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Inhibin Reduces Spermatogonial Numbers in Testes of Adult Mice and Chinese Hamsters

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ABSTRACT. Bovine follicular fluid (bFF) injected ip in mice during 2 days (65,000 U inhibin/day, 1 U inhibin the activity in 1 μg bFF protein) caused a significant decrease in the numbers of A_4, intermediate (In), and B spermatogonia to 91%, 74%, and 67% of the control values, respectively. The numbers of undifferentiated spermatogonia remained unchanged. These injections suppressed peripheral FSH levels to 6% of the control values, suggesting that FSH might be the modulator of the effects on spermatogenesis.

However, in the Chinese hamster, intratesticular injections of bFF during 4 days (6500 U inhibin/day into one testis) also caused a significant decrease in the numbers of A_3, In, B_1, and B_2 spermatogonia to 86%, 61%, 55%, and 94% of the control values, respectively. Similarly, treatment with a partially purified inhibin preparation from rat Sertoli cell-conditioned medium (rSCCM) during 4 days (Mono Q fraction; 1512 U inhibin/day; 37.8 μg protein) caused a significant decrease in the numbers of A_3, In, B_1, and B_2 spermatogonia to 90%, 87%, 66%, and 93% of the control values, respectively. Treatment with a highly purified inhibin preparation from rSCCM during 4 days (30K inhibin; 750 U inhibin/day; 100 ng protein) significantly decreased the numbers of In and B_1 spermatogonia to, respectively, 87% and 91% of the control values. These effects were limited to the testis into which the material was injected; the contralateral testis or testes injected with control fluid always showed normal numbers of spermatogonia. This implies that the effects on the seminiferous epithelium are not FSH mediated.

Intratesticular injections of bFF or pure inhibin did not affect the number of undifferentiated spermatogonia. However, the Mono Q fraction caused a significant increase in the numbers of undifferentiated spermatogonia in stages IV–VII of the cycle, suggesting the presence of a mitogenic factor for undifferentiated spermatogonia in rSCCM which is not present or is counteracted in bFF.

The results suggest that inhibin may have a role in the regulation of spermatogonial development in the adult animal. (*Endocrinology* **125:** 1899–1903, 1989)

INHIBIN is a glycoprotein hormone "which inhibits gonadotropin production and/or secretion, preferentially that of FSH" (1, 2). While it has been demonstrated that inhibin plays a key role in the regulation of peripheral FSH levels during the early stages of spermatogenesis in the rat (3–8), the physiological role of inhibin as a feedback regulator of FSH secretion in the adult animal remains to be determined (9, 10). Large amounts of inhibin have been found in testicular homogenates of adult rats (4, 6). Hence, it is possible that inhibin fulfills a function in the adult testis not involving its feedback action on FSH secretion. In fact, several pieces of evidence have suggested paracrine roles for inhibin and/or inhibin-related peptides. Both the Leydig cells (11) and spermatogonia (8) have been suggested as intratesticular targets for inhibin.

To gain more insight into the role of inhibin on adult spermatogenesis, we examined the effects of administration of bovine follicular fluid (bFF) and partially and highly purified inhibin preparations from rat Sertoli cell-conditioned medium (rSCCM) on spermatogonial development in adult mice and Chinese hamsters.

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Materials and Methods

Animals

Male Chinese hamsters (12 weeks old) and Cpb-N mice were obtained from the Animal Breeding Station HSD/CPB (Zeist, The Netherlands). Mice were mated at random in our laboratory, and their offspring (12 weeks old) were used in this investigation. All animals were kept under controlled conditions of light (lights on, 0445–1900 h) and temperature (22–24 C).

Experimental protocols

Exp I. The aim of this experiment was to investigate whether systemic injections of bFF could affect spermatogenesis of adult mice.

Charcoal-treated bFF (1 U inhibin/μg protein; 65 μg protein/μl) was prepared as previously described (12). Saline (0.9% NaCl) was used as control material. The plasma of an ovariectomized cow (bPcas) was used as an additional control material, since its protein concentration is similar to that of follicular fluid (13), and no inhibin activity is present.

Mice were injected ip with bFF, saline, or bPcas. Groups of three mice were injected twice daily with bFF (0.5 ml each time), during 1 (24 h bFF) or 2 days (48 h bFF). One group of three mice was injected twice on the first day and once on the second day (0.5 ml each time; 32 h bFF). Groups of four mice were injected with control material twice daily (0.5 ml each time) during 2 days. Eight hours (32 h bFF) or 16 h (24 h bFF, 48 h bFF, and control groups) after the last injection of bFF or control material, animals were killed for testicular histology.

