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Original article

F5-peptide enhances the efficacy of the non-hormonal male contraceptive adjudin☆,☆☆

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ABSTRACT

Objective: The bioavailability of the non-hormonal male contraceptive adjudin is low in rats due to the blood-testis barrier (BTB). This study was designed to examine if F5-peptide, an endogenously produced reversible BTB modifier, could enhance the bioavailability of adjudin to affect spermatogenesis and provide a contraceptive effect in rats while reducing systemic toxicity.

Study Design: We overexpressed F5-peptide in adult male rats (n=10 rats; with 3 or 4 rats for each of the three different experiments noted in the three regimens) by intratesticular injection of a mammalian expression vector pCI-neo (pCI-neo/F5-peptide) *vs.* empty vector alone (pCI-neo/Ctrl) to be followed by treatment with adjudin by oral gavage at a dose of 10 or 20 mg/kg. The status of spermatogenesis was assessed by histological analysis and dual-labeled immunofluorescence analysis on Day 16. To assess fertility, we allowed treated males (n=3–4 rats) to mate with mature female rats (n=3–4) individually, and assessed the number of pups on Days 23, 36 and 82 to assess fertility and reversibility.

Results: All 4 treated rats overexpressed with F5-peptide and low-dose adjudin were infertile by Day 36, and half of these rats were fertile by Day 82, illustrating reversibility. However, overexpression of F5-peptide alone (or low-dose adjudin alone) had no effects on fertility in n=3 rats. These findings were consistent with the histology data that illustrated the BTB modifier F5-peptide promoted the action of adjudin to induce germ cell exfoliation, mediated by changes in cytoskeletal organization of F-actin and microtubules across the epithelium, thereby reducing the systemic toxicity of adjudin.

Conclusion: In this proof-of-concept study, it was shown that overexpression of the F5-peptide prior to administration of adjudin to rats at a low (and ineffective dose by itself) was found to induce reversible male infertility.

Implications: Overexpression of F5-peptide, an endogenously produced biomolecule in the testis known to induce BTB remodeling, enhanced the contraceptive effect of adjudin in rats, supporting proof of concept studies of BTB disrupters in men.

1. Introduction

Hormonal contraceptives for men [1,2] based on the use of a transdermal gel [3–5] or an injectable [6] with progestogen and testosterone for co-administration to healthy men was found to be effective. However, side effects (e.g., acne, injection site pain, and mood change and increased libido) have led to an early termination of the study [6]. Nonetheless, it is likely that these issues will be resolved by altering the frequency of administration and/or dosing regimens. Nonetheless, these possible side effects, which may still arise following long-term use of hormonal male contraceptives, have prompted investigators to identify alternative non-hormonal male contraceptives [7,8].

Adjudin, 1-(2,4-dichlorobenzyl)-*1H*-indazole-3-carbohydrazide, is a promising non-hormonal male contraceptive under development [9]. For instance, studies have shown that adjudin at 37.5–50 mg/kg b.w. (via oral gavage) was effective to induce transient male infertility in rats [10] and rabbits [11]. However, a narrow margin between efficacy and safety was found based on a 29-day subchronic toxicity study in rats [12]. For instance, the no-observable-adverse-effect level (NOAEL) for adjudin in female rats was at 50 mg/kg/day [12]. But the NOAEL in males could not be established because three out of ten rats had signs

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of skeletal muscle degeneration/atrophy, and one rat exhibited signs of chronic-active portal inflammation and mid-zonal vacuolization of minimal severity, even though their tubules were all devoid of germ cells except spermatogonia and Sertoli cells and no deaths were recorded [12]. This narrow margin between the efficacy and safety of adjudin is the result of its poor bioavailability in the testis because of the blood-testis barrier (BTB) [10]. In short, the BTB blocks the entry of adjudin to the adluminal compartment to exert its effects to induce germ cell exfoliaton that leads to male infertility [9]. Furthermore, the presence of drug transporters such as active drug pumps (e.g., P-glycoprotein) in the testis also reduce the bioavailability of adjudin [13], thereby narrowing the margin between its safety and efficacy. It is likely that the effective dose of adjudin would require a >10-fold reduction in effective dosing to avoid systemic toxicity by improving its bioavailability. A possibility is to modify the BTB function considerably but transiently, to avoid long-term damage to testis function, and to improve adjudin accessibility to the testis.

