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REDUCED SEMINIFEROUS TUBULE DIAMETER IN MICE NEONATALLY EXPOSED TO PERFUME

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Summary: Odors play important roles in rodent social behavior and reproduction. Pheromones, acting through vomeronasal organ, influence pubertal development, estrus cycling and implantation in female mice. Therefore, we examined whether constant exposure to strong odor (perfume) could influence postnatal development of male reproductive organs. A breeding pairs of mice (one male and one female) were constantly exposed to perfume in cages with filter tops for one week before mating, throughout pregnancy and until weaning of the pups. No difference was observed in litter size or time between pregnancies. Male pups were sacrificed at 6, 16, 19 and 50 days of life and their testes fixed in Bouin's solution. Separate group of sexually mature (60 days old) male mice was exposed to perfume for 12 days and blood collected on 4, 8 and 12 day of exposure. Immunohistochemical staining using antibodies against 3ß-HSD, antimullerian hormone and proliferating cell nuclear antigen did not reveal major differences in postnatal development of testicular cells. Testosterone levels in blood collected from sexually mature mice were lower in a group exposed to perfume in comparison to control group, but difference was not statistically significant. However, seminiferous tubule diameter was reduced ($p<0.05$) in group of male mice exposed perinataly to perfume in comparison to control group. Although the present study did not reveal a major effect of constant exposure perfume on reproductive function in mice, reduced tubule diameter could suggest moderate changes in hormone levels such as FSH, which is known to influence Sertoli cell proliferation.

Key words: pheromones - analysis; perfume - adverse effects; testis - growth and development - cytology; mice

Introduction

Odors, or more specifically pheromones, play important roles in rodent reproduction. Pheromones and functional vomeronasal organ are necessary for mating behavior, sex discrimination, and also aggressive behavior in rodents. Exposure to adult male pheromones triggers early onset of puberty in juvenile female mice and similarly, exposure to male pheromones would induce cycling in anestric female mice (reviewed in (1-3). Pheromones act through vomeronasal organ, which has direct connections to hypothalamus through accessory olfactory bulb (3). Pheromones have, at least in rodents, profound effects on hormone secretion. One of the most interesting effects of pheromones is so called Bruce effect (4). Female mice exposed to different male during first four days of pregnancy (before implantation) will abort as a consequence of exposure to pheromones of male that did not impregnate her. In this case, male pheromones influence dopamine release from hypothalamus, which inhibits prolactin secretion from pituitary and these reduced levels of prolactin could not sustain corpus luteum, what would finally cause miscarriage (3). Pheromones could also influence sperm motility in male mice as reported in study by Koyama and Kamimura (5). Pheromones in vomeronasal organs act through specific receptors, divided into two distinct families, V1R and V2R and are encoded by approximately 300 genes (6-8). Although several pheromones were identified in recent years, the chemical structure of many pheromones is not yet known. Some studies have shown that VNO neurons are very sensitive to different chemical stimuli and furthermore, they also respond to some volatile substances (9-11).

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Postnatal development of the testis is a critical period for function of this organ in adult life (12). Sertoli cells proliferate only for a limited period during prenatal and postnatal life and by day 18 postnatally, when Sertoli cell proliferation cease, their numbers are final for the rest of life in most animals (13). Sertoli cell proliferation is hormonally regulated; FSH was reported to stimulate Sertoli cell proliferation while thyroid hormones T3 and T4 are most likely involved in cessation of Sertoli cell proliferation (13, 14). During first postnatal days, fetal Leydig cells disappear from the testis and are gradually replaced by adult type of Leydig cells, which differ from their fetal counterparts in many aspects. Although these processes are not well understood, it is most likely that this change of Leydig cell populations is also influenced by reproductive hormones (15, 16). Therefore, the proper function of hypothalamic – pituitary – gonadal axis in this period is extremely important for proper development of testis and any potential disturbances in this period could have profound effects on function of adult testis.

