

Sexual Function and Fertility in Hypophysectomized Adult Male Rats After Pituitary Grafting: Part I

Hipofizektomi Yapılan Erişkin Erkek Sıçanlarda Pituitier Greftleme Sonrası Seksüel Fonksiyon ve Fertilité

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Abstract: Male rats underwent hypophysectomy and graft procedures, and a normal control group (C) was also established. In the hypophysectomy-plus-pituitary graft (HG) group, a suspension of neonatal rat pituitary cells (cortical cells in the hypophysectomy-plus-cortical graft [CG] group) was implanted into the third ventricle. Four weeks later, the surviving hypophysectomy only H (n=21), HG (n=21), and CG (n=21) rats were evaluated for copulation/ejaculation/fertility. Endocrine target organs were histologically examined, and serum levels of luteinising hormone (LH) and testosterone were compared among the four groups. LH response to endogenous (cold stress) and exogenous (gonadotropin-releasing hormone injection) stimuli was also assessed in the experimental groups. The HG group had higher LH than the H and CG groups (0.89 ± 0.2 pg/ml versus 0.58 ± 0.2 pg/ml and 0.47 ± 0.3 pg/ml, respectively; $p < 0.05$), but lower LH than controls ($p < 0.05$). The LH response to exogenous and endogenous stimuli was greater in the HG than in the H and CG rats ($p < 0.05$). Only control rats had detectable testosterone. HG rats (52.4%) were more sexually active than H (5.3%) and CG (5.5%) animals ($p < 0.001$). Histologically, grade 3 thyroidal and testicular atrophy predominated in the H and CG groups (17 [89.5%] and 15 [83.3%] rats, respectively), whereas grade 2 atrophy predominated in the HG animals (17 [81%]). Implantation of neonatal pituitary grafts into the third ventricle of hypophysectomized rats caused LH secretion to rise, but not enough to stimulate testosterone secretion.

Key words: Hypophysectomy, pituitary transplantation, sexual function, LH, testosterone, fertility

Özet: Erkek sıçanlara hipofizektomi ve greft uygulamaları yapıldı ve normal kontrol grubu (C) çalışmaya dahil edildi. Hipofizektomi sonrası hipofiz grefti (HG) uygulanan grupta, yenidoğan hipofiz hücrelerinden hazırlanan süspansiyon, hipofizektomi sonrası kortikal greft (CG) uygulanan grupta ise kortikal hücreler içeren süspansiyon üçüncü ventrikül içine enjekte edildi. 4 hafta sonra, yaşayan sadece hipofizektomili (H, n=21), hipofiz greftli (HG, n=21), ve kortikal greftli (CG, n=21) sıçanlar kopülasyon/ejakülasyon/fertilité için değerlendirildi. Endokrin hedef organlar histolojik olarak incelendi. Luteinizan hormon (LH) ve testosteron serum düzeyleri 4 grup arasında karşılaştırıldı. Deney gruplarında, ayrıca endojen (soğuk stres) ve eksojen (gonadotropin releasing hormon injeksiyonu) stimuluslara LH sekresyon cevabı değerlendirildi. HG grubunda LH düzeyi H ve CG gruplarından daha yüksekti (0.89 ± 0.2 pg/ml versus 0.58 ± 0.2 pg/ml and 0.47 ± 0.3 pg/ml, respectively; $p < 0.05$), ancak kontrol grubundaki LH düzeyinden daha düşüktü ($p < 0.05$). HG grubunda, endojen ve eksojen stimuluslara LH sekresyon cevabı, H ve CG grubundaki sıçanlardan daha yüksekti ($p < 0.05$). Sadece kontrol grubu sıçanlarda tespit edilebilen düzeyde testosteron vardı. HG grubundaki sıçanlar (%52.4) seksüel olarak H (%5.3) ve CG (%5.5) gruplarındaki sıçanlardan daha aktifti ($p < 0.001$). H ve CG gruplarında histolojik olarak grade 3 tiroidal ve testiküler atrofi hakimdi (sırasıyla 17 [89.5%] ve 15 [83.3%] sıçan). HG grubunda ise tersine grade 2 atrofi ön plandaydı (17[81%]sıçan). Hipofizektomili sıçanların 3. ventrikülleri içine yenidoğan hipofiz greftlerinin implante edilmesi, LH sekresyonunda artmaya yol açar. Ancak bu artış testosteron sekresyonunu stimüle etmek için yeterli değildir.

