A novel non-surgical method to reduce fertility using the rat as a model

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Abstract

Overpopulation of pets, livestock, and some species of wildlife is a world-wide problem that could be addressed by reducing or eliminating fertility. Although surgical sterilization, immunocontraceptive vaccines, and chemical castrations are available, their limitations include cost, training, equipment, efficacy, and morbidity. Therefore, there is a need for a novel non-surgical method of sterilization for control of animal overpopulation. The goal of the present study was to develop a novel non-surgical sterilization technique employing a lipid-based nanocomplex carrying a cytotoxin, guided to specific gonadal cells by an antibody against anti-Mullerian hormone receptor type II. An intravenous injection of the antibody-guided lipid-nanocomplex negatively impacted aspects of reproduction in rats, including sperm production, estrous cyclicity and testicular and ovarian morphology, without affecting overall health within a 4-week interval after treatment. These results are promising initial steps toward developing a novel non-surgical method of sterilization that may change control of pet and wildlife overpopulation.

Keywords: Non-surgical sterilization, anti-Mullerian hormone, nanocomplex, nanoparticle

Introduction

Overpopulation of pets, livestock, and some species of wildlife is a world-wide problem and contributes to the spread of disease in humans, pets, and other domestic species. For example, the spread of rabies,[1,2] brucellosis,[3,4] leptospirosis,[4,5] *Neospora caninum,*[6] toxoplasmosis,[6,7] and tick-borne diseases such as Lyme disease, ehrlichiosis, anaplasmosis, and babesiosis,[8] have all been attributed to animal overpopulation. Further, decimation of native environments and indigenous wildlife, as well as destruction of local flora and fauna can occur as a result of feral cats,[9] deer,[10] feral horses,[11] and feral goats.[12]

Hunting,[12,13] euthanasia,[14] birth control,[15,16] and sterilization[17,18] are currently used to control animal overpopulation. While hunting and euthanasia can be effective, preventing animals from reproducing is a better, more socially and ethically acceptable option. Although there are contraceptive methods available, such as the zona pellucida vaccine,[19] effects are temporary and vaccines need to be given every 2 - 3 years. In contrast, sterilization can permanently reduce fertility. Currently, the most effective method of sterilization involves surgical removal of ovaries and testes which is expensive, requires surgical equipment and trained personnel, and has inherent risks of morbidity and mortality. Therefore, there is a need for a novel non-surgical method of sterilization to control animal overpopulation. The goal of the current study was to develop a non-surgical sterilization technique employing a nanocomplex.

The non-surgical technique that we have developed consists of an antibody-guided, lipid-based nanocomplex that carries a cytotoxin.[20,21] This nanocomplex is guided to specific cells in the gonads by an antibody against the anti-Mullerian hormone receptor type II (AMHRII). The AMHRII is expressed in the adrenal glands, pancreas, and spleen (among other tissues),[22] but is almost exclusively expressed within the gonads, specifically by granulosa and theca cells in the ovary, and Sertoli and Leydig cells in the testes. The antibody-guided nanocomplex is engineered to carry the cytotoxin saporin,[23] which induces apoptosis in the above-listed gonadal cells; these "nurse cells" are essential for oocytes and sperm to develop, undergo meiosis and survive.[24-33] They also produce the hormones estrogen and testosterone, which promote reproductive behavior.

We hypothesize that the above described gonadal "nurse cells" are destroyed after administration of the nanocomplex, and that this cellular destruction will cause disruption in ovarian follicular development in females and spermatogonia development in males, ultimately causing infertility.

Preliminary data in rats collected 24 hours after injection of the nanocomplex indicated that apoptosis was occurring in these gonadal cells, particularly in males.[34] The goal of the current study was to expand upon the results of our previous pilot work to determine the extent of damage in the gonads during a 4 week interval after nanocomplex injection.