Exp II. The aim of this experiment was to investigate whether the effects caused by bFF were mediated by suppressed FSH levels or whether bFF could affect spermatogenesis directly. Therefore, the material was injected intratesticularly. As mice could not be used for this purpose due to their small testis size [testis weight, 86 ± 2 mg/testis (mean ± SEM) n = 8], adult Chinese hamsters were used instead (testis weight, 1.00 ± 0.05 g/testis; n = 8). The same bFF preparations and control materials were used as those used for the mice.

Under light ether anesthesia the hamsters received intratesticular injections of bFF, saline, or bPcas. Groups of three hamsters were injected once daily with bFF (0.1 ml each time) during 2 (48 h bFF), 3 (72 h bFF), or 4 days (96 h bFF). Groups of four hamsters were injected with control material once daily during 4 days (0.1 ml each time). Twenty-four hours after the last injection of bFF or control material, animals were killed for testicular histology. The contralateral testes of animals injected with bFF during 4 days were used to evaluate whether the effects caused by bFF were local or mediated by FSH.

Exp III. To find out whether the effects were indeed caused by inhibin and not by other factors also present in bFF (14–18), a partially purified inhibin preparation from rSCCM (Mono Q fraction) was used in this experiment. The effects on spermatogenesis in adult Chinese hamsters were investigated.

The partially purified inhibin preparation (40 U inhibin/μg protein; 378 ng protein/μl) was isolated from rSCCM as described previously (19). Tris buffer (Tris-HCl, pH 7.9) was used as control material.

Groups of four Chinese hamsters received intratesticular injections, under light ether anesthesia, of the Mono Q fraction or control material once a day during four days (0.1 ml each time). Twenty-four hours after the last injection of the partially purified inhibin preparation or control material, animals were killed for testicular histology. The contralateral testes of those injected with the Mono Q fraction were used as the controls for the local effect of the material.

Exp IV. Finally, in this experiment the effects of a highly purified inhibin preparation (30K inhibin) on spermatogenesis of adult Chinese hamsters were evaluated.

The highly purified inhibin preparation (7500 U inhibin/μg protein; 4 ng protein/μl) was isolated from rSCCM as previously described (19). The vehicle (0.04% BSA in saline) was used as control material.

Groups of five Chinese hamsters received intratesticular injections, under a light ether anesthesia, of 30K inhibin (25 μl each time) once a day during 4 days. As testes contralateral to those injected with bFF or the Mono Q fraction were not affected by these treatments (see *Results*), contralateral testes were used here for control injections. These were given at the same time as the injections of 30K inhibin (25 μl each time). Twenty-four hours after the last injection of inhibin or control material, animals were killed for testicular histology.

Histological studies

Seminiferous tubules were prepared according to the method of Clermont and Bustos-Obregon (20), fixed in Bouin's fluid, stained with Harris' hematoxylin, and mounted *in toto*. Spermatogonial cell types and epithelial stages in the mouse and the Chinese hamster were classified according to the methods of Oakberg (21), de Rooij (22), and Oud and de Rooij (23). Differentiating [A_1, A_2, A_3, A_4, intermediate (In), and B spermatogonia in the mouse, and A_1, A_2, A_3, In, B_1, and B_2 spermatogonia in the Chinese hamster] and undifferentiated spermatogonia and Sertoli cells were counted in the various stages of the seminiferous epithelium. Counts were made using an ocular grid (area of 144 μm^2) with a ×100 oil immersion objective placed over the domed center of the tubule. All spermatogonia having at least half of their nucleus within the frame were counted. The numbers found were related to the number of Sertoli cells present in the same areas. For each cell type a total area containing 1000 Sertoli cells, corresponding to about 7000 μm^2, was studied (for each animal, 50–100 segments of seminiferous tubules, 0.5–1.0 cm long, were used).

Hormone estimations

In vitro inhibin bioassay. For the determination of the endogenous inhibin concentration of Chinese hamster testes, homogenates were prepared from testes of four untreated adult Chinese hamsters, as described previously (4), and the cytosols were tested in the inhibin bioassay. Inhibin bioactivity was determined by *in vitro* suppression of basal FSH release from dispersed rat pituitary cells (24). Charcoal-treated bFF was used as a standard of inhibin activity.

