In vitro fertilizing capacity of sperm from FSH-treated photoinhibited Djungarian hamsters (*Phodopus sungorus*)

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Abstract

In hypogonadal male Djungarian hamsters FSH alone can induce normal spermatogenesis. However, for the induction of mating behavior, supplementation with testosterone is necessary. We have here investigated, by *in vitro* fertilization, whether sperm produced by photoinhibited hamsters treated with FSH alone can fertilize without testosterone. Photoinhibited hypogonadal male Djungarian hamsters were injected daily with human FSH (10 IU; Fertinorm) for 5–7 weeks. The hormone stimulated re-growth of the testes. Neither body weight nor the weights of the androgen-dependent organs – epididymides, prostate, accessory glands – showed significant differences from photoinhibited controls; furthermore FSH treatment did not raise intratesticular or serum testosterone levels. In eleven out of the twelve FSH-treated photoinhibited hamsters, elongated spermatids were found in the testes; in five out of nine epididymides, sperm was found by histological examination. In two out of the twelve FSH-treated hamsters, the amount of sperm in the caudal part of the epididymis was sufficient for *in vitro* fertilization with oocytes collected from superstimulated females. These spermatozoa fertilized 16–29% of the oocytes. Spermatozoa from photostimulated controls produced similar levels of fertilization.

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Introduction

The role of follicle-stimulating hormone (FSH) in the regulation of spermatogenesis in the adult mammal is still controversial. In primates, the reinitiation of quantitatively normal spermatogenesis with either testosterone alone or FSH alone is possible. However, both hormones appear to be considerably more potent in maintaining than in reinitiating the spermatogenic process (Weinbauer & Nieschlag 1993, Sharpe 1994). Data from studies in rodents suggest that FSH is not required for spermatogenesis. In adult rats either being treated with a gonadotropin-releasing hormone (GnRH) antagonist (Rea et al. 1986) or immunized against GnRH (Awoniyi et al. 1989, 1992a), testosterone is able respectively to reinitiate or maintain qualitatively normal spermatogenesis. Other studies in rats support the concept of a synergistic effect of testosterone and FSH (Rea et al. 1987, Weinbauer & Nieschlag 1993, Sharpe 1994, McLachlan et al. 1995). Direct support for a role of FSH in the reinitiation of spermatogenesis was obtained in another rodent, the Djungarian hamster (*Phodopus sungorus*). In this seasonally breeding species, short photoperiods induce secondary hypogonadism and testicular involution. In the maximally regressed testis, the cellular population of the seminiferous tubules consists mainly of spermatogonia, some primary spermatocytes, and Sertoli cells (Bergmann 1987), a situation similar to that seen in hypophysectomized hamsters (Niklowitz et al. 1989). In these photoinhibited hamsters, FSH alone can restore spermatogenesis to a qualitatively normal extent in the absence of steroidogenically active Leydig cells and thus at very low testosterone concentrations. Luteinizing hormone (LH) alone, however, causes full redifferentiation of Leydig cell function with minor effects on tubular function (Niklowitz et al. 1989).

Spermatozoa produced by FSH-treated photoinhibited hamsters are released from the testes and reach the epididymis (Lerchl et al. 1993). The supplementation of testosterone at the end of the FSH treatment induces mating behavior and results in normal pregnancy rates of mated females without increasing intratesticular testosterone. Thus it remains unclear as to whether FSH alone produces ‘fertile’ sperm, or whether the late addition of testosterone is required to render the sperm viable. The present study was designed to provide further evidence that sperm produced by hypogonadal hamsters treated with FSH alone are capable of fertilization without supplementation with testosterone.
Materials and Methods

Animals

Male and female Djungarian hamsters (Phodopus sungorus) were raised in our colony and kept in cages with one to three littermates under long photoperiods with 16 h of light per day at a temperature of 22 ± 2 °C. Food and water were available ad libitum. For details of breeding conditions see Lerchl (1995). All experiments were in accordance with local guidelines and with German law on the care and use of laboratory animals.

Supерovulation

Females ranging in age from 3 to 4 months and exhibiting a normal estrous cycle (Schlatt et al. 1993) were given 10 IU FSH (Fertinorm; Serono, Freiburg, Germany) in 100 µl saline i.p. in the afternoon (1600 h) of day 1 during diestrus I. After 50 h the females were injected i.p. with 10 IU human chorionic gonadotropin (hCG; Pregnesin; Serono) in 100 µl saline in the afternoon of day 3 (1800 h). Oocytes were collected 15–17 h after injection of hCG in the morning of day 4 (0900–1100 h). During the period of superstimulation the females were kept together with vasectomized males. This procedure resulted in higher numbers of harvested eggs than without males.