We have identified F5-peptide produced locally at the Sertoli cell-spermatid interface [14,15], that is apical ectoplasmic specialization (apical ES, a testis-specific anchoring junction type [16]) during the epithelial cycle of spermatogenesis [17]. This peptide effectively induces BTB remodeling, making the barrier "leaky" transiently [15,18]. This peptide is derived from laminin-γ3 chain expressed by elongated spermatids at the apical ES via the action of MMP-2 (matrix metalloprotease 2) [19,20], and it facilitates sperm release during spermiation at stage VIII of the epithelial cycle [18,21]. In this context, it is of interest to note that while laminin-γ3 is broadly expressed in skin, heart, lung, and testis and tightly associated with the apical surface of ciliated epithelial cells in lung, oviduct, epididymis and ductus deferens [19], it is not known if F5-peptide can be generated in other organs via the action of MMP-2 as reported in the testis [15,18,20]. Since the disruptive effects of the F5-peptide on Sertoli cell BTB function is reversible, we thought it pertinent to perform a proof-of-concept study to examine if the F5-peptide that is capable of making the BTB "leaky" can facilitate the entry of a low dose of adjudin, which by itself has no notable phenotypes in the testis, into the adluminal compartment to induce germ cell exfoliation, causing reversible infertility, since the F5-peptide is endogenously produced and side effects, if any, should be minimal.

2. Materials and methods

2.1. Animals

Sprague-Dawley adult male rats at ~250–300 g b.w. and adult female rats at ~200–250 g b.w. were obtained from Charles River Laboratories (Kingston, NY). The use of rats and recombinant DNA materials for all pertinent experiments in this report was approved by the Rockefeller University Institutional Animal Care and Use Committee (IACUC) with Protocol Numbers 12-506 and 15-780-H. At specified time points, rats kept in a gas chamber in a group of two were euthanized by CO_2 asphyxiation using slow displacement (20%-30%/min) of chamber air from a compressed CO_2 cylinder. Testes were removed, weighed and used for specific experiments.

2.2. Treatment of rats with Adjudin, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide

Adjudin has been investigated as a non-hormonal male contraceptive drug [9,10]. Herein, we examined if overexpression of the endogenously produced BTB modifying F5-peptide that renders the BTB "leaky" transiently [14,15] could considerably increase the effectiveness of adjudin by improving its bioavailability in the testis. Adjudin was prepared and used to treat rats as described [10].

2.3. Overexpression of the F5-peptide in adult male rat testes

F5-peptide was a BTB modifier which was shown to have effects to perturb spermatogenesis *per se* [14,15]. However, due to the limited transfection efficiency, at ~60%, its anti-fertility effects had not been examined [14] since rodents remained fertile with just 10% spermatogenic outputs of normal rats/mice [22]. To overexpress the F5-peptide in testes *in vivo*, adult rats (~250–300 g b.w.) were transfected with either pCI-neo/Ctrl (control, empty vector only) or pCI-neo/F5-peptide (pCI-neo plasmid containing the full-length F5-peptide clone) plasmid DNA via intratesticular injection as described [14] using the F5-peptide cDNA [14,15]. In brief, a recently developed transfection reagent specifically designed for *in vivo* transfection known as *in vivo*-jetPEI® (Polyplus) was mixed with plasmid DNA (15 mcg/testis) according to the manufacturer's instructions [14]. The transfection routinely led to a 7-fold increase in the F5-peptide mRNA steady-state level when quantified by qPCR and a ~60% increase in its protein level, which is considerably higher than the regular transfection medium, at ~25%, that was previously used [14].

2.4. Treatment regimens

We had used two similar Regimens in this report with the first one noted in Fig. 1A & C, and the second in Fig. 2A. The Regimen noted in Fig. 2A was used for all the mechanistically based studies reported in Figs. 2–3 and Figs. S1-S3. The differences between the two Regimens were minor. In the first Regimen reported in Fig. 1A & C, three transfections for overexpression of F5-peptide were used, to be followed by three treatment of rats with adjudin at 20 mg/kg b.w. (via oral gavage), so that distinctive phenotypes were clearly noted across the entire testis. In the second Regimen reported in Fig. 2A, only two transfections for F5-peptide overexpression to be followed by two adjudin treatments (10 mg/kg b.w., via oral gavage) were used to avoid excessive damage in the seminiferous epithelium regarding relative distribution of several target proteins between treatment groups, thereby masking differences that could be detected between treatment groups based on results of pilot experiments.