Materials and methods

Experimental Animals

Eight male and eight female, 7-week-old Sprague-Dawley rats were obtained from Charles River Laboratory (Shrewsbury, MA). For 1 week prior to injections, all rats were acclimated to the animal colony to reduce non-specific stress. All rats were pair-housed in light (12:12 hour lighting schedule with lights on during working hours) and temperature (21 - 24°C) controlled rooms, in shoe-box type cages with corncob bedding, and Enviro-Dri® (Biological Associates, Melbourne, Australia) nest material for enrichment. Food (2018 Teklad global 18% protein rodent diet, Envigo, Cambridge, UK) and water were provided *ad libitum*. Males and females were kept in the same room within the animal colony. All animals were maintained in accordance with the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals and all animal procedures were approved by the Institutional Animals Care and Use Committee of Tufts University (protocol numbers G2018-158 and G2018-101).

Nanocomplex Production

Cationic lipidoids, cholesterol, and dioleyl phosphatidylethanolamine (DOPE) were mixed in a chloroform solution. After evaporating the organic solvent, the thin film formed was rehydrated with sterilized PBS and dispersed by probe sonication. Phospholipids like DOPE are used to provide support to the lipid bilayer and to facilitate endosomal escape during cell internalization. Cholesterol is relatively more hydrophobic and is able to stabilize the supramolecular structure and promote the membrane fusion process, which could enhance delivery efficiciency.[35] Ultrasonication was used to facilitate dispersion of the lipid mixture in aqueous solution to produce lipidoid nanoparticles, which in most cases are liposomes (vesicular structures with lipid bilayers and an aqueous interior). To the rehydrated formations, protein (Saporin Cytotoxin, Sigma Aldrich, St. Louis, MO) was added in pre-optimized ratios and was complexed with lipoid nanoparticles through supramolecular interactions (hydrogen bonding, electrostatic interactions and hydrophobic interactions).[20] Lipidoid-protein nanocomplexes were vortexed for another 60 minutes at room temperature and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol) 2000]-anti-anti-Mullerian hormone receptor type II (DSPE-PEG 2000-anti-AMHRII) was mixed and incorporated into the lipid/saporin nanocomplex for targeted protein delivery. The AMHRII antibody was first covalently conjugated to the macromolecular lipid, DSPE-PEG 2000, through the cyanur-amine conjugation reaction, a widely used technique for protein/antibody labelling.[36,37] Due to its amphiphilic nature, the DSPE-PEG 2000-AMHRII can easily incorporate into cholesterol/DOPE/cationic lipidoid nanoparticles, primarily through a hydrophobic interaction between the hydrophobic domain of the nanoparticle and the dioleoyl-tail of DSPE-PEG 2000-AMHRII. The antibody was covalently conjugated onto the nanoparticles and saporin was loaded through supramolecular interactions; therefore, labeling/binding was expected to be stable, as free saporin cannot be efficiently internalized by cells. Furthermore, we have used similar methods for complexation and delivery of other proteins, both *in vitro* and *in vivo*.[38]

Nanocomplex Injections

Eight-week-old animals were randomly allocated to one of two treatment groups: injection with either sterile saline (male n = 4, female n = 4) or nanocomplex (male n = 4, female n = 4). All injections were administered IV into the lateral tail vein, using a 24-gauge over-the-needle catheter. All test rats received 8.2 nmol of the nanocomplex in 0.1 ml of sterile saline, followed by a 0.1 ml sterile saline flush to ensure that nanocomplexes were delivered. All control rats received a 0.1 ml dose of sterile saline, followed by a 0.1 ml sterile saline flush. Rats were weighed twice weekly after injections and were

maintained in the animal colony for 4 weeks.

Estrous cycle

Beginning 2 weeks after injection, female animals were evaluated once daily (between 0900 and 1100). Vaginal lavage was performed by manually restraining the rats and placing a small pipette containing 0.25 ml (maximum volume) of sterile saline into the caudal vagina. The saline was gently introduced into the vagina and immediately aspirated. Each aspirate was examined using an Olympus BX50 microscope (Olympus Corporation of the Americas Headquarters, Center Valley, PA) equipped with a Photometrics CoolSNAP HQ2 video camera (Photometrics, Tucson, AZ) interfaced with MetaMorph® (Nashville, TN) software.