Culture media

All chemicals were obtained from Sigma, Deisenhofen, Germany, unless stated otherwise. A modified Tyrode’s medium (Bavister 1969) was made by adding 30 mg sodium bicarbonate, 1·1 mg sodium pyruvate, 10 mg glucose, and 25 mg BSA to 10 ml Tyrode’s solution. For sperm capacitation, heat-inactivated human follicular fluid (hFF; 20%) was substituted for BSA. The pH of the medium was 7·8 and the osmolarity 345 mOsm. On the evening prior to oocyte collection, culture dishes (Nunc) were prepared and placed with the remaining media for equilibration in an incubator (37 °C) gassed with 5% CO₂.

In vitro fertilization (IVF)

The IVF working protocol was modified as described by Parkening (1990). In the morning of day 4 (0730 h) one male was killed and the epididymis dissected and placed into a culture dish without medium. An incision was made into the cauda epididymis and a droplet of sperm was transferred to medium containing hFF under oil. After 5 min, 20 µl aliquots were removed for checking of motility and concentration. After 30 min, the sperm were diluted to a final concentration of 500 000/ml in the IVF culture dish with medium under oil. The diluted sperm were preincubated for 1–3 h before the addition of oocytes.

For oocyte preparation (0900–1100 h) the superstimulated females were killed and ovaries and oviducts were dissected and transferred to medium without oil. Oviducts were placed in fresh medium. Under a dissecting microscope, the wall of the ampulla was ruptured, allowing the oocytes within their cumulus mass to exude into the medium. Using sterile glass capillary tubes the oocytes were transferred to the IVF culture dish containing the sperm and incubated for 9–10 h.

In the evening of day 4 (1900–2000 h) the oocytes were washed once (media under oil) and transferred to culture dishes containing fresh media (under oil). In the morning of day 5 the success of fertilization was checked by counting the oocytes cleaved to the two-cell stage or from the appearance of the pronuclei.

Experimental design

FSH kinetics

Previous experiments have revealed that the injection of 5 IU FSH did not lead to a clear elevation of serum FSH in all treated hamsters over a time-period of 24 h. In the present study, therefore, 10 IU FSH in 0·2 ml saline or the vehicle only were injected s.c. into 52 photoinhibited male hamsters (testes not palpable) which had been kept for 8 weeks in short photoperiods of 8 h light:16 h darkness (8L:16D). At each time-point, four to five animals were killed 0, 3, 6, 9, 12 and 24 h after the injection (0900 h), trunk blood was collected, and serum was stored at −20 °C until assayed for FSH.

Phase I: Photoinhibition

Male adult hamsters, 3–4 months of age, were transferred from long photoperiods (16L:8D, lights on at 0400 h) to short photoperiods (8L:16D, lights on at 0800 h). Body weight and testicular size were monitored at 2-weekly intervals. Testicular size was estimated by palpation, a method which had been verified in former studies (Hoffmann 1973, 1978). After 8 weeks in short photoperiods 24 hamsters with completely regressed testes and 12 long-day controls with large testes were transferred to individual cages and used for phase II.

Phase II: FSH stimulation

Twelve photoinhibited hamsters under short photoperiods were injected daily with 10 IU FSH in 0·2 ml saline s.c. between 0800 and 1000 h for 5 or 7 weeks. Twelve short-day controls and twelve long-day controls were injected daily with the vehicle only. Body weight and testicular size were monitored at weekly intervals. Depending on their gonadal status, these animals were selected to be used in phase III.

Phase III: IVF

Depending on whether it was possible to obtain sperm from the cauda epididymis, the sperm of FSH-stimulated photoinhibited hamsters were tested for...
their IVF capacity after 5 or 7 weeks of FSH treatment. For each IVF experiment, the eggs of eight superstimulated females were collected and, equally distributed, incubated with the sperm of FSH-stimulated hamsters and the sperm of long-day controls. The male hamsters were killed by decapitation, trunk blood was collected and serum was stored at −20 °C until assayed for testosterone. The epididymides were removed, weighed, and examined for sperm in the caudal tubules. The epididymides not used for IVF were immersion-fixed in Bouin’s solution. Testes, accessory glands, and prostates were removed and weighed. One testis was frozen on dry ice and stored at −20 °C until assayed for testosterone. The other testis was fixed in Bouin’s solution.

Histological analysis
After at least 48 h of fixation the testes and epididymides were embedded in paraffin following dehydration. Sections of 5 µm thickness were cut and stained with hematoxylin/eosin. All the tubules of at least ten testicular or epididymal cross-sections were recorded for the presence or absence of sperm.