2.5. Fertility test

To assess the fertility status of treated male rats (n=4 rats), fertility test was performed as earlier described using the same number of virgin female rats (n=4 rats) [10]. At birth, the number of pups from each mother rat and the sex of each pup was recorded. Changes in the gross morphology of pups were also noted.

2.6. Other sections of the **Materials and Methods**, including details on the immunological analysis (IF) or dual-labeled IF and general methods, in sections 2.7–2.11., can be found in Supplemental Material section.

3. Results

3.1. Overexpression of the F5-peptide in the testis in vivo lowers the dose response for adjudin-induced germ cell exfoliation and reversible male infertility in rats

Using the regimen shown in Fig. 1A (*top left panel*), adult rat testes were transfected with 15 mcg plasmid DNA/testis of either pCI-neo (empty vector, Ctrl (control)) or pCI-neo containing the F5-peptide cDNA (pCI-neo/F5-peptide) on Day 1, 2 and 6. These rats were then treated with either adjudin at 20 mg/kg b.w. (via oral gavage) *vs.* vehicle (0.05% methylcellulose in Milli Q water, wt./vol.) on Day 2, 4 and 7 before their termination on Day 16. Overexpression of F5-peptide was confirmed by q-PCR that monitored the steady-state mRNA level of this small peptide (Fig. 1A, *right panel*). The status of spermatogenesis in these four groups of rats, including the three control groups (i.e., pCI-