Tissue collection and processing

Rats were euthanized by CO_2 asphyxiation as recommended by the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition (https://www.avma.org/KB/Policies/Documents/euthanasia.pdf). Gonads were excised and after removing epididymides, were preserved in 10% formaldehyde for histological processing. In all males, the tail of the epididymis was removed along with approximately 6 mm of ductus deferens and placed in a 1.5 ml microcentrifuge tube containing normal saline. These samples remained at room temperature for approximately 6 hours. Then,100 µl of fluid was aspirated from the tube, ~50 µl placed onto a slide and examined using an Olympus BX50 microscope (Olympus Corporation of the Americas Headquarters) equipped with a Photometrics CoolSNAP HQ2 video camera (Photometrics, Tuscon, AZ) interfaced with MetaMorph® (Nashville, TN) software. Quantification of sperm cell count was performed using ImageJ software (NIH, Bethesda, MD) multipoint cell counter with ROI manager. Two images (10 X objective + 10 X eyepiece = 100 X magnification) per subject were quantified per high-power field and mean counts used for analyses.

Hematoxylin and eosin staining

Formalin-fixed paraffin embedded gonadal tissue was sliced from the central axis of each ovary and testis (six sections from the right and left gonad of each animal were sampled), at five µm per section and mounted onto microscope slides. Slides were stained with hematoxylin (Gill 3 Hematoxylin, StatLab, McKinney, TX) and eosin (Eosin Y, StatLab) in an automated stainer (Tissue-Tek, Sakura Finetek Inc., Torrance, CA) using a standard protocol. Tissue preparation and staining was performed by the Histopathology Section at the Cummings Veterinary School.

Image production and analysis

Bright field images were obtained of the ovaries at 2 X magnification and the testes at 20 X magnification using an Olympus BX41 microscope, with an attached Olympus Q-Color 5 camera (Olympus Corporation of the Americas Headquarters) interfaced with QCapture (QImaging, Surrey, BC, Canada) software. Quantification of spermatogonia and primary spermatocyte counts was performed using ImageJ software (NIH, Bethesda, MD) multipoint cell counter with ROI manager. Twenty images per subject were quantified and mean counts were used for analysis.

Statistical analysis

All data were analyzed using an unpaired Student's *t*-test. Significance was defined as $p < 0.05$.

Results

Body weight and wellbeing

All animals gained weight and appeared healthy throughout the study. As expected, male rats consistently weighed more and gained weight at a slightly faster rate than female rats.[39] There were no significant differences in weight gain as a measure of overall health between nanocomplex-treated and saline-treated controls.

Estrous cycle

Nanocomplex-treated female rats had vaginal cytologies with many cells and mixed cell types (including cornified, intermediate, parabasal and white blood cells), making it difficult to determine stages of the estrous cycle (Figure 1). All but one saline-treated control had vaginal cytology that indicated normal cyclicity,[40,41] whereas the remaining one had constant diestrus, consistent with pseudopregnancy.[42]

Epididymal sperm count

Nanocomplex-treated males had lower epididymal sperm count (Figure 2) compared to saline-treated controls (unpaired *t*-test, $t = 3.062$, $df = 14$, $p < 0.010$) and appeared to have fewer live (motile) sperm than controls (Figure 3).

Spermatogonia and primary spermatocyte counts

Spermatogonia (panel A; unpaired *t*-test, $t = 26.9$, $df = 158$, $p < 0.01$) and primary spermatocytes (panel B; unpaired *t*-test; $t = 21.92$, $df = 158$, $p < 0.01$) counts were lower (Figure 4) in nanocomplex-treated rats compared to saline-treated control rats. Under bright-field microscopy, spermatogonia were small, located close to the basement membrane of seminiferous tubules and often flattened on the side facing the basement membrane. Primary spermatocytes were large, round cells in a layer next to spermatogonia.

Testis histology

Seminiferous tubules of nanocomplex-treated males appeared smaller than saline-treated controls (Figure 5). In addition, there appeared to be atypical luminal contents (no sperm or undeveloped sperm, acellular material and immature germ cells), decreased interstitial cells, and disruption of general cohesion of cells in nanocomplex-treated males.

Ovary histology

Ovarian stroma of nanocomplex-treated females had notable hyperplasia and appeared larger than saline-treated controls, with some evidence of increased vasculature (Figure 6). In nanocomplex-treated females, stromal architecture appeared mildly edematous with decreased, incohesive interstitial tissue and there appeared to be increased numbers of corpora lutea and tertiary follicles.