Perfumes are ubiquitously used chemicals with strong odor in humans that act through main olfactory epithelium. However, many different substances with strong odors influence also vomeronasal organ and/or accessory olfactory bulb and this could modulate secretion of pituitary hormones. Therefore, the aim of the present study was to determine whether constant exposure to perfume could influence reproductive function and pre- and postnatal development of the testis, possibly by disturbing normal pheromonal signaling.

Material and methods

Animals

Sexually mature (60 – 70 days old) BALB/c male and female mice were bred in cages with filtertops. Minisart filters (Sartorius, Goetingen, Germany) were soaked with perfume of undisclosed producer and placed in the cage with individual male and female mice. Minisart filters were chosen as preferred way of exposure as they are encapsulated in plastic frame that prevented direct access of animals to perfume, but enabled constant evaporation of perfume into environment. Every third day, exactly 0.5 ml of widely used perfume from undisclosed producer was added to the filter. Male and female mice were kept under influence of perfume for one week separately, after which one male and one female mouse was joined in a single cage, containing one Minisart filter soaked with perfume. Mice were exposed to perfume throughout pregnancy and lactation period until weaning at 21 days. Pups were sacrificed at 6, 16, 19 and 50 day of age. 50 days old mice were exposed to perfume only until weaning. 6 days old mice were sacrificed by CO_2 exposure followed by cervical dislocation. Mice old 16, 19 and 50 days were anaesthetized by a mixture of ketamine (2.5mg/animal), Xylazine (0.25mg/animal) and Acepromazine (0.05mg/animal) and perfused with Bouin's solution. Testes were extracted and post-fixed in Bouin's solution for 4 to 20 hours depending on size. Subsequently, testes were processed into paraffin wax using standard procedures. Separate group of 5 sexually mature males was exposed to perfume for 12 days and blood was collected from saphenous vein on day 4, 8 and 12 of experiment. For all experiments, control groups were bred in identical conditions in filter top cages but in a separate room to prevent any cross-exposure to perfume. All animal work was done according to EU directive and NIH guidelines and with permission from Veterinary commission of Slovenia.

Immunohistochemistry

Sections (5 microns) were mounted on slides coated with 3-aminopropyl triethoxy-silane (TESPA; Sigma, Taufkirchen, Germany) and dried overnight at 50°C. Before incubation with primary antibodies, sections were dewaxed, rehydrated in graded ethanols, washed in water and phosphate buffer saline (PBS) followed by blocking endogenous peroxidase by incubating the section for 30 min in 1% H_2O_2 in PBS. Sections used for immunostaining with antisera against antimullerian hormone (AMH) and PCNA were subjected to antigen retrieval by microwaving in 0.01M citrate buffer (pH 6.0) on full power for 20 min, and thereafter left standing for 20 min without disturbance. Sections were then washed for 5 min in PBS and blocked using normal rabbit (3ß-HSD and AMH) or mouse (PCNA) serum (Dako, High Wycombe Bucks, UK) diluted 1:5 in PBS. Polyclonal rabbit antibodies directed against AMH (gift from Dr. Nathalie Josso, France) were used at a dilution of 1:100, polyclonal rabbit antibodies against 3ß-HSD (gift from Dr. Ian Mason, Edinburgh, Scotland) were used at a dilution of 1:500 and monoclonal mouse antibodies against PCNA (Dako) were used in 1:100 dilution. All primary antibodies were diluted in PBS containing 20% normal goat (AMH, 3ß-HSD) or rab-

