsiveness of mast

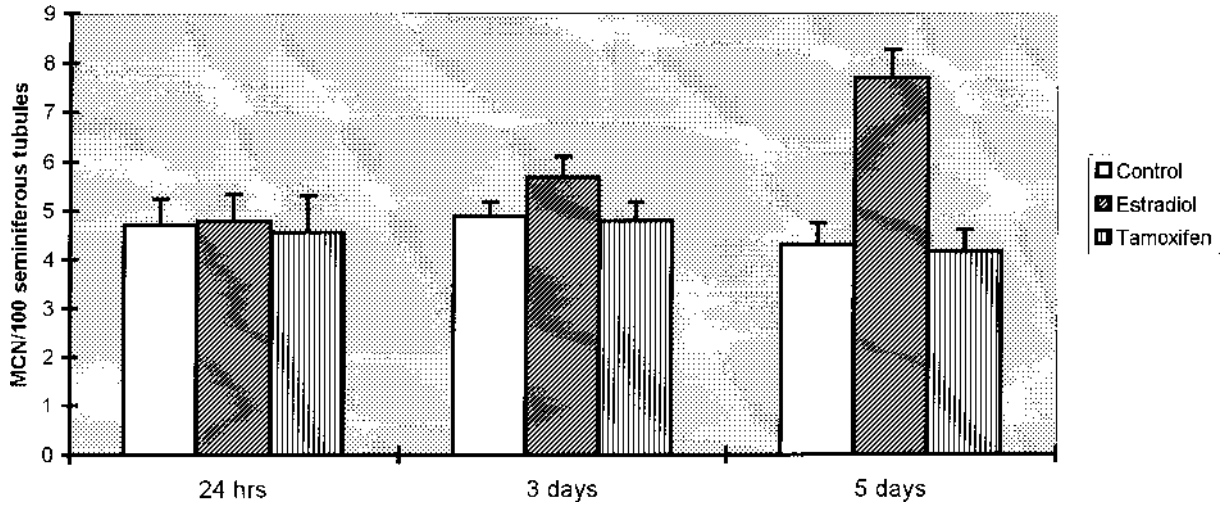
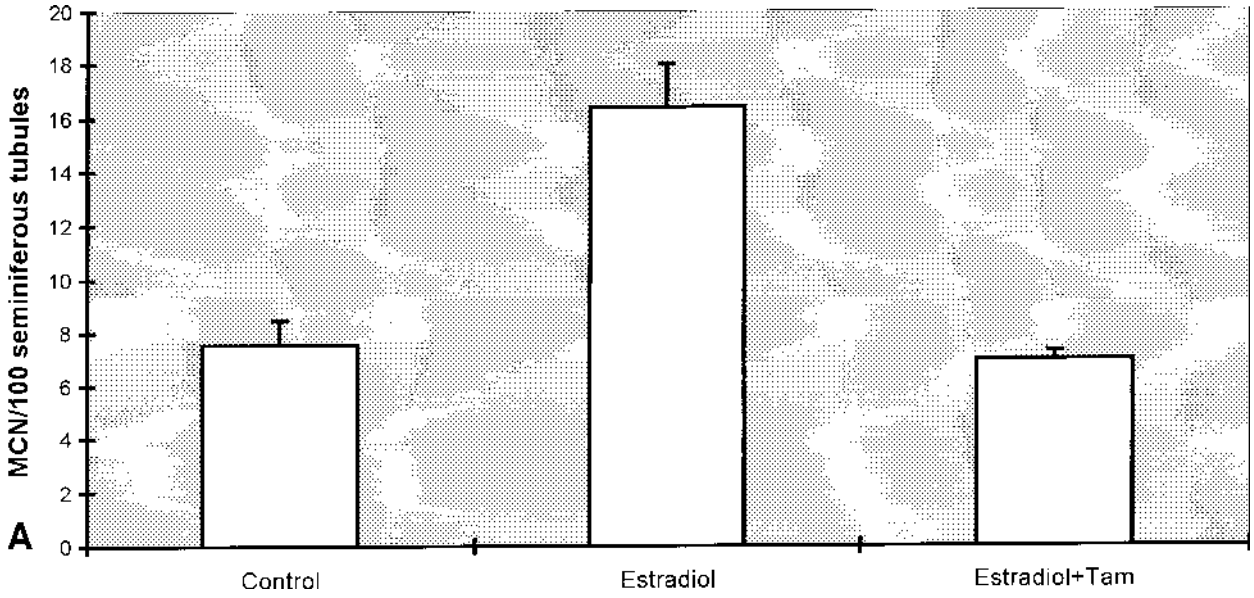