RIA of FSH. Peripheral FSH concentrations in the mouse were estimated by RIA, (24) using NIDDK rat FSH RP-1 as a standard. Blood samples were collected in heparinized tubes, 16 h after the last injection of bFF or saline. Plasma (three and four samples for the bFF- and saline-treated groups, respectively) was stored at −20 C until further processing for the RIA.

Statistical analysis

The significance of differences between mean values of different treatment groups in Exp I, II, and III was estimated using Student's t test (under assumption of normality). The matched pair Student's t test was used in Exp IV. Differences were considered significant when $P < 0.05$.

Results

Effect of systemic injection of bFF on spermatogonial numbers in the adult mouse

Although Student's *t* test may not be completely reliable with the small numbers of mice used in this pilot experiment, the results strongly suggest an effect of bFF on the numbers of differentiating spermatogonia.

The first effect became clear after 24 h of treatment. By that time the numbers of In and B spermatogonia were lowered to 89% and 91%, respectively, of the control values (saline-injected group; Fig. 1). When the treatment was given for 2 days, the effect became more pronounced; the numbers of In and B spermatogonia were 74% and 67% of the control values, respectively (Fig. 1). Furthermore, degeneration of these cells could clearly be seen (Fig. 2). A slight decrease in the numbers of A_4 spermatogonia to about 91% of the control values was also detected. The numbers of A_1, A_2, and A_3 spermatogonia were not affected by treatment at all times studied (results not shown).

Injections of bFF never affected the number of undifferentiated spermatogonia scored in the same epithelial stages as the differentiating spermatogonia (results not shown).

Treatment with bPcas did not have any effect on spermatogonial numbers compared to that in the saline-injected group (Fig. 1).

The peripheral FSH levels in mice treated with bFF during 2 days were significantly lowered to 6% of the control values [162 ± 21 ng FSH/ml (n = 3) *vs.* 4521 ± 1298 ng FSH/ml in the control group (n = 4); mean ± SEM); $P < 0.01$].

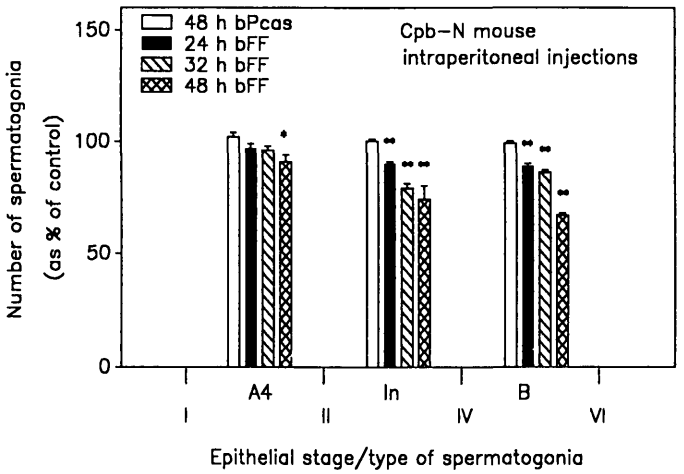

FIG. 1. Effect of systemic injection of bFF or bPcas on the numbers of differentiating spermatogonia in the adult mouse. Results have been expressed relative to those determined in saline-injected animals (mean ± SEM; n = 3 or 4). *, $P < 0.05$; **, $P < 0.01$ (by *t* test). The mean number of spermatogonia in each treatment group was calculated from the numbers of the different types of spermatogonia per 1000 Sertoli cells found per animal (an area containing 1000 Sertoli cells corresponds to about 7000 μm^2). For each animal, 50–100 segments of seminiferous tubules (0.5–1.0 cm long) were used.