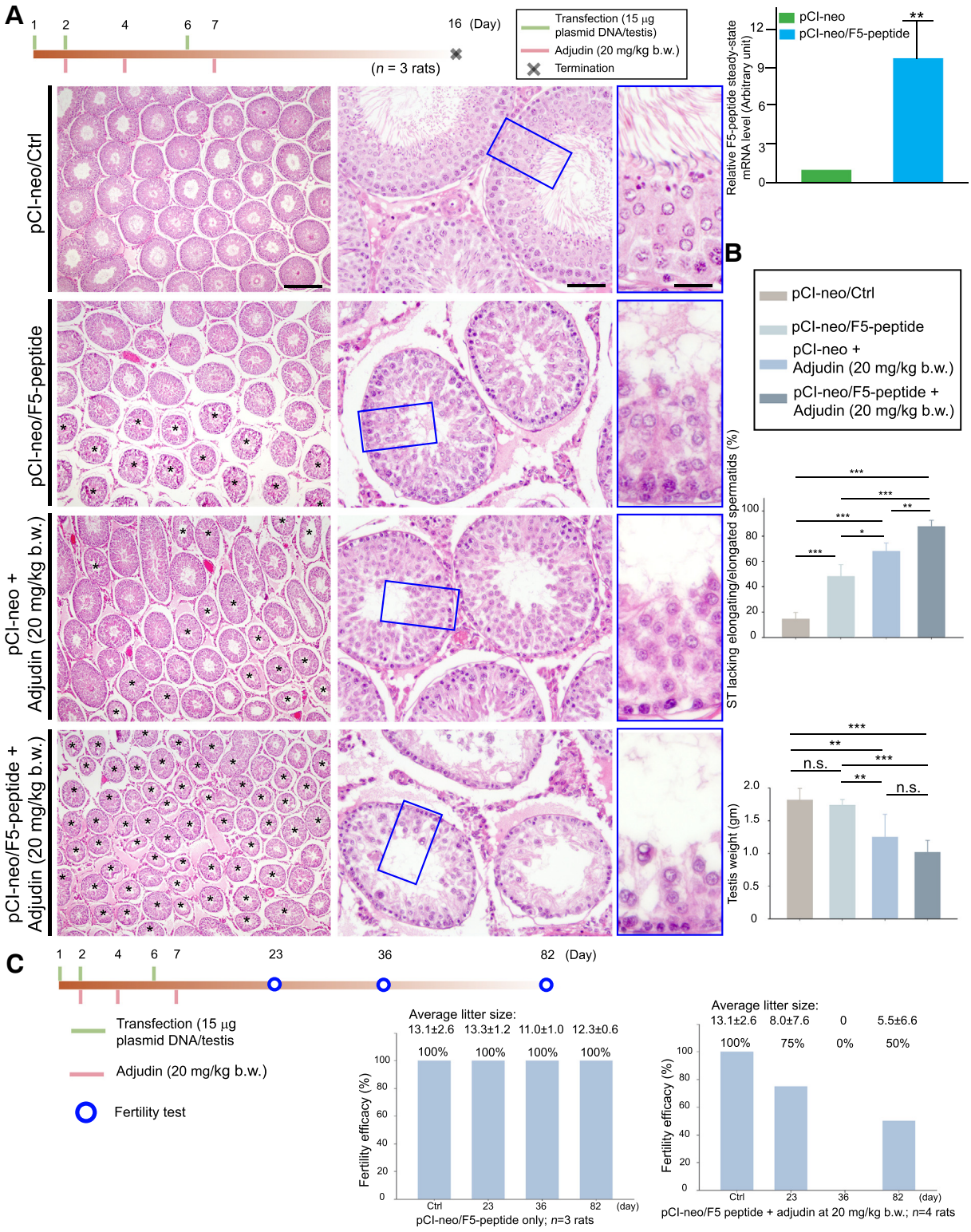

Fig. 1. A study to assess the promoting effects of the F5-peptide on the efficacy of adjudin as a male contraceptive. (A) Regimen for the experiment of $n=3$ rats in each time point. Control groups included: (i) rats transfected with pCI-neo empty vector DNA alone (pCI-neo/Ctrl), (ii) rats transfected with pCI-neo/F5-peptide alone, and (iii) rats treated with adjudin (20 mg/kg b.w., via oral gavage) alone. The treatment group was rats transfected with pCI-neo/F5-peptide and adjudin (20 mg/kg b.w.). The right panel in (A) is the composite data of $n=3$ experiments of qPCR that quantified the steady-state mRNA level of F5-peptide. **p<.01 by Student's *t*-test. The lower panel in **(A)** shows representative micrographs from an experiment using cross-sections of paraffin embedded testes, and stained with H&E. All testes from control and treatment groups were processed simultaneously in a single experimental session to avoid inter-experimental variations, and this experiment was repeated three times using $n=3$ rats, which yielded similar results. Seminiferous tubules devoid of elongating and/or elongated spermatids were labeled with *. Scale bar, 800 μm, which applies to the micrographs in the left column; 80 μm, middle column; 40 μm, right column, which also apply to similar micrographs in **(A)**. **(B)** Defects in spermatogenesis are summarized in these two bar graphs to indicate the relative number of seminiferous tubules devoid of elongating/elongated spermatids. Rats receiving three transfections of the empty vector (pCI-neo) served as the control. Statistical analysis was performed using ANOVA. *p<.05; **p<.01; ***p<.001; n.s., not significantly different. **(C)** Bar graphs showing results of the fertility tests in pCI-neo/F5-peptide or pCI-neo/F5-peptide + adjudin (20 mg/kg b.w.) *vs.* control group (pCI-neo/empty vector). Regimen is shown in the top panel. Average litter size at specified time point was also indicated.