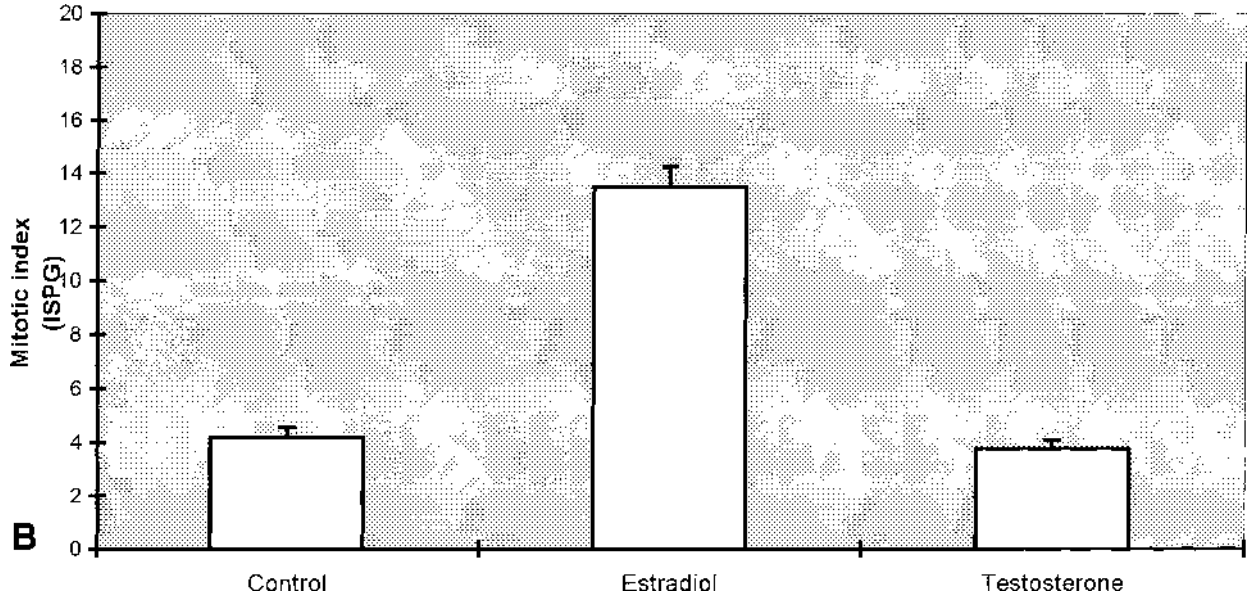
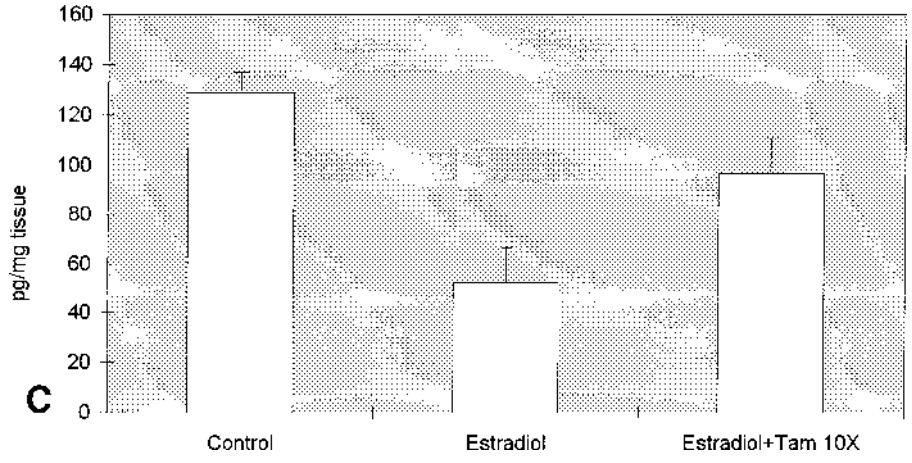