bit (PCNA) serum. Sections were incubated with primary antibodies overnight at 4°C in humid chamber. The following day coverslips were removed, sections washed twice in PBS (5 min each wash), incubated for 30 min with goat anti-rabbit (AMH, 3ß-HSD) or rabbit anti-mouse (PCNA) immunoglobulins (Dako) diluted 1:100 in PBS and then washed again in PBS (2 times 5 min). For detection of bound antibodies, sections were first incubated with rabbit (AMH, 3ß-HSD) or mouse (PCNA) peroxidase-antiperoxidase complex (Dako) for 30 min and washed 2 times in PBS (5 min each). Color reaction product was developed by incubating sections in a mixture of 0.05% (w/v) 3,3'-diaminobenzidine tetra-hydrochloride (DAB, Sigma) in 0.05M Tris-HCl, pH 7.4 and 0.01% hydrogen peroxide. After 5-15 min, sections were washed in distilled water, counterstained with hematoxylin, dehydrated in graded ethanols, cleared in xylene and coverslipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). Specificity of the antibodies was controlled by using non-immune rabbit serum instead of primary antibodies.

Tubule diameter measurements

Testes from 50 days old mice (5 experimental and 5 control mice), exposed neonatally to perfume, were cut on a microtome at 5 μm and stained with hematoxyline and eosin using standard procedures. Seminiferous tubule diameter was measured at 40x magnification using measuring eyepiece. Diameter of 50 randomly chosen tubules was measured in each testis from each animal. Only tubules considered perfectly round by eye examination were used for diameter measurement to make sure there was no error due to oblique cutting of tubules.

Testosterone measurements

Blood was collected from all animals from saphenous vein at 12.00 am exactly. Heparinized blood was centrifuged at 2000 rpm for 3 minutes. Plasma was removed and stored at -20°C until use. Testosterone was measured at Department for clinical biochemistry, Clinical center, Ljubljana using Testosterone direct radioimmunoassay kit from Diasorin (Saluggia, Italy).

Statistical analyses

Microsoft excel was used for all statistical analyses. ANOVA was performed to determine whether there are significant differences between groups. Where implicated, student T-test was additionally used to confirm difference between two groups and $p<0.05$ was considered as significant.

Results

Litter size and time between pregnancies

All together, 8 females with total of 16 litters were included in control group and 7 females with total of 16 litters were included in experimental group. Both litter size and time between pregnancies did not differ significantly between control and experimental groups. Average litter size in control group was 7.19 ± 0.58 and 8.06 ± 0.71 (mean ± S.E.) in perfume exposed group. Time between pregnancies was 35.87 ± 4.40 days in control group and 32.24 ± 2.25 days in perfume exposed group (mean ± S.E.).

Immunohistochemical staining

Testes from 6 days old animals were used for immunohistochemical staining with antibodies against antimullerian hormone and 3ß-HSD. No major difference was observed between control and perfume exposed groups (Fig. 1). Testes from 16 and 19 days old mice were used for immunohistochemical staining with antibodies against 3ß-HSD (not shown) and PCNA (Fig. 2). Again, no obvious difference was observed between control and perfume exposed groups.

Seminiferous tubule diameter

Measurement of seminiferous tubule diameter revealed small, but statistically significant ($p = 0.013$) difference between control and experimental group. Tubule diameter was 169.45 ± 1.48 μm in control group and 164.52 ± 1.30 μm in perfume exposed group (mean ± S.E.).

Testosterone measurements

Testosterone levels measured in plasma were lower in experimental group in comparison to control group, although the difference did not reach statistical significance. Testosterone levels in control group were 23.6 ± 8.3 nMol/L and 13.88 ± 7.25 nMol/L in perfume exposed group (mean ± S.E.).