Discussion

In the current study, an IV injection of an antibody-guided (AMHRII antibody) lipid-nanocomplex carrying the cytotoxin, saporin, negatively impacted aspects of reproduction in both male and female rats, without affecting general health and wellbeing, for 4 weeks after treatment.

There were no significant effects of treatment (compared to saline-treated controls) on body weight gain in either males or females during the 4-week duration of monitoring. In contrast, in studies comparing sham-operated and surgically gonadectomized rats,[39] there were differences between the two groups in weight gain. Castrated males lost more weight than sham-operated males and never regained that difference in weight.[39] Furthermore, females had opposite results, with greater weight gain in ovariectomized rats than sham-operated controls, particularly during the first 30 days after surgery.[39] An intriguing possibility is that, unlike surgical gonadectomy, the nanocomplex has not removed all support cells from the gonads at 4 weeks after treatment. This could leave behind cells still producing scant amounts of hormones, possibly accounting for the lack of differences in weight gain. Overall, at this dose, the nanocomplex procedure did not appear to impact general health of animals, at least within the first 4 weeks after treatment, as indicated by similar weight gains in both groups.

Vaginal cytologies from nanocomplex-treated females were difficult to evaluate, as there were often many cell types in each aspirate, with no clear dominant cell type. In contrast, three of four saline treated-control females had normal estrous cycles that could be easily identified by evaluating the cell

types on vaginal cytology.[40,41] Reproductively senescent, aged rats commonly have irregular estrous cycles as demonstrated by similar vaginal cytologies with many numbers of mixed cell types, presumably due to age-related dysregulation of the hypothalamic-pituitary-gonadal axis.[43,44] Vaginal cytologies in the current study may have been due to the nanocomplex destroying granulosa cells sufficient to interfere with normal hormone production, particularly of estrogen and anti-Mullerian hormone, effectively beginning reproductive senescence.

There were significantly fewer sperm in samples from nanocomplex-treated males compared to saline-treated controls. In addition, there appeared to be more motile, or live, sperm per sample in saline-treated control males. Because Sertoli cells are essential for sperm development and survival, [28,30-33] this significant decrease in sperm numbers was attributed to destruction of Sertoli cells by the nanocomplex as hypothesized. It takes approximately 8 weeks for sperm to be produced and travel to the tail of the epididymis; therefore, some sperm may still be in the seminiferous tubules and epididymis of treated rats after 4 weeks, albeit in much lower numbers than in controls.

Additionally, there were significantly fewer spermatogonia and primary spermatocytes in the seminiferous tubules of nanocomplex-treated males compared to saline-treated controls. Furthermore, based on histological examination, architecture was disrupted in the testes of nanocomplex-treated males. Tubules appeared smaller compared to saline-treated control males, and there appeared to be less cohesion between the cells within the seminiferous tubules, with elongated spermatids mingling with spermatogonia or primary spermatocytes, rather than appearing in well-organized rings at the lumen of the tubule. Based on these changes, we inferred that Sertoli cells had been negatively impacted or eliminated by the nanocomplex, as these cells form "channels" in which sperm develop in an ordered fashion, progressing from spermatogonia at the base of the seminiferous tubule, to elongated spermatids or sperm at the lumen.[28,31] Moreover, abnormal seminiferous tubule formation with decreased numbers of spermatogonia, were also present in a rat model of testicular dysgenesis syndrome,[45] suggesting that fertility was likely decreased in the current study.

Ovaries of treated rats also had irregular morphology, including notable hyperplasia, with decreased, incohesive interstitial tissue that appeared mildly edematous. Ovarian hyperplasia with minimal interstitial tissue has been demonstrated as part of a progression toward ovarian atrophy in a rodent model of early reproductive senescence.[46] Additionally, the ovary-intact rodent model of menopause has similar morphologic changes to those in the current study, including retention of ovarian tissue prior to the progression to complete ovarian atrophy.[43] Thus, morphologic changes in the current study were consistent with onset of reproductive senescence following treatment with the nanocomplex.