Anahtar kelimeler: Hipofizektomi, hipofiz transplantasyonu, seksüel fonksiyon, LH, testosteron, fertilité

INTRODUCTION

It has been known for years that tissues transplanted to the central nervous system (CNS) can survive (12,25,30,44,47). Many studies have demonstrated the ability of neurons from the CNS of neonatal and fetal mammals to survive, differentiate, and function in the host brain (13,18,19,33,44). Early grafting studies showed that pituitary grafts transplanted to various parts of the body survived but displayed no hormonal function (1,16,24,27,38,41). Investigations of target tissue cytology have demonstrated some improvement in pituitary function after intracerebral implantation of grafts into hypophysectomized rats (3,11,17,31,46). In these animals, pituitary hormone levels and target cell cytology indicate that estrogen cycles improve under hypothalamo-hypophyseal axis control (26). Fetal pituitary tissue transplantation into the third ventricle of hypophysectomized rats affects pituitary hormone function at various levels (37), but the functional effects of these grafts have not been researched in detail.

The aim of this study was to assess the neuroendocrine (pituitary hormone levels and histological evaluation of endocrine target organs), sexual, and reproductive function effects of neonatal pituitary grafts transplanted into the third ventricle of hypophysectomized adult rats. We also examined the effects of endogenous (cold) and exogenous (gonadotropin-releasing hormone [GnRH]) stimuli on neuroendocrine function after pituitary grafting.

MATERIALS AND METHOD

The study design was reviewed and approved by the Animal Care and Use Committee of Uludag University School of Medicine. One hundred forty adult male Sprague-Dawley (SD) rats (250-300 g) were placed in individual cages and kept in a temperature-controlled environment (24-25)°C on a 14:10-hr light/dark cycle. Twenty SD females were used for fertility testing, and were kept under the same conditions but did not undergo any of the surgical or implantation procedures. All the male animals were fed standard rat chow and 5% dextrose-saline. Each received a subcutaneous (s.c.) injection of 20-µg adrenocorticotrophic hormone (ACTH; Sinacten depot, 1 mg/ml, Ciba, Germany) every 3 days throughout the experiment. No surgical procedures were performed on the control rats (C group, n=10), as they were used to assess normal

pharmacological hormone levels and histology. The other animals underwent hypophysectomy only (H group, n=21 survivors), hypophysectomy-plus-cortical graft (CG group, n=21 survivors), or hypophysectomy-plus-pituitary graft (HG group; n=21 survivors).

Hypophysectomy

Prior to surgery, the animals were fasted overnight but were allowed free access to water. Each rat was pre-medicated with 0.05 mg atropine s.c. plus a 10 mg/kg intramuscular (i.m.) injection of dexamethasone, and was then anaesthetised with an intraperitoneal injection of 30-mg/kg sodium thiopental and intubated (PE250 polyethylene tubing). The animal was placed in the supine position, the surgery site was cleansed with 10% povidone iodine, and topical anaesthetic (10% Xylocaine Spray, Astra Södertalje, Sweden) was applied. A 2-cm supralaryngeal midline incision was made in the anterior neck. The sternohyoid and omohyoid muscles were bluntly dissected and the trachea and oesophagus were retracted slightly with a hand-held retractor. Then, using a surgical microscope (Carl Zeiss 99, Germany), we were able to visualise the base of the cranium and the sphenoccipital fissure. A 2-mm burr hole was made in the sphenoccipital fissure using a saline-cooled high-speed drill (Aesculap, Microtron, GD412, Germany). Through this hole, we carefully elevated the inner table of the cranium using a microdissector, and then made a large opening in the dura. The entire pituitary gland was removed using blunt dissection, suction, and irrigation, and the bone defect was covered with bone wax. Finally, we closed the incision with silk sutures and administered 25 mg cephalosporin i.m.