RIAs
The concentration of serum FSH was estimated by double-antibody techniques as described previously (Lerchl et al. 1993). Precision was estimated with pooled control sera and was 15.1% at 1.8 ng/ml, 8.7% at 6.6 ng/ml, and 12.3% at 28 ng/ml. The minimal detectable concentration was 1.0 ng/ml. All samples were run in one assay. Samples of homogenized testes and serum were ether extracted and analyzed for testosterone by an RIA method which has been described previously (Lerchl & Nieschlag 1995). Precision was 19.5% at 26 pg/tube, 7.7% at 92 pg/tube, and 9.4% at 273 pg/tube. The minimal detectable concentration was 8.5 pg/tube. Serum and testes samples were run in single assays. Values were corrected for recoveries.

Statistical analysis
Parameters were subjected to one-way ANOVA followed by least square difference multiple range test with 95% confidence intervals (Statgraphics V. 5-0; STSC, Inc.). Data are expressed as means ± s.e.m.

Results
The subcutaneous injection of FSH into photoinhibited hypogonadal hamsters led to a significant elevation (P<0.0001) of serum FSH over a time-period of 24 h in all treated animals in comparison with vehicle-treated controls (Fig. 1). As presented in Fig. 2, there was a clear reduction in body weight after transfer to short photoperiods. Up to week 15 there was no sign of spontaneous recrudescence. At no time-point did the vehicle-injected short-day hamsters show significant differences in body weight from the FSH-injected photoinhibited hamsters.

While all hamsters maintained in long photoperiods retained large palpable testes, photoinhibition led to a clear reduction of testicular volume to a non-palpable stage. The FSH injectionstimulated the regrowth of the testes, in some hamsters up to the full long-day size. The five hamsters which had been treated with FSH for 7 weeks showed no further testicular growth during the last 2 weeks of injections.

The positive effect of FSH on testicular volume was confirmed by the measurement of testicular weight at the end of the experiment (Table 1). The paired testes weight of FSH-treated photoinhibited hamsters was significantly elevated above that of the vehicle-treated short-day hamsters. However, none of the other weight parameters – neither for accessory glands, prostates nor epididymides – showed significant differences between the short-day groups. The concentrations of intratesticular and serum testosterone were also not different (Table 1). In eleven out of twelve FSH-treated short-day hamsters, elongated spermatids were found in the testes (long-day controls: 12/12; short-day controls: 0/12). In five out of nine epididymides (the epididymides of three animals were damaged during IVF search for sperm and not used for histological examination) sperm was found by histological
examination of the tubules of these FSH-treated animals. However, only in two out of these five positive epididymides was the amount of sperm in the caudal part of the epididymis sufficient for IVF.

Table 2 shows the fertilization rate of oocytes from superstimulated females with sperm collected from the males. Spermatozoa collected from 5- and 7-week FSH-treated hamsters fertilized, respectively, 16% and 29% of the oocytes. Sperm from photostimulated long-day hamsters had been incubated in parallel with a resulting range of fertilization between 8% and 50%. Within the FSH-treatment group there was no statistical difference in testosterone concentrations between those animals with or without sperm in the epididymides.

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Discussion

The fertilization capacity of sperm obtained from FSH-treated hamsters could be achieved at low levels of testosterone. FSH did not raise intratesticular testosterone concentrations or serum testosterone and had no influence on the weight of the androgen-dependent accessory glands or prostates. The stimulating effect on the testis cannot be attributed to spontaneous recrudescence (Stetson et al. 1977, Hoffmann 1979) since there was no increase in body weight or testicular weight of the vehicle-treated short-day controls.

Only in two out of twelve FSH-treated photoinhibited hamsters was the amount of sperm in the caudal part of the epididymis sufficient for IVF. However, if sperm were found macroscopically in the epididymal tubules they were able to fertilize hamster eggs in vitro. During their passage through the epididymis spermatozoa acquire the capacity to move and fertilize eggs. By far the most important hormone affecting the epididymis is testosterone (for review see Cooper 1995). An androgen-free experimental situation may allow qualitative reinitiation of spermatogenesis, but may hinder the maturation and storage of sperm in the epididymis of the FSH-treated short-day hamsters. At all events, the results presented here demonstrate that such sperm may have full fertilizing capacity even in an epididymis not exposed to testosterone.