In conclusion, the current study demonstrated that 4 weeks after injection with AMHRII antibody-guided lipid nanocomplex, fertility in rats was likely reduced, as evidenced by decreased sperm production, decreased spermatogonia and primary spermatocyte counts in males, disruptions in estrous cyclicity in females, as well as changes in both testicular and ovarian architecture. Future studies are needed to extend the duration of treatment monitoring to determine impacts on fertility, especially in the male, where the testes could produce sperm from residual germ cells for up to 60 days after injection. In addition, hormonal assays are essential for a more comprehensive understanding of the effects of the nanocomplex on fertility. Lastly, breeding experiments will be necessary to determine if the nanocomplex treatment has definitively reduced or eliminated fertility.

Results of the current study are promising initial steps toward developing a novel non-surgical method of sterilization. This technique could be refined for use as a novel rodent model of menopause and may change our approach to controlling pet and wildlife overpopulation.

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Conflict of interest

The authors have no conflicts of interest.

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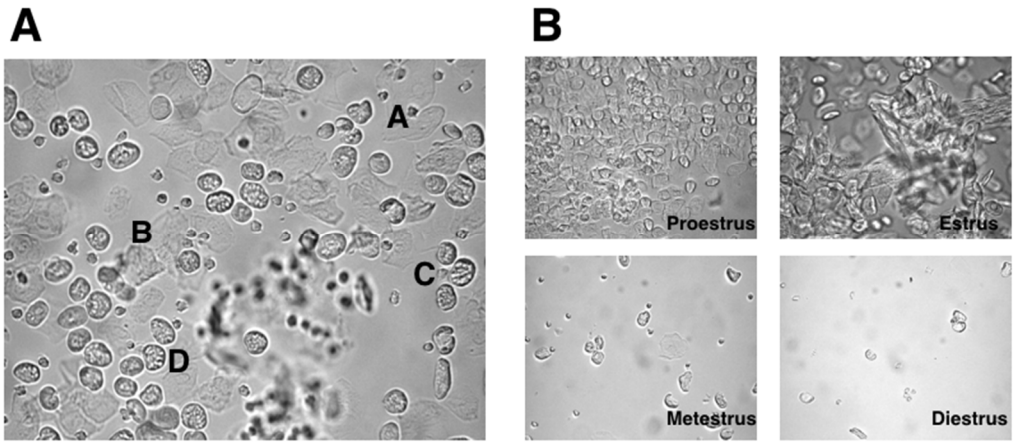

Figure 1. Representative image (40 X objective) of the effect of intravenous nanoparticle administration on estrous cyclicity (panel A). Vaginal smears of nanoparticle injected rats displayed many cells and mixed cell types, including white blood cells (A), cornified cells (B), parabasal cells (C), and intermediate cells (D), making it difficult to determine stages of the estrous cycle definitively. Panel B displays representative images of normal direct cytology for comparison.

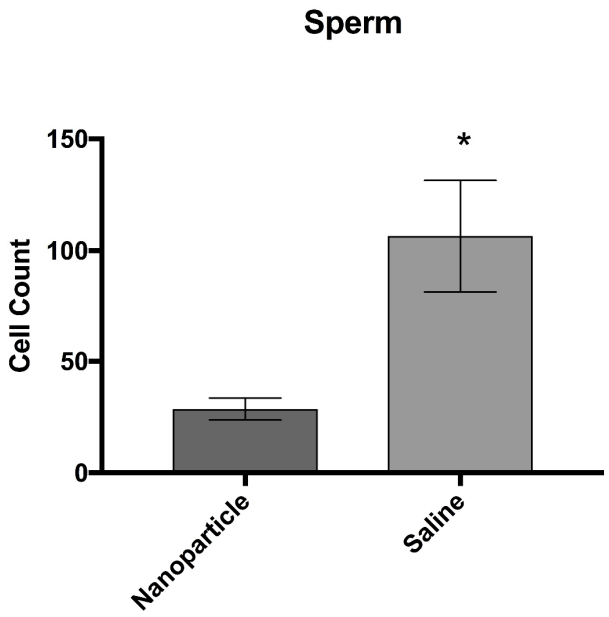

Figure 2. Effect of intravenous nanoparticle administration on epididymal sperm count when compared to saline injected controls (unpaired *t*-test, t = 3.062, df = 14, *p < 0.01). Values are presented as mean +/- SEM. The x-axis denotes nanoparticle injected animals vs saline injected controls, and the y-axis denotes cell count.