Preparation of Graft Solutions

Neonatal male SD rats (1-5 days) were decapitated and their pituitary glands were immediately removed using aseptic technique. The tissues were collected into chilled (4 °C) sterile saline. The pooled glands were then minced with sterile surgical instruments and dispersed mechanically in 10 ml volumes of Dulbecco's modified Eagle's medium (Life Technologies, Inc., Bethesda, MD, USA), which was buffered to pH 7.3 and contained 0.1% trypsin (Sigma, St. Louis, MO, USA) and collagenase III (3.5 mg/ml) (Sigma). Dispersion was allowed to continue for 1 hour at 37 °C, after which the suspension was washed three times in trypsin-free Dulbecco's medium and then re-suspended in

lactated Ringer's solution. At this stage, the cell suspension was ready for intracerebral implantation. The viability fraction and yield were estimated using a hemocytometer, tissue culture, and trypan blue exclusion. The yield from this step was $0.5-2 \times 10^6$ cells per 20 μ l of graft solution, and viability was 80-90%. The graft solution was used within a maximum of 4 hours of its preparation. For the cortical graft suspension, neonatal rat cerebral cortical tissue was processed using the same steps outlined above.

Grafting

One day after the hypophysectomy procedure, the animals in the HG and CG groups were pre-medicated and re-anaesthetised with sodium thiopental, as detailed above. Each rat was placed in a stereotactic frame (Harvard Apparatus, MI, USA) in the prone position. After the skin was cleansed with 10% povidone iodine, a 1-cm midline scalp incision was made, the galea was retracted, and a dental drill was used to make a hole at the bregma. Once the dura was carefully pierced, we used a Hamilton syringe to slowly inject 20 μ l of either pituitary or cortical graft solution ($0.5-2 \times 10^6$ cells) into the third ventricle (7 mm deep to the bregma) of rats in the HG and CG groups, respectively. The needle was withdrawn 2 minutes after the injection, and the hole in the cranium was covered by bone wax to prevent leakage of cerebrospinal fluid. The incision was then closed and the animal was given 25 mg cephalosporin i.m.

Testing of Sexual Function and Fertility

The rats that survived 4 weeks were also observed for their level of sexual activity. Each male rat was housed for one night with three females whose vaginal smears confirmed they were in pro-oestrus. A vaginal smear from each female was examined early the next morning, and presence of sperm was regarded as positive for copulation and ejaculation. The test was repeated twice for each male. To assess male fertility, each female that had been bred by the male was housed individually and followed for pregnancy.

Weight Loss

All the animals were weighed preoperatively, at 24 hours post-surgery, and at 4 weeks after the surgery. Weight changes in the groups were compared and analysed.

Blood Sampling for Hormone Levels

Twenty-one rats in each of the H, HG, and CG

groups survived 4 weeks. For exogenous and endogenous stimulation testing, each group was divided into three subgroups of seven rats each. Each subgroup was then designated as baseline, exogenous stimulation, or endogenous stimulation. The rats were anaesthetised with ether atmosphere, and the right jugular vein and carotid artery were catheterised using PE50 polyethylene tubing. The tip of each catheter was passed subcutaneously to an exit site on top of the head. The jugular vein was used for GnRH administration, and the carotid artery for blood sampling. The rats were recovered after 4 hours of anaesthesia, and the collected blood samples were analysed for LH and testosterone levels.

Samples from the three baseline subgroups were drawn at resting conditions and at the same time of day for all rats (1400-1500 hours). In the stimulation subgroups, blood samples were drawn just before and 45 minutes after application of the endogenous stimulus (cold stress, which involved lowering the cage temperature to $+4^\circ\text{C}$), and just before and at 10 and 20 minutes after the exogenous stimulus (1,000 ng/kg intravenous GnRH). In the rats in the baseline subgroups, blood sampling in control and all experimental groups was done after decapitation to minimise the stress effect. In the rats in the stimulation subgroups, blood was drawn from the arterial catheter at the times detailed above. The serum was quickly separated off and then stored at -70°C for later radioimmunoassay (RIA). LH was measured with a rat kit for this hormone (RPA552-Biotrak cellular communication assays, Amersham, UK), and total testosterone was measured by chemoluminescence, with immolate obtained from the pharmacology laboratory.