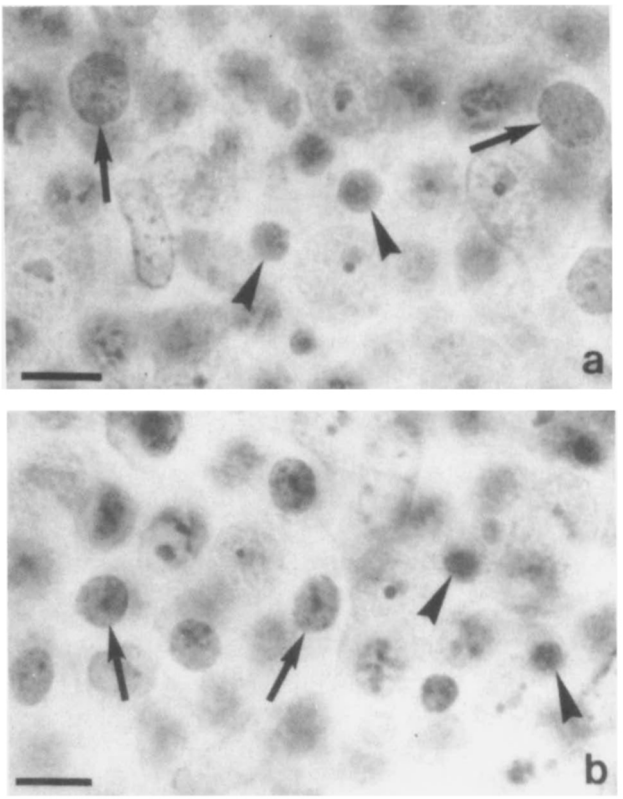

FIG. 2. Micrograph of mouse seminiferous tubules mounted *in toto*, showing degenerations of the In (a) and B spermatogonia (b) after bFF treatment during 2 days. The degenerating spermatogonia (*arrowheads*) were found adjacent to normal spermatogonia (*arrows*). Magnification, ×1068; *bar* = 9 μm.

Effect of intratesticular injection of bFF on spermatogonial numbers in the adult Chinese hamster

The effects of intratesticular injections of bFF were comparable to those observed after systemic injection of the material in the mouse. In the treated testis, the numbers of differentiating spermatogonia were clearly affected after 72 h of treatment (Fig. 3). At this time, the numbers of In and B_1 spermatogonia were lowered to 96% and 89% of the control values, respectively (saline-injected group). The degenerative process became more clear with increasing duration of the treatment, and by 4 days even the numbers of B_2 spermatogonia, which originally were not influenced by the treatment (99% of the control values after 72 h of treatment), were also lowered to 94% of the control values. At this time the numbers of In and B_1 spermatogonia were decreased to 61% and 55%, respectively, of the control values. The treatment also affected the numbers of A_3 spermatogonia, which by 4 days were decreased to 86% of the control values. No effect was found on A_1 and A_2 sper-

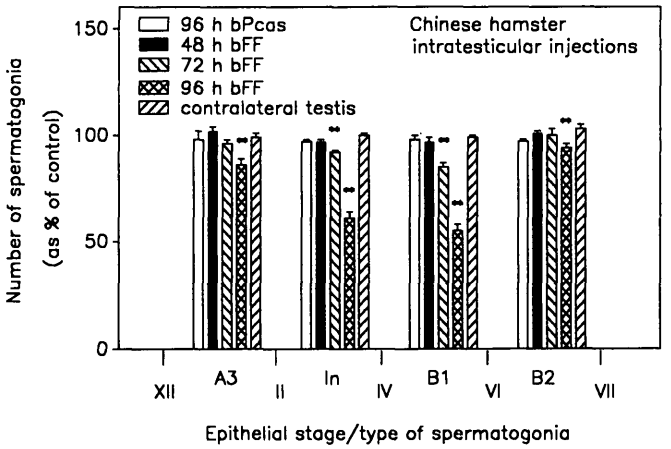

FIG. 3. Effect of intratesticular injection of bFF or bPcas on the numbers of differentiating spermatogonia in the adult Chinese hamster. Results have been expressed relative to those obtained in saline-injected animals (mean ± SEM; n = 3 or 4). **, $P < 0.01$ (by t test). The mean number of differentiating spermatogonia in each treatment group was calculated as described in Fig. 1.

matogonia (results not shown). The numbers of undifferentiated spermatogonia scored in the same stages as the differentiating spermatogonia were not affected by the treatment at any time studied (results not shown). All counts made in testes contralateral to those injected with bFF for 4 days were not significantly different from counts made in animals injected with saline (Fig. 3).

The inhibin activity measured in testicular homogenates of normal adult Chinese hamsters was 800 ± 50 U inhibin/testis [950 ± 37 U inhibin/g testicular tissue (mean ± SEM); n = 4].