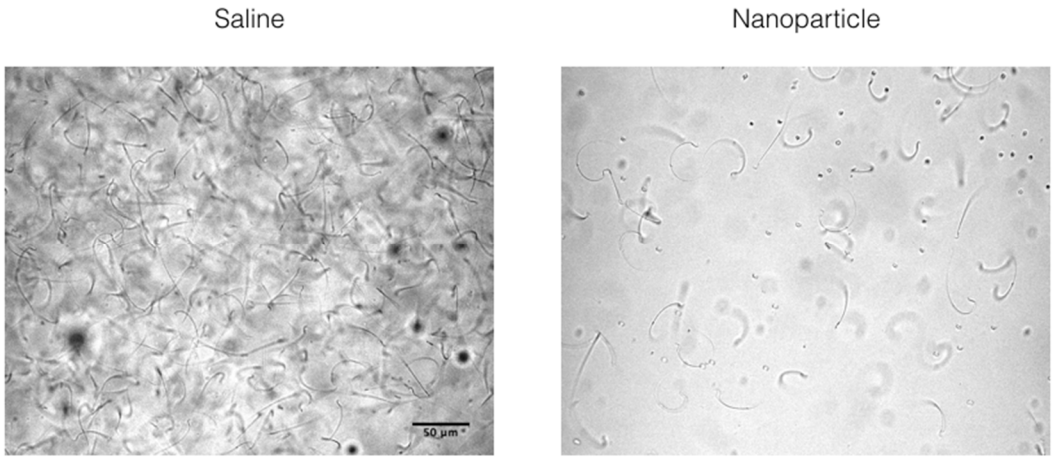

Figure 3. Representative images (10 X objective) displaying the effect of intravenous nanoparticle administration on epididymal sperm numbers when compared to saline injected controls.

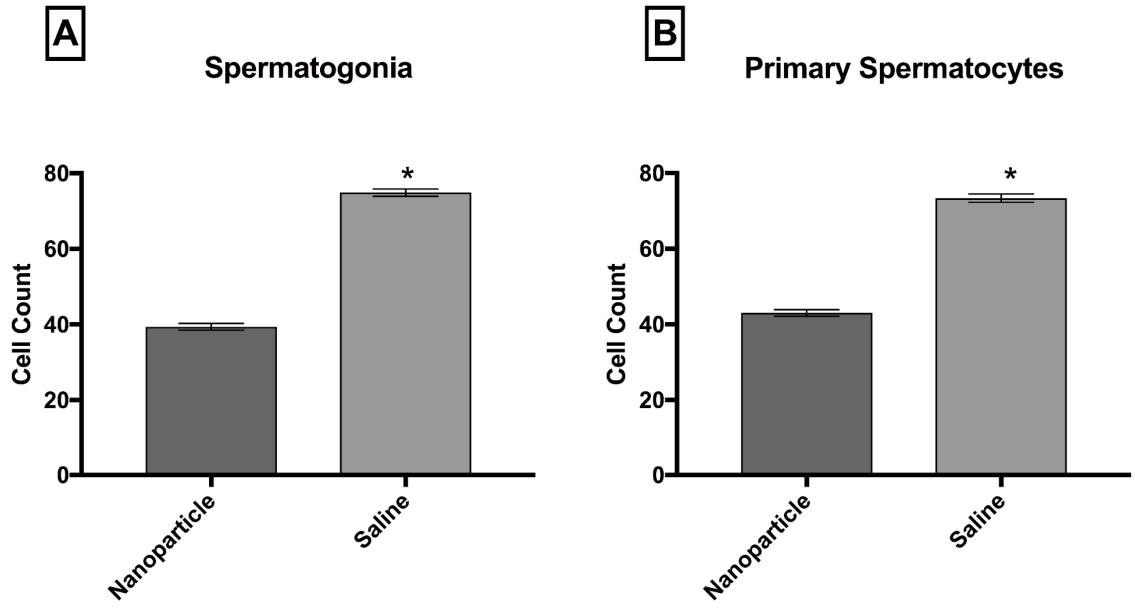

Figure 4. Effect of intravenous nanoparticle administration on spermatogonia (panel A; unpaired *t*-test, t = 26.9, df = 158, *p < 0.01) and primary spermatocyte (panel B; unpaired *t*-test; t = 21.92, df = 158, *p < 0.01) cell counts when compared to saline injected controls. Values are presented as mean +/- SEM. The X-axis denotes nanoparticle injected animals versus saline injected controls, and the Y-axis denotes cell count.