Parkening (1990) developed a method for IVF of Djungarian hamster oocytes and investigated the development of early embryos in culture. He reported that this species responds unhomogeneously to superstimulation with gonadotropins. Females of the same weight and the same stage of the estrous cycle, given a particular regimen of gonadotropins, may ovulate two or three oocytes or ten to fifteen or may not ovulate at all. The mean number of oocytes shed in cyclic Djungarian hamsters is reported to be slightly more than five oocytes for both ovaries combined, reflecting a failure of some early preovulatory follicles to ovulate (Wynne-Edwards et al. 1987). The present investigation confirms the difficulties in superstimulation of Djungarian hamsters. Unlike mice or Syrian hamsters, even littermates respond with an extremely random pattern of ovulation. The authors found it helpful if the superstimulated females were kept together with vasectomized males during the period of gonadotropin treatment. In these females, the ampulla and the whole oviduct seemed to be more swollen and it appeared to be easier to identify the cumulus mass with oocytes within the ampulla.

The present study was the logical continuation of two previous investigations by our laboratory. In 1989 Niklowitz et al. showed for the first time that treatment with FSH alone induced qualitatively full restoration of testicular tubular function in photoinhibited hamsters, while FSH had no direct effect on Leydig cell steroidogenesis, i.e. neither increasing intratesticular testosterone nor enhancing 3β-hydroxysteroid dehydrogenase activity. However, FSH enhanced the in vitro response of interstitial cells to hCG: LH alone caused full redifferentiation of Leydig cells with minor effects on spermatogenesis. Lerchl et al. (1993) demonstrated that spermatozoa produced by these FSH-treated photoinhibited hamsters were released and reached the epididymis. When photoinhibited hamsters received FSH plus late testosterone – to induce copulatory and ejaculatory behavior – mating behavior could be induced which resulted in normal pregnancy rates of mated females without increasing intratesticular testosterone. In fact, mammalian spermatogenesis may proceed with low amounts of androgens (for review see Weinbauer & Nieschlag 1990). The present study provides further evidence that the treatment of hypogonadal hamsters with FSH alone may restore fertility. In the Djungarian hamster, FSH may not only reinitiate the spermatogenic process but, in addition, the rapid increase in serum FSH after photostimulation is the primary signal for initiating testicular development in this seasonally breeding species (Milette et al. 1988). Interestingly, in contrast to other rodents, LH, or testosterone alone, is only marginally effective in restoring spermatogenesis (Niklowitz et al. 1989). In rats with intact pituitary glands, testosterone is able to maintain quantitatively normal spermatogenesis (Robaire et al. 1979, Sharpe et al. 1988, Awoniyi et al. 1992a) or can restore full fertility (Awoniyi et al. 1992b), suggesting that FSH is not required for spermatogenesis under normal conditions of high intratesticular testosterone concentrations. Other findings

### Table 2: IVF capacity of sperm from FSH-treated hypogonadal hamsters and controls

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Photoperiod</th>
<th>Weeks of treatment</th>
<th>Oocytes fertilized</th>
<th>Percentage of fertilized oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>16L:8D</td>
<td>7</td>
<td>5/10</td>
<td>50</td>
</tr>
<tr>
<td>Vehicle</td>
<td>16L:8D</td>
<td>7</td>
<td>1/13</td>
<td>8</td>
</tr>
<tr>
<td>Vehicle</td>
<td>16L:8D</td>
<td>7</td>
<td>3/20</td>
<td>15</td>
</tr>
<tr>
<td>FSH</td>
<td>8L:16D</td>
<td>5</td>
<td>2/7</td>
<td>29</td>
</tr>
<tr>
<td>FSH</td>
<td>8L:16D</td>
<td>7</td>
<td>3/19</td>
<td>16</td>
</tr>
</tbody>
</table>
support the involvement of FSH in the spermatogenic process of the adult rat. In hypophysectomized rats, germ cell development was supported qualitatively by FSH up to step 7 of spermiogenesis (Bartlett et al. 1989), in GnRH antagonist-treated animals, the complete process of spermatogenesis was maintained qualitatively by FSH (Chandolia et al. 1991). In hypophysectomized and Leydig cell-depleted rats, FSH failed to exert a stimulatory effect on spermatids (Kerr et al. 1992), supporting a concept of synergism between FSH and testosterone. However, the evidence for independent or synergistic effects of testosterone and FSH depends on the experimental model. In the photoinhibited Djungarian hamster, testosterone alone is unable to restore spermatogenesis, whereas FSH alone reinitiates spermatogenesis in a qualitatively normal manner. The sperm produced under this condition reach the epididymis and have IVF capacity.

From an ecophysiological point of view it seems to make sense that the processes of sperm production and sexual maturation are separated in seasonal breeders. The presence of the full spectrum of sexual behavior is a waste of energy as long as no sperm are available. These different requirements may explain the obvious differences between the actions of FSH and LH on the testes of seasonally breeding mammals.

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