Effect of intratesticular injection of the Mono Q fraction from rSCCM on spermatogonial numbers in adult Chinese hamsters

When the inhibin-containing Mono Q fraction was used, comparable effects were obtained as after the bFF treatment. A significant lowering of the numbers of A_3, In, B_1, and B_2 spermatogonia to, respectively, 90%, 87%, 66%, and 93% of the control values was found (Fig. 4a). In contrast to what was found with bFF the number of undifferentiated spermatogonia was also affected by this treatment. Their numbers were significantly increased in stages IV–VI and VI–VII of the epithelial cycle to 176% and 145%, respectively, of the control values (Fig. 4b). Counts in testes contralateral to those injected with the Mono Q fraction were not different from those in the control testes (Fig. 4a).

Effect of intratesticular injection of 30K inhibin from rSCCM on spermatogonial numbers in adult Chinese hamsters

As with the Mono Q fraction, the numbers of In and B_1 spermatogonia were significantly lowered in the inhibin-treated testis (Table 1). However, the numbers of A_3 and B_2 spermatogonia were unchanged compared to the control values. Also, the numbers of undifferentiated spermatogonia were not affected by the treatment (Table 1).

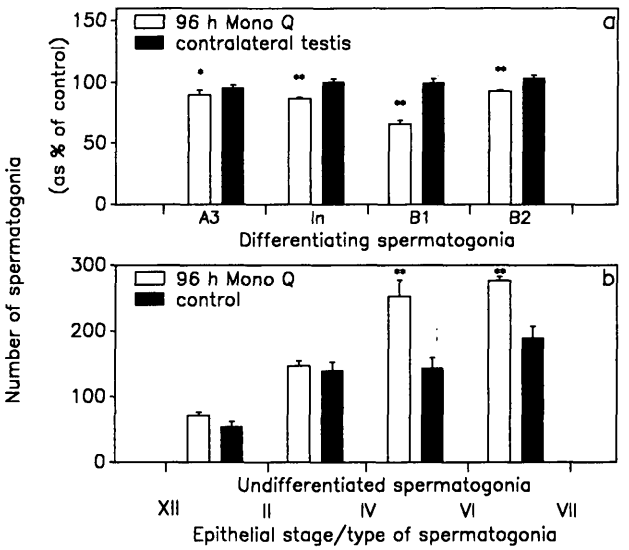

FIG. 4. Effect of intratesticular injection of a partially purified rat inhibin preparation (Mono Q fraction) on the numbers of differentiating (a) and undifferentiated (b) spermatogonia in the adult Chinese hamster. The numbers of differentiating spermatogonia have been expressed relative to those obtained in Tris buffer-injected controls (mean ± SEM; n = 4). *, $P < 0.05$; **, $P < 0.01$ (by t test). The numbers of undifferentiated spermatogonia are expressed as mean numbers of spermatogonia per 1000 Sertoli cells. The mean number of spermatogonia in each treatment group was calculated as described in Fig. 1.

TABLE 1. Effect of intratesticular injection of highly purified 30K rat inhibin on the numbers of differentiating and undifferentiated spermatogonia in the adult Chinese hamster

Cell type (stage of the cycle)	Contralateral testis	30K Inhibin
Differentiating spermatogonia		
A_3 (XII–II)	266 ± 3	263 ± 4
In (II–IV)	514 ± 6	446 ± 6*
B_1 (IV–VI)	1049 ± 9	959 ± 2*
B_2 (VI–VII)	2084 ± 40	2076 ± 13
Undifferentiated spermatogonia		
XII–II	58 ± 6	54 ± 6
II–IV	136 ± 6	124 ± 22
IV–VI	168 ± 15	165 ± 23
VI–VII	203 ± 24	211 ± 21

The mean number of spermatogonia in each treatment group was calculated as described in Fig. 1. Values are the mean ± SEM (n = 5).
* $P < 0.01$ (by matched pair t test).

Discussion

The present results demonstrate that in the adult testis, increased levels of inhibin cause a decrease in the

numbers of differentiating spermatogonia at particular stages of the epithelial cycle. As the systemic injections of bFF in mice also caused a significant decrease in peripheral FSH levels, the most likely explanation seemed to be that these effects were mediated by the suppression of FSH. However, comparable effects were obtained when bFF- or inhibin-containing preparations were administered intratesticularly. Furthermore, in Chinese hamsters, no effect on the numbers of differentiating spermatogonia were seen in the testes contralateral to those injected with bFF or the Mono Q fraction. This indicates that inhibin has a local effect on spermatogonial development in the adult animal. Whether inhibin exerts its effects directly on the spermatogonia or through an action on Sertoli or interstitial cells is at this time unknown.