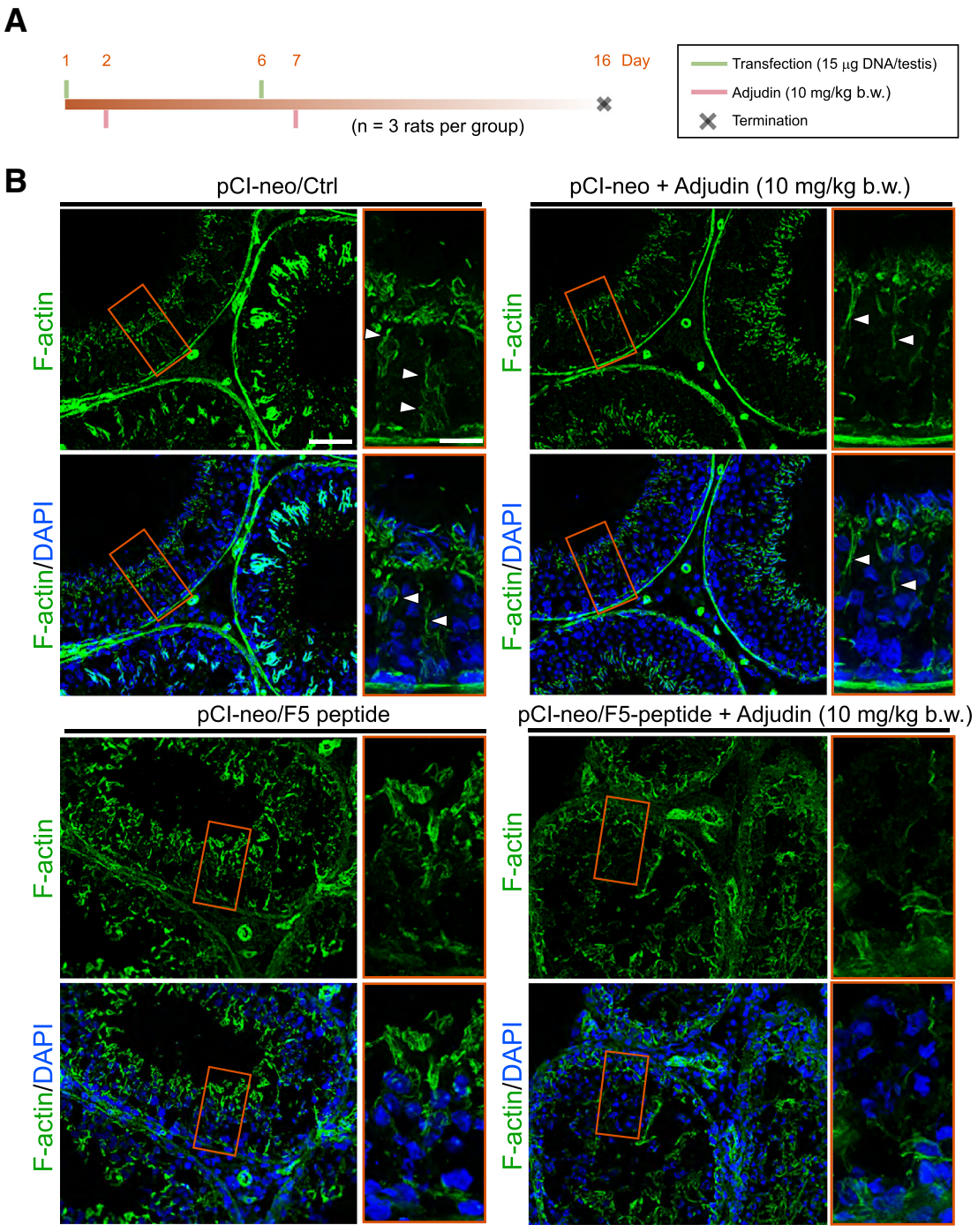

Fig. 2. Overexpression of the F5-peptide promotes the disruptive effects of adjudin on F-actin organization in adult rat testes. **(A)** Regimen used for this experiment with adult Sprague-Dawley rats (~270–300 g b.w.). This regimen was similar to Fig. 1 except that adjudin was used at 10 mg/kg b.w., which by itself had no detectable effects on the phenotypes in the epithelium as reported [10]. Also, two consecutive transfections were used in this regimen on Days 1 and 6, to be followed by two adjudin treatments. Rats from all groups were terminated on Days 16. **(B)** F-actin in the epithelium was visualized by FITC-conjugated phalloidin (green fluorescence). In testes of the control group, F-actin appeared as track-like structures at stage VIII of the seminiferous epithelium (white arrow heads), but also tightly associated with elongating spermatids in stage V-VI tubules and the apical ES at spermatid heads; similar to adjudin (10 mg/kg b.w. by oral gavage) treated rats. The F5-peptide (pCI-neo/F5-peptide) had some disruptive effects on F-actin organization across the epithelium. However, the F5-peptide considerably promoted the adjudin disruptive effects on F-actin organization when it was overexpressed in testes from rats treated with adjudin at low dose. Selected areas of micrographs are boxed in red and magnified on the right. Scale bar, 80 μm; 40 μm in insets, which apply to corresponding micrographs.