Fig. 5. Effect of estradiol-17β and estradiol-17β given in combination with tamoxifen (100-fold in excess) on MCN in the February frog testis of hypophysectomized animals 24 h, 3 days, and 5 days after the last injection (see Materials and Methods). Vertical bars indicate values ± SD.


MCN/100 seminiferous tubules
20
18
16
14
12
10
8
6
4
2
0
A
Control
Estradiol
Estradiol+Tam


Mitotic index (ISPG)
20
18
16
14
12
10
8
6
4
2
0
B
Control
Estradiol
Testosterone


pg/mg tissue
160
140
120
100
80
60
40
20
0
C
Control
Estradiol
Estradiol+Tam 10X

cells to steroid hormones has been established in the female rat uterus during the estrous cycle (Gibbons and Chang, '72; Krishna and Terranova, '85; Shinohara et al., '87) as well as in the peritoneum (Modat et al., '82). Although the mechanism of the changes of MCN is still not clear, it is generally accepted that estradiol acts on mast cells (Modat et al., '82; Krishna and Terranova, '85). Indeed, estradiol increases histamine and serotonin secretion while tamoxifen inhibits it, and estrogen receptors in mast cells have been detected (Vliagoftis et al., '92). Our data indicate that the MCN increase depends on estrogens, keeping in mind that estrogens are produced by the vertebrate testis (Chieffi, '66), where estrogen receptors have been found (Mak et al., '83a,b; Fasano et al., '89a). Interestingly, Varriale et al. ('86) and Fasano et al. ('89b) reported that in the male frog, *Rana esculenta,* plasma 17β-estradiol peaks in early spring and remains at significantly high concentrations until nearly summer. In this respect, we can speculate that the peak of MCN recorded in May is due to the plasma estradiol.

Increased MCN after estradiol administration was observed also in the testis of the lizard *Podarcis s. sicula* Raf (Minucci et al., '95) and in the rat. Neonatally estrogen-treated rats show considerable numbers of mast cells (Gaytan et al., '86), and increase of MCN is not induced after neonatal androgen treatment (Gaytan et al., '89). Similarly, in frogs, while androgen treatment does not increase MCN, estradiol does, and its activity may depend on the modification of an inhibitory factor present in the testicular interstitium, as suggested by Gaytan et al. ('90a,b). Several lines of evidence indicate that high testosterone levels may be related to a low MCN. Ethylene dimethane sulphonate (EDS), which specifically destroys Leydig cells, induces mast cell appearance in rats, frogs, and lizards (for review see Minucci et al., '92) after cellular destruction. Moreover, treatments which provoke a decrease of testosterone levels have been proved to increase MCN in *Rana esculenta* (Di Matteo et al., '92).

In the present study, testis pieces incubated with estradiol show decreased testosterone production and increased MCN, with these effects counteracted by tamoxifen. Furthermore, the increase of MCN during the cycle occurs also in December, when a decrease of androgen levels without a concomitant estradiol peak is observed (Fasano et al., '89a; Paolucci et al., '92). However, mast cells are present in the interstitial compartment of testes in EDS-treated rats supplemented with testosterone (Sharpe and Donachie, '88), but, due to the presence also of eosinophil leucocytes (Zaidi et al., '88), it has been suggested it is an inflammatory reaction because of the damaged interstitial tissue (Gaytan et al., '90a,b).

An alternative hypothesis besides mast cell migration is that estrogen treatment elicits proliferative activity of immature mast cells. This is supported by the experiment in HPX animals in which estradiol increases MCN, with this effect counteracted by tamoxifen. It is interesting to note that estradiol induces cellular proliferative activity both in vivo and in vitro (Schuchard et al., '93); therefore, it may act directly on putative testicular mast cell precursors and/or stimulating growth factors. In this respect, our data also show that the primary spermatogonial mitotic index strongly increases in the testes incubated with estradiol but not in estradiol plux tamoxifen–treated testes. A strong mitogenic activity of primary spermatogonia has been detected during the annual reproductive cycle (Rastogi et al., '85) in the periods of the year characterized by the estradiol peak (Varriale et al., '86; Fasano et al., '89b).

In conclusion, the present results indicate that estradiol increases both MCN and the primary spermatogonial mitotic index in the *Rana esculenta* testis. The above effects, together with the inhibition of androgen production (Fasano et al., '89a), are counteracted by tamoxifen, supporting the existence of intratesticular (autocrine/paracrine) mechanisms of action.

Fig. 6. **A:** In vitro effect of estradiol-17β and estradiol-17β given in combination with tamoxifen (Tam) (100-fold in excess) on MCN in February frog testis. Vertical bars indicate values ± SD. **B:** In vitro effect of estradiol-17β and estradiol-17β given in combination with tamoxifen (100-fold in excess) on mitotic index of primary spermatogonia (ISPG) in February frog testis. Vertical bars indicate values ± SD. **C:** Testicular androgen levels of February animals after 24 h of incubation with estradiol-17β at 10^{6} M or estradiol-17β at 10^{-6} M + tamoxifen (Tam) 10^{-4} M. Vertical bars indicate values ± SD.

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