The effects of administration of inhibin are delayed in the Chinese hamster compared to those in the mouse (first effect seen after 72 *vs.* 24 h). This may be due to the differences in the cell cycle duration of the differentiating spermatogonia in both animals (57–60.5 h in the Chinese hamster *vs.* 27–30.5 h in the mouse) (25, 26).

The effects of inhibin seem to be dose dependent, as in the Chinese hamster intratesticular injections of 6500 U inhibin/day (bFF) caused a greater decrease in spermatogonial numbers than 1512 U inhibin/day (Mono Q fraction) or 750 U inhibin/day (30K inhibin). In the normal testis, the inhibin content was 800 ± 50 U inhibin. Hence, immediately after bFF injection, the inhibin content of the testis may have increased to about 8-fold the normal value, whereas with the Mono Q fraction or the highly purified inhibin preparation the increase may only have been around 3- to 2-fold, respectively. However, it cannot be excluded that bFF affects spermatogenesis in a different way than the purified preparations from rSCCM (24, 27).

Franchimont *et al.* (8) showed a decrease in testicular DNA synthesis in immature rats after treatment with a partially purified inhibin preparation from ram rete testis fluid. Their inhibin preparation was shown to affect DNA synthesis of In and B spermatogonia *in vivo* as well as *in vitro*. The researchers postulated that their inhibin preparation and the testicular chalone of Clermont and Mauger (28) were similar. However, the differences between the testicular chalone, which only acts on the proliferative activity of type A spermatogonia (28, 29), and the preparation of Franchimont *et al.* (8), which only influences the DNA synthesis of differentiating spermatogonia, suggest that other factors, possibly inhibin, were involved in the decrease in spermatogonial DNA synthesis. Although the researchers did not find any effect in adult rats, the local effect of inhibin on spermatogonial development described by Franchimont *et al.* (8) is reminiscent of our data. The effect on DNA synthesis may precede the degeneration of spermatogonia described in the present study. Alternatively, the decrease in testicular DNA synthesis may also be the result of degeneration of spermatogonia. The fact that Franchimont *et al.* (8) did not find an effect on adult spermatogenesis might be explained by the different sources of inhibin used and the different protocols applied.

The differentiating spermatogonia present in stages I–VI of the epithelial cycle seem to be the most sensitive to increased levels of inhibin. The decrease in the numbers of B_2 spermatogonia in the Chinese hamster is clearly due to the decrease in the numbers of their precursors, the B_1 spermatogonia, at an earlier time and do not reflect a sensitivity of these cells for inhibin.

It is not known as yet whether the production of inhibin varies with the stage of the epithelial cycle, as has been shown for various other Sertoli cell products (30). However, in the rat mRNA expression of the α- and βB-subunits of inhibin varied significantly during the cycle of the seminiferous epithelium (31). mRNA levels of both subunits were minimal in stages II–VI and VII–VIII. If the inhibin production is indeed low or absent in these stages, it is plausible that increased inhibin levels there could affect the seminiferous epithelium.

Spermatogonial degeneration of certain types of differentiating spermatogonia, such as A_1, A_2, and A_3 spermatogonia in the rat (32) or In spermatogonia in the ram and the bull (33), is a physiological phenomenon. The extent of these degenerations is strictly correlated to the density of the spermatogonia and enables the epithelium to regulate the density of the spermatocytes (34). In view of the present results, it is tempting to speculate that in a normally functioning adult testis, inhibin has a role in the regulation of spermatogonial numbers.

It is remarkable that the Mono Q fraction caused an increase in the numbers of undifferentiated spermatogonia in stages IV–VII after 4 days of treatment. Since the effects of injection with bFF or the Mono Q fraction may be caused by different factors present in both preparations, it is likely that a factor in the Mono Q fraction, which is not present in the highly purified inhibin preparation and is absent or counteracted in bFF, enhances the proliferative activity of undifferentiated spermatogonia. Several growth factors have been described in the testis, such as the seminiferous growth factor (35) and Sertoli cell-secreted growth factor (36, 37). It is, however, not known whether these factors affect spermatogonial multiplication or differentiation. Studies are currently underway to isolate and characterize the mitogenic factor in the Mono Q fraction.

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