neo/Ctrl (negative control), pCI-neo/F5-peptide alone, and adjudin at low dose group (20 mg/kg b.w.)) *vs.* the treatment group (i.e., pCI-neo/F5-peptide/adjudin (20 mg/kg b.w.)) were examined, and results are shown in the *lower panel* Fig. 1A. Control (Ctrl) rats transfected with pCI-neo (empty vector) alone had no effects on spermatogenesis, and the low-dose adjudin (20 mg/kg b.w.) treated group had moderate defects in spermatogenesis, such as elongating/elongated spermatid loss that affected only ~60% of tubules (Fig. 1A; B) consistent with an earlier study [10]. In the pCI-neo/F5-peptide group, ~50% of tubules had signs of elongating/elongated spermatid loss, consistent with an earlier report [14] due to the limited transfection efficiency of the transfection medium (Fig. 1A, B). However, in pCI-neo/F5-peptide + adjudin (20 mg/kg b.w.)

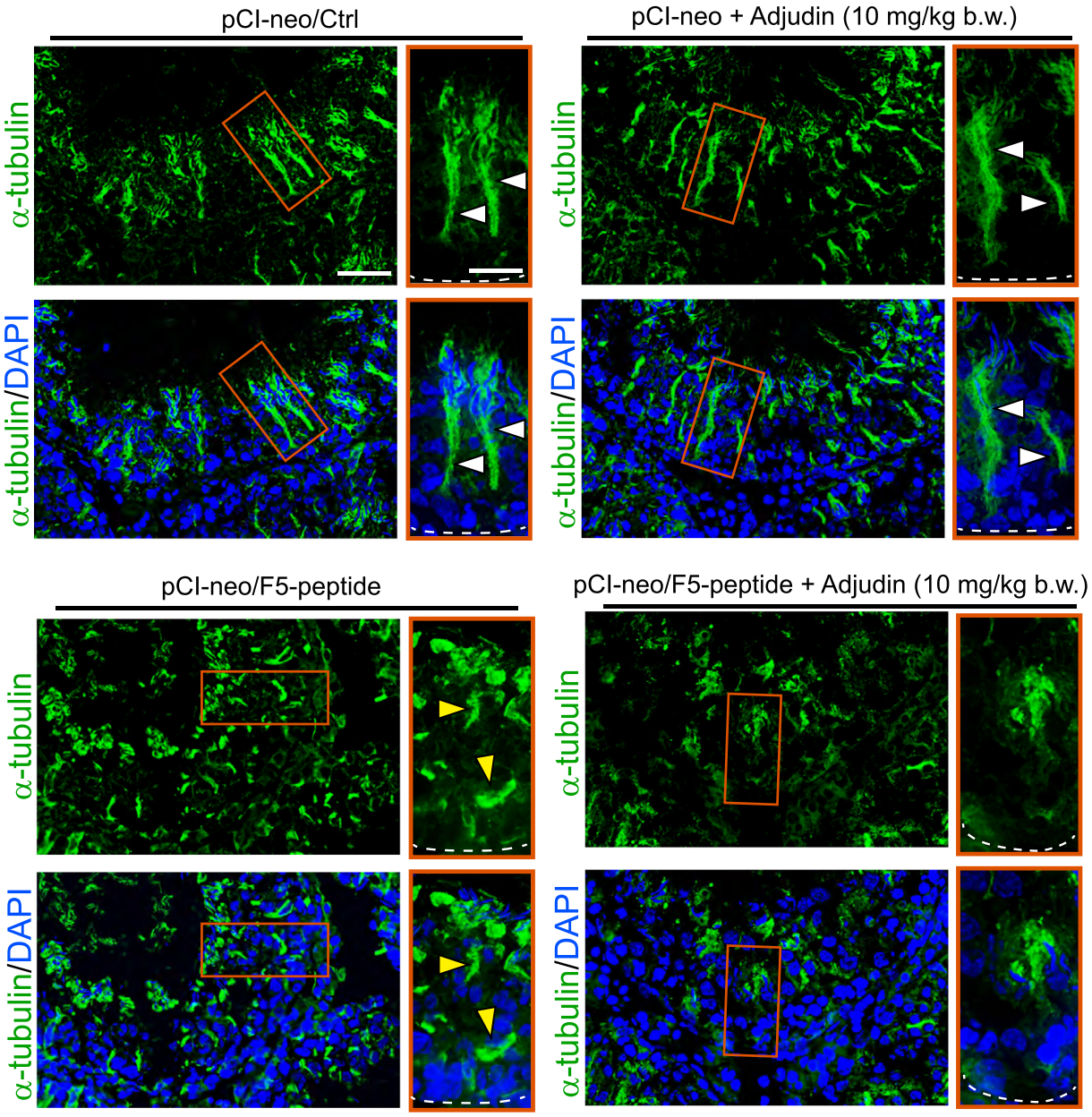

Fig. 3. Overexpression of the F5-peptide promotes the disruptive effects of adjudin on MT-based cytoskeletal organization in the seminiferous epithelium of adult rat testes. Microtubules (MTs) were visualized by staining with α-tubulin (note: α- and ß-tubulins that assemble the α-/ß-tubulin dimers are the building blocks of MTs [37]). As noted in control testes (overexpressed with pCI-neo empty vector), MTs were organized as track-like structures (green fluorescence for α-tubulins, annotated by white arrowheads) that lie perpendicular to the basement membrane (annotated by a dashed white line), stretching across the entire epithelium to support cell (e.g., spermatids) or organelle (e.g., phagosomes, residual bodies) transport during the epithelial cycle. Treatment of rats with adjudin at 10 mg/kg b.w. by oral gavage was found to have no effects on the organization of MT-based cytoskeleton in the epithelium of testes, similar to control rats. However, overexpression of the F5-peptide in the testis perturbed the organization of MT-based tracks, causing considerable truncations, and virtually no track-like structures were noted except some short fragments of MT-based tracks (annotated by yellow arrowheads), some of which lie parallel to the basement membrane. However, overexpression of the F5-peptide and adjudin at 10 mg/kg b.w. had additive effects (since adjudin at 10 mg/kg b.w. alone had no effects) to perturb MT-based cytoskeletal organization since even short fragments of MT-based tracks were not found across the epithelium. Scale bar, 60 μm; 40 μm in insets, which also apply to corresponding micrographs.