Histopathological Evaluation

After hormonal and sexual function/fertility testing at 4 weeks, all the H, HG, and CG animals were decapitated and endocrine target organs including the thyroid, adrenal gland, and testes were rapidly removed and fixed in 10% formalin solution for later histopathological examination. The skull base of each decapitated rat was evaluated under light microscopy, and animals that had pituitary tissue remaining were excluded from the experiment. The collected tissues were embedded in paraffin blocks, which were then cut into 5- μ m sections and stained with hematoxylin-eosin for light microscopic evaluation. The findings were graded according to Smith's criteria (0 = no atrophy; 3 = all accepted pathological features of atrophy) (Table 1).

Table 1: The evaluation criteria for endocrine target organ histology in hypophysectomized rats as described by Smith (45)

Target organs	Atrophy finding	Atrophy levels
Thyroid gland	Tall epithelial cells and vacuolated colloid in follicles, The epithelial cells are not as tall as normal, vacuolation of the colloid can still be seen, Flattened epithelial cells and complete absence of vacuolation in the colloid	0,1,2,3
Adrenal gland	Significant loss of body weight, Abnormal appearance of the cortex, with a pale peripheral and a dark deep zone, Small cortical cells,	0,1,2,3
Testes	Opacity of the thickened capsule in the atrophied organ, Greatly reduced tubule diameter, Absence of spermatogenesis	0,1,2,3

Statistics

Statistical analysis was done using the Mann-Whitney U and Wilcoxon tests. P values <0.05 were considered to indicate statistical significance.

RESULTS

Sixty-seven animals (67/140, 47.9%) were lost during the experiment for various reasons. Most deaths (42/67, 62.7%) occurred between 1 and 3 days after hypophysectomy surgery. Thirteen rats (13/67, 19.4%) died just after the implantation stage, three (3/67, 4.5%) died during catheterisation, eight (8/67, 11.9%) died between 3 and 10 days after hypophysectomy/graft implantation, and one (1/67, 1.5%) died during the functional testing phase. The most frequent cause of death was respiratory tract obstruction due to copious secretions. The other causes were haemorrhage from the base of the pituitary stalk, infection, and unknown reasons.

Five of the surviving hypophysectomized rats (5/63, 7.9%) that had remnant pituitary tissue were excluded from the experiment (two [2/21, 9.5%] H group and three [3/21, 14.3%] CG group rats). All rats in the HG group were confirmed to have undergone total hypophysectomy.

Sexual Function and Fertility

None of the 21 H group survivors that were tested for copulation bred the females in the first test. In the second test, the vaginal smear of only one of the six female rats that were caged with the two rats with grade 2 testicular atrophy showed a few sperm, but she did not become pregnant.

Similarly, none of the 21 CG survivors tested for copulation bred the females in the first test. On the second test for one of the CG rats with grade 2 testicular atrophy, the vaginal smear from one of the three females in the cage showed a few sperm, but no pregnancy resulted.

In the four HG group rats with grade 3 testicular atrophy, no copulation was recorded. In the 17 rats with grade 2 atrophy, copulation occurred in 8 cages on the first test (8/24 female rats bred in 8 cages), and in 3 different cages in the second test (4/9 female rats bred in 3 cages). In 10 of the cages there was no copulation on either test (0/30 female rats in 10 cages). In total, 11 of the 21 HG group rats (52.4%) were positive on sexual function testing. However, the smears in all the copulation cases showed azoospermia, and no pregnancy resulted. Comparison showed significantly more sexual activity in the HG group than in the H or CG groups (p<0.001).

Weight Loss

Most animals lost weight in the immediate postoperative period, and then slowly gained or lost weight in rough proportion to their hormonal levels. Comparing weights at the beginning and end of the experiment, the HG group showed the least weight loss (p<0.05) (Figure 1).