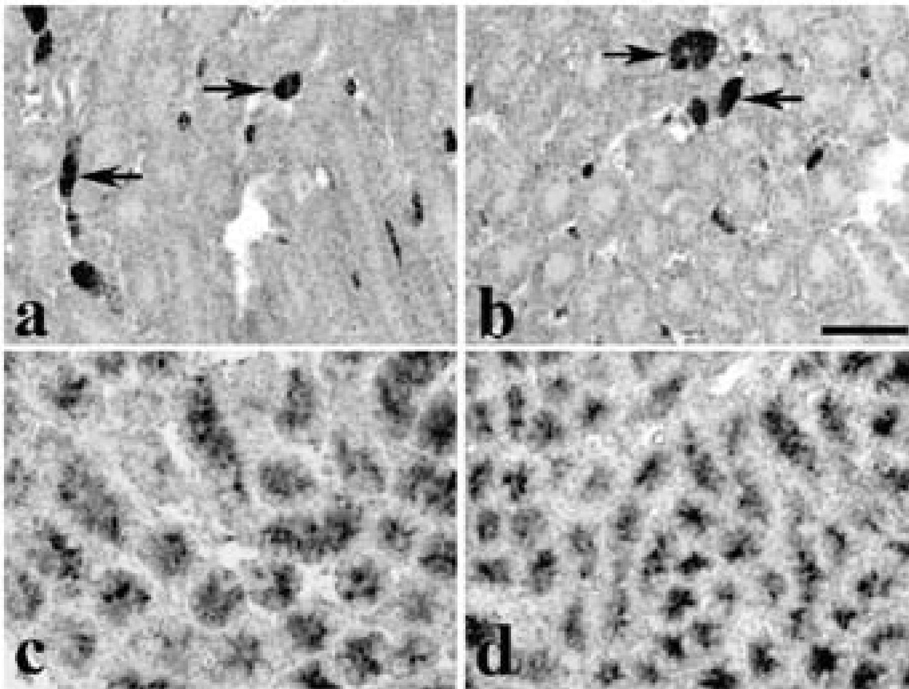

Figure 1: Immunostaining with antibodies against 3ß-HSD (a, b) revealed the presence of grouped fetal Leydig cells (arrows) and there was no difference observed between control (a) and perfume exposed (b) group at 6 days of age. Immunostaining using antibodies against AMH (c, d) showed that majority of Sertoli cells within the testicular cords still expressed AMH at 6 days of age and again, there was no difference between perfume exposed (d) and control (c) group of mice (bar = 50μm).

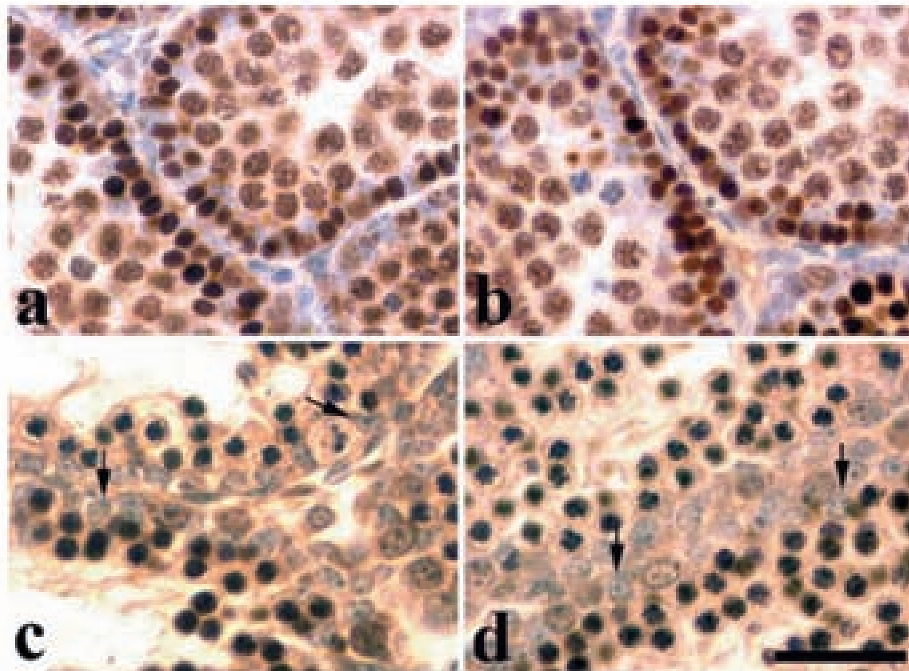

Figure 2: Immunostaining with antibodies against PCNA revealed positive reaction in majority of tubular cells including Sertoli cells from both control (a) and perfume exposed (b) testes at 16 days of age. At 19 days of age, Sertoli cells (arrows) were negative for PCNA expression in both control (c) and perfume exposed (d) groups suggesting that Sertoli cell proliferation stopped (bar = 50 μm).