group, >95% of tubules had no elongating/elongated and round spermatids nor spermatocytes (Fig. 1A), and had the most severe tubule damage (Fig. 1B). We next performed fertility test to assess the effects of these treatments on the fertility using the Regimen shown in the top panel of Fig. 1C on Days 23, 36 and 82. We found that transfection of the testis with the F5-peptide alone (pCI-neo/F5-peptide), similar to using adjudin alone at 20 mg/kg b.w. as earlier reported [10], did not affect the fertility of the treated rats (Fig. 1C) since an earlier study has shown that spermatogenesis at 10% capacity was sufficient to maintain fertility in rodents [22]. Thus, in order to suppress male fertility in rodents, a drug has to be able to induce defects in spermatogenesis in >95% of tubules. Indeed, the combined use of the F5-peptide and adjudin at 20 mg/kg b.w. was effective in inducing male infertility (Fig. 1C). These findings thus confirm the notion that the use of the F5-peptide that caused the BTB to become "leaky" promoted the entry of adjudin into the adluminal compartment to induce spermatid exfoliation, thereby enhancing its efficacy. In brief, data shown in Fig. 1A-C have unequivocally demonstrated that co-administration of low-dose adjudin (20 mg/kg b.w., by oral gavage) alongside with overexpression of the endogenously produced BTB modifier F5-peptide effectively induced transient infertility which was also reversible based on histological analysis and fertility test. This was possible since F5-peptide was highly effective to induce transient

"opening" of the BTB as recently reported [14,15], thereby adjudin (even administered at low dose via oral gavage) was able to enter the adluminal compartment to induce germ cell exfoliation as noted in Fig. 1A-B. As such, F5-peptide lowered the effective dose of adjudin and either treatment alone (i.e., overexpression of F5-peptide or adjudin at low-dose) did not cause the typical changes noted following overexpression of F5-peptide and low-dose adjudin.