Hormone Assays

The RIA hormone findings for each group are listed in Table 2. Testosterone levels were undetectable in the H, HG, and CG groups. The LH levels in the controls were significantly higher than the H and CG levels (2.35 ± 1.2 pg/ml versus 0.58 ± 0.2 pg/ml and 0.47 ± 0.3 pg/ml, respectively;

Table 2: Serum LH levels detected at 4 weeks after hypophysectomy/implantation procedures.

Group	Luteinising Hormone Levels	
	Mean ± SEM (pg/ml)	Min-Max (pg/ml)
Control	2.35 ± 1.2	1.5 - 5.3
Hypophysectomy only	0.58 ± 0.2	0.0 - 0.9
Cortical graft	0.47 ± 0.3	0.1 - 0.9
Pituitary graft	0.89 ± 0.2	0.5 - 1.3

p<0.001). The HG animals' LH levels (0.89 ± 0.2 pg/ml) were higher than the levels in the H and CG groups, but were still significantly lower than control levels (p<0.05). In the HG group, LH rose to 40% of control LH levels (p<0.05). There was no statistical difference between the LH levels in the CG and H groups (p>0.05). The LH responses to the endogenous and exogenous stimuli are depicted in Figures 2 and 3. The responses in the HG group were greater than those observed in the H and CG groups, according to analysis with the Wilcoxon test (p<0.05).

Histopathological evaluation

In the H group (final n=19), 17 rats (89.5%) exhibited grade 3 thyroidal and testicular atrophy and 2 rats (10.5%) showed grade 2 atrophic changes. Testicular examination in the latter two rats revealed anatomical abnormalities and just a few sperm cells. Evaluation of the adrenal glands revealed grade 3 atrophy in 13 (68.4%) and grade 2 atrophy in 6 rats (31.6%). Four of the six rats with grade 2 atrophy had cysts in the adrenal medulla.

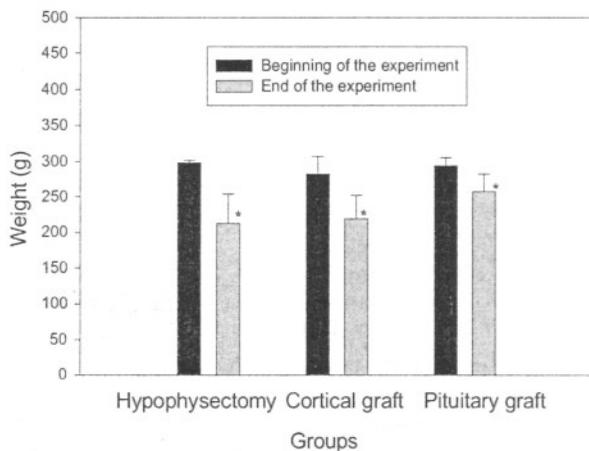


Figure 1: Animal weights at the beginning and at the end of the experiments.

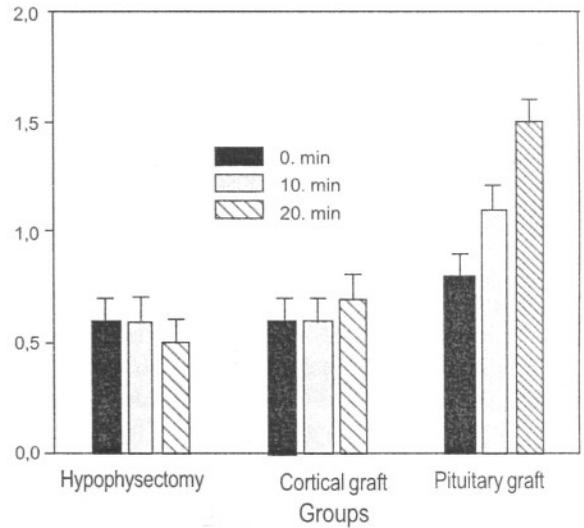


Figure 2: Luteinising hormone response induced by GnRH in a subgroup (n=7) from each of the experimental groups. GnRH was injected intravenously (1000 ng/kg), and blood samples were drawn from the arterial catheters at times pointing zero (0), 10, and 20 min after injection. Serum samples were separated and stored at 70°C until they were analysed. Data were presented as mean ± standard error of the mean (SEM) of 7 measurements. *, P<0.05; significantly different from the group's own basal (0) levels.