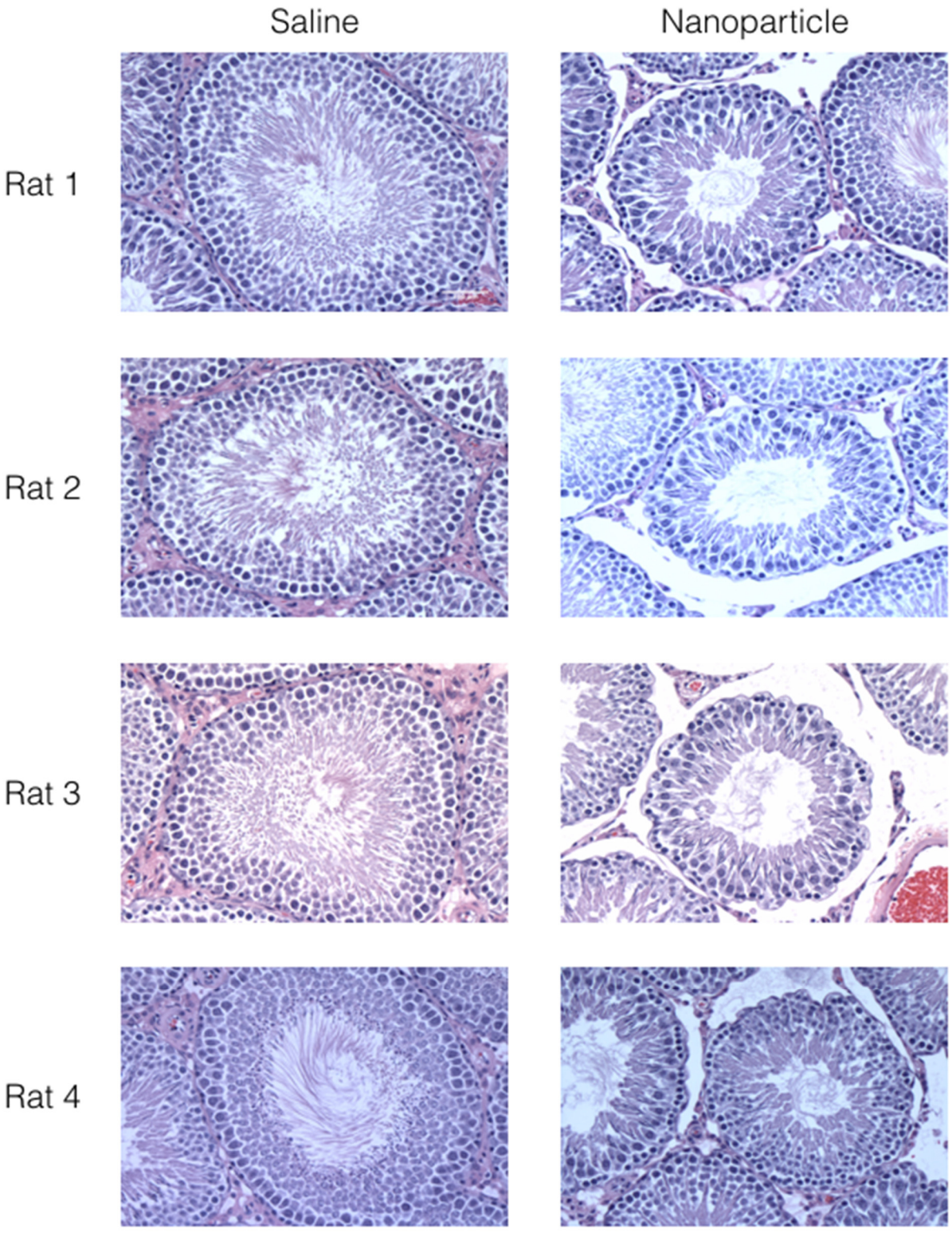

Figure 5. Hematoxylin and eosin stained sections of testes (20 X objective) displaying the effect of intravenous nanoparticle administration on seminiferous tubule morphology when compared to saline injected controls.