Discussion

In many mammals, smell and not vision is the main sensory input influencing animal life. In rodents, odors are important for animal orientation, recognizing their environments and also for social interactions. Social interactions and especially sexual encounters are thought to be mainly influenced by pheromones that are detected by vomeronasal organ (reviewed in 2, 3). Although it is not clear whether pheromones are important also in primates and especially humans, they have profound roles in regulating reproductive function in rodents. Pheromones produce several significant effects in female mice such as Whitten, Bruce and Vanderbergh effects (reviewed in 3), at least partially through modulating LH and prolactin secretion (3). Two classes of putative pheromone receptors have been

identified and it is now established that rodents poses about 300 different pheromone receptors, suggesting that many different substances could acts as pheromones (6-8, 17). Although pheromones and odors are usually different substances, it is possible that some strong odors might also act through vomeronasal organs or influence pheromone receptors, and some studies have shown that volatile compounds indeed stimulate vomeronasal neurons (10, 11). Pheromones are necessary for normal sexual behavior and reproductive function in mouse and functional vomeronasal organs are needed for normal mating and aggressive behavior in mice. In our study, pairs of male and female mice were housed together in presence of strong odor from perfume to examine whether constant exposure to volatile odor could influence reproductive performance. Neither litter size nor time between pregnancies differed significantly between control and perfume exposed mice, suggesting that perfume used in our experiment did not disturb normal pheromonal signaling through vomeronasal organ, at least not enough to disturb normal reproductive behaviors such as estrus detection and mating in mice.

Vomeronasal neurons project directly to the accessory olfactory bulb, which has connections to the hypothalamus, a master center for regulation of most endocrine processes in mammalian organism (1, 3). Some pheromone effects are directly connected to differential secretion of pituitary hormones such as LH, FSH and prolactin (18-20). For example, Bruce effect is a result of reduced prolactin levels that prevent implantation and reduced prolactin secretion is caused by increased dopamine secretion from hypothalamus (20). Pheromone effects are best known in female rodents. However, since pheromones in females could modulate gonadotropin and prolactin secretion, we hypothesized that constant exposure to strong odors might also influence testis development in postnatal period. This is a period when important changes occur in testis, with both Sertoli and Leydig cells differentiating into adult cells. Pituitary hormones are involved in these processes and careful hormonal regulation is necessary to ensure proper development and differentiation of different testicular cells (12, 15). In the present study, both Sertoli and Leydig cell differentiation was monitored by immunochemical staining with markers for either Sertoli or Leydig cell differentiation. No changes were observed either in AMH or PCNA immunostaining, suggesting that both postnatal maturation of Sertoli cells and cessation of their proliferation were not greatly affected by constant exposure to strong odor. Similarly, the intensity of staining as well as numbers of Leydig cells (as determined by qualitative observations) was not obviously different between experimental groups. However, interestingly, seminiferous tubule diameter was moderately, although statistically significantly reduced in animals perinataly exposed to perfume. Reduced tubule diameter could reflect either reduced Sertoli cell number or defective spermatogenesis. We did histological analyses of testes at 50 days of age, 2 days after first wave of spermatogenesis should be completed. No differences in spermatogenesis between both groups were observed, suggesting that first cycle of spermatogenesis was normally completed in both groups of animals as we observed tubules of all stages in all 50 days old samples. Therefore, reduced tubule diameter could reflect reduced numbers of Sertoli cells. Sertoli cells proliferate only during postnatal life and their proliferation normally cease by day 18 (13). To determine whether there are any differences in Sertoli cell proliferation, we used immunohistochemical staining with antibodies against PCNA, marker of proliferating cells (21). On day 16 postnatally, all Sertoli cells were still positive for this marker and on day 19, all Sertoli cells were negative. This would suggest either that there is no change in timing of cessation of Sertoli cell proliferation or changes were so subtle that we did not observe them with experimental design. Since differences in tubule diameter were small, it is possible that only short difference, perhaps few hours, occurs in cessation of proliferation, or, perhaps more likely, that the rate at which Sertoli cells proliferate was slightly different between experimental groups. This later possibility might be more plausible knowing that FSH, secreted from pituitary has effect on Sertoli cell proliferation and several studies have shown that pheromones in mice could modulate FSH secretion (22, 23). However, in the present study we did not measure FSH levels to be able to confirm any changes in secretion of this hormone.