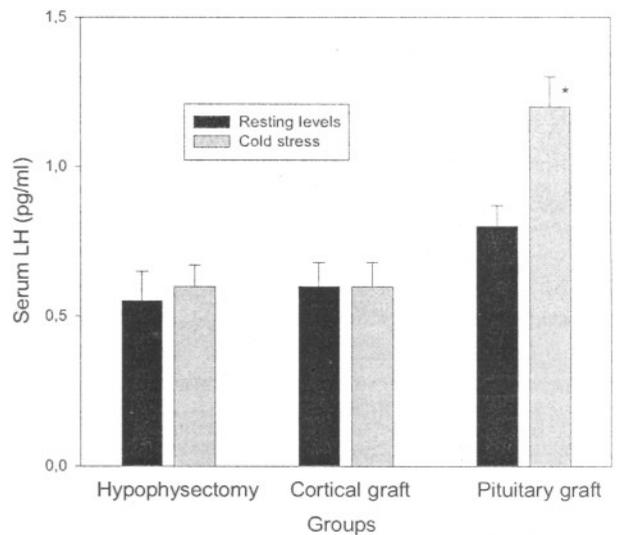


Figure 3: Luteinising hormone response to endogenous stimulation (cold stress) in a subgroup (n=7) from each of the experimental groups. The cold stress group animals were kept at +4 °C for 45 min. At the end of 45 min, all animals were decapitated and blood samples were taken for serum LH determination. Serum of samples were separated and stored at 70°C until they were analysed. Data were presented as mean ± SEM of 7 measurements. *, P<0.05; significantly different from the control group value.

In the CG group (final n=18), 15 rats (83.3%) had grade 3 thyroidal and testicular atrophy, and 3 (16.7%) showed grade 2 atrophy. Testicular evaluation in the latter three animals showed anatomical abnormalities and just a few sperm cells. Adrenal gland examination revealed grade 3 atrophy in 12 rats (66.7%), and grade 2 atrophy in 6 (33.3%).

In the HG group (n=21), 4 rats (19%) had grade 3 thyroidal, adrenal, and testicular atrophy, and 17 (81%) showed grade 2 atrophy in these tissues. No sperm cells were present in the animals with grade 3 testicular atrophy. The rats with grade 2 testicular atrophy showed abnormal anatomy, but had a few sperm.

DISCUSSION

Research has shown that when the neurohormonal link between the brain and the pituitary gland is interrupted by stalk section, or by destruction of the arcuate nucleus or the median eminence region of the hypothalamus, pituitary gonadotropin secretion is essentially abolished and gonadal function arrested (6,42,43,45). Some experimental studies have attempted to implant fetal grafts of the pre-optic area (POA) containing GnRH with the aim of improving sexual function (4,22,23,34,39). The results have shown that transplantation of this tissue into the third ventricle of hypogonadal mice increases pituitary gonadotropin levels and results in the formation of connections between the graft and the median eminence. If these axonal connections function properly, follicle-stimulating hormone (FSH) from the pituitary, and serum FSH, LH, and testosterone levels all rise to different degrees, leading to spermatogenesis and development of interstitial cells in the testicles (21,22,28,32,34,40). However, the extent to which the restored hormone levels affect sexual function and fertility has not been investigated in depth. As outlined above, our purpose in this study was to learn more about these changes.

The issue of where graft tissue should be placed in order to maintain the normal structure of the pituitary was first investigated by Halasz et al., who determined that transplants to the hypothalamic area maintain pituitary hormone function (26). More recently, Knigge showed that implanting grafts into the third ventricle in mice resulted in weight gain. The same study revealed that the transplanted tissue must have contact with cerebrospinal fluid in order to survive (31). The consensus is that the most

important factor with regard to function in pituitary graft experiments is the ability of graft cells to connect with the median eminence (3,5,26,27,34).