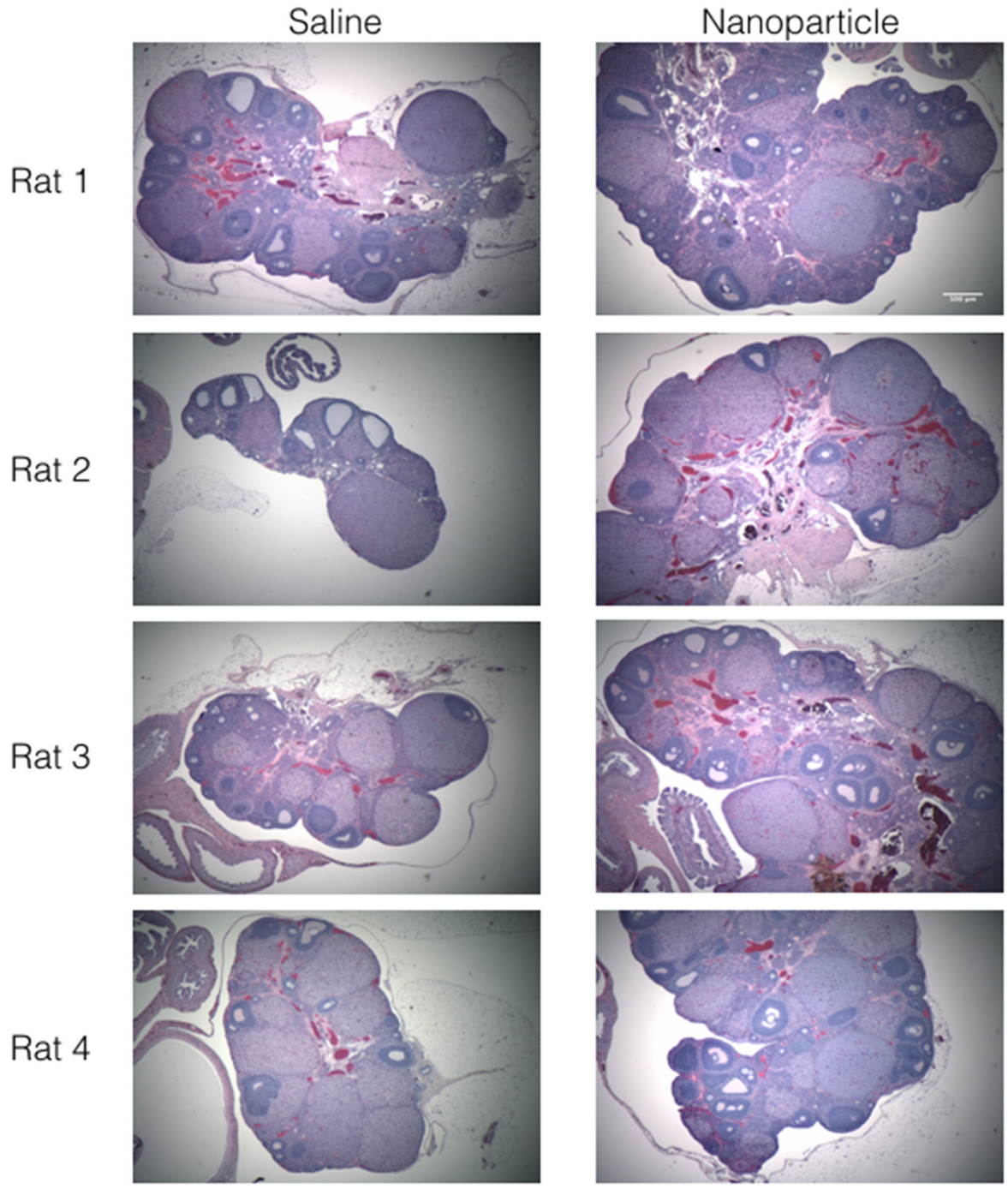

Figure 6. Hematoxylin and eosin stained sections of ovary (2 X objective) displaying the effect of intravenous nanoparticle administration on ovary morphology when compared to saline injected controls.