In conclusion, our study demonstrated that there is no major effect of constant exposure to perfume on mouse reproductive performance. This could suggest that perfumes do not contain substances that would strongly influence function of vomeronasal neurons or perhaps that vomeronasal organ does not importantly affect secretion of any reproductive hormones in normal breeding conditions. However, small difference in seminiferous tubule

diameter suggests that constant exposure to perfume might produce moderate effects on postnatal development of reproductive organs.

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ZMANJŠAN PREMER SEMENSKIH CEVK PRI MIŠKAH, IZPOSTAVLJENIH PARFUMU OB ROJSTVU DO ODSTAVITVE

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Povzetek: Vonji imajo pri glodavcih zelo pomembno vlogo pri socialnem obnašanju in obnašanju, povezanem z razmnoževanjem. Feromoni, ki jih organizem zaznava z vomeronazalnim organom, vplivajo na začetek pubertete, delovanje spolnega ciklusa in vsaditev zarodkov pri miših. Zaradi pomembne vloge vonjev za delovanje spolnega sistema smo v opisani raziskavi ugotavljali, ali lahko stalna izpostavljenost močnemu vonju ga oddaja parfum, vpliva na razvoj moških spolnih organov po rojstvu. Pari miši (en samec in ena samica) so bili stalno izpostavljeni parfumu v kletkah s filtrskimi pokrovi, in sicer en teden pred parjenjem, med parjenjem in do odstavitve mladičev. Med kontrolno (skupina brez parfuma) in poskusno skupino ni bilo razlik v številu živorojenih mladičev. Moške potomce smo žrtvovali na 6., 16., 19. in 50. dan starosti in njihova moda učvrstili v bouinovem učvrščevalcu. Posebna skupina spolno zrelih živali (starih 60 dni) je bila izpostavljena parfumu 12 dni, v tem času pa smo jim 4., 8., in 12. dan odvzeli kri. Imunohistokemično barvanje z uporabo protiteles proti antimulerjevemu hormonu, 3beta-hidroksi steroidni dehidrogenazi in antigenu delečih se celic ni pokazalo nobenih očitnih razlik v razvoju mod med kontrolno in poskusno skupino. Raven testosterona je bila v skupini, izpostavljeni vplivu parfuma sicer nižja kot v kontrolni skupini, vendar pa razlika ni bila statistično značilna. Ugotovili pa smo statistično značilno razliko v premeru semenskih cevk pri 50 dni starih živalih. Premer je bil manjši ($p<0.05$) pri samcih, ki so bili v obdobju po rojstvu izpostavljeni vplivu parfuma v primerjavi s samci iz kontrolne skupine. Čeprav v opisani raziskavi nismo ugotovili pomembnih razlik v dozorevanju mod po rojstvu med kontrolno in poskusno skupino pa manjši premer semenskih cevk pri samcih izpostavljenih vplivu parfuma, kaže na možnost motnje v delovanju nekaterih hormonov, kot je FSH, ki vplivajo na deljenje sertolijevih celic in s tem na kasnejši premer semenskih cevk v zgodnjem obdobju po rojstvu.

Ključne besede: feromoni - analize; parfum - škodljivi učinki; testis - citologija - rast in razvoj; miši