Theoretically, a certain amount of time is required in order for implanted cells to take hold, and for a connection to be established between the grafted tissue and the host brain. During this period, the endocrine target organs in the hypophysectomized host may atrophy and signs of hypopituitarism may appear. El Shennawy et al. stated that replacement therapy must be administered in this interval (15). In the post-hypophysectomy period, apoptosis tends to be particularly striking in the adrenal glands on days 3-7, and atrophy may be evident by 15 days (8,45). Atrophy of the adrenal cortex after hypophysectomy can be prevented by exogenous ACTH administration (9). In our study, we used this method to successfully prevent early-stage adrenal cortical apoptosis.

Many published studies have evaluated solid versus suspension grafts (2,5,36). Baker-Cairns et al. compared intraventricular and intraparenchymal transplanted solid and suspension grafts, and found that the latter were highly vascularised and included many more donor vessels than the solid grafts (5). In our study, we prepared cell suspensions following the method of Maxwell et al., and tested them in *in vitro* cell cultures before they were used (37).

To prevent stress during blood sampling, we used carotid artery catheterisation as described by Du Ruisseau et al., and collected blood via these catheters (14). Our findings showed that the LH levels in the HG group were significantly higher than the levels in the H and CG groups. Also, the serum LH levels in the HG rats reached 40% of control levels. These findings are in accordance with those of Maxwell et al. (37). However, the fact that only our control rats had detectable levels of testosterone suggests that the LH increase in the HG animals was not adequate to stimulate normal testosterone secretion. A higher level of stimulation may be achieved by implanting a larger amount of graft tissue. Although 52.4% of the surviving HG group rats engaged in copulation, azoospermia likely explains the fact that no pregnancies resulted.

We examined the groups' LH responses to exogenous and endogenous stimuli in order to more specifically assess graft function. The HG rats' response to exogenous stimulus (GnRH) was

statistically significant at 10 and 20 minutes after injection ($p < 0.05$). Similarly, this group's response to the endogenous stimulus (cold stress) was also significant ($p < 0.05$). Studies have shown that, although the effect of induced LH secretion in hypogonadal mice does not differ from that of natural LH, synthetic GnRH injections generate less pituitary LH secretion than occurs in normal rats (29). Charlton et al. reported that more than one daily injection of GnRH for 5 days in hypogonadal male rats markedly elevated pituitary FSH secretion, but did not change the weight of the seminal vesicle (10). Gibson et al. found similar results with fetal POA grafts implanted into the third ventricle of hypogonadal mice. In their experiment, progesterone administered 2 months after grafting produced increased serum LH levels in only 30% of the mice (20).

One immunochemical study investigated GnRH-producing cells in the graft, and the link between the graft and the median eminence. The investigators were unable to demonstrate an association between the GnRH cell count and the level of connection with the median eminence using the negative and positive feedback determination technique (23). In more recent studies, these same authors have reported that not all GnRH cells in the graft are able to project axons to the median eminence, and that the GnRH cell count required to restore the hypothalamic-testicular axis is actually lower than previously estimated. Further, they have shown that the scar tissue in hypogonadal mice with damaged arcuate nuclei blocks the formation of these axonal connections (23).

Reports state that the most important factors in pituitary graft survival and function are the vascularity of the graft tissue, scars resulting from transplantation, and tissue rejection (5,23,35). Baker-Cairns et al. noted that the antigen-presenting capability of the microglial cells surrounding the donor vessels make them potential immune targets for graft rejection. The same authors suggested that replacing the donor vessels with host vessels should reduce the potential for graft rejection, and that the increased vascularity of donor tissue should increase the probability of graft survival (5).

In our study, transplantation of neonatal pituitary cell suspension grafts into the third ventricle of hypophysectomized male rats led to a rise in LH levels. Although this significant increase in LH improves sexual function to some extent, it appears to have no effect on testosterone secretion or fertility.

Elevating LH levels by injecting more cells into a larger area may have a greater impact on these functions.

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