3.2. Overexpression of the F5-peptide promotes the disruptive effects of adjudin on actin-based cytoskeletal organization in the testis, leading to germ cell exfoliation

We next investigated the possible mechanism by which the combined use of the F5-peptide and adjudin (at a low dose) that perturbed spermatogenesis, thereby disrupting fertility in adult rats. The regimen used for this study is shown in Fig. 2A. In this study, we had reduced the frequency of transfection with pCI-neo plasmid DNA (either pCI-neo/Crl (i.e., empty vector) or with the F5-peptide (pCI-neo/F5-peptide)) from thrice to twice, and the adjudin dose was lowered from 20 to 10 mg/kg b.w. (via oral gavage). This regimen was selected based on results of pilot experiments so that the synergistic effects of the F5-peptide that promoted the disruptive effects of adjudin on the epithelium were considerably more obvious for examination. As shown in Fig. 2B, the organization of F-actin across the epithelium in rats treated with adjudin at 10 mg/kg b.w. was indistinguishable from control testes. For instance, F-actin was expressed at the apical ES, tightly associated with elongating/elongated spermatids, and appeared as "track-like" structures across the epithelium (annotated by white arrowheads) and also prominently expressed at the basal ES/BTB in adjudin-treated rats at 10 mg/kg b.w., similar to the phenotype in control testes. However, following overexpression of the F5-peptide, the organization of F-actin across the epithelium was considerably disrupted since F-actin was no longer expressed at the apical and basal ES/BTB, and no distinguishable track-like structures across the seminiferous epithelium were noted (Fig. 2B). The disruptively organized F-actin was even more remarkable in testes overexpressed with the F5-peptide and adjudin (also at dose of 10 mg/kg b.w.) since F-actin was randomly expressed across the epithelium with fewer apical ES structures around to support spermatid adhesion, and most tubules were devoid of spermatids (Fig. 2B *vs.* Fig. 1A). Additional investigations were performed to understand the underlying mechanism by which F5-peptide promoted the disruptive effects of adjudin at low dose that led to reversible infertility in the treated rats. This included an examination of actin regulatory proteins (Fig. S1), and actin nucleation proteins (Fig. S2). These changes in turn, affected the function of the apical ES (Fig. S3A) and basal ES (Fig. S3B), thereby perturbing germ cell adhesive and BTB function as noted in data shown in Figs. 1 and 2.

3.3. Overexpression of the F5-peptide promotes the disruptive effects of adjudin on microtubule (MT) organization, leading to male infertility

In control testes, MTs are organized into track-like structures that support the transport of spermatids and organelles (e.g., residual bodies, phagosomes, Golgi apparatus and others) across the seminiferous epithelium (see white arrowheads) (Fig. 3). When rats were treated with adjudin at 10 mg/kg b.w., there were no noticeable effects on MT organization, since the track-like structures that laid perpendicular to the basement membrane (annotated by a dashed white line) were similar to those in control testes (Fig. 3). Overexpression of the F5-peptide alone did perturb the organization of MT-based track-like structures since these tracks were truncated and no longer aligned perpendicular but parallel (see yellow arrowhead) to the basement membrane, consistent with findings of an earlier report [14]. However, the F5-peptide was found to considerably promote the disruptive effects of adjudin at a dose that had no effects on MT organization per se, causing more remarkable disruptive changes on MT organization where MTs were grossly disrupted across the entire epithelium in rats treated with pCI-neo/F5-peptide + adjudin (10 mg/kg b.w.) *vs.* pCI-neo/F5-peptide (Fig. 3).

4. Discussion

Blood-tissue barriers, including the BTB, are crucial guardians of tissue homeostasis in multiple organs [23–29]. They maintain a specialized microenvironment behind the barrier by regulating the paracellular and transcellular transport of ions, electrolytes, nutrients, water, biomolecules, hormones, and paracrine factors. However, blood-tissue barriers also pose a major obstacle to the delivery of drugs, often limiting their bioavailability [30,31]. This thus poses a major hurdle for the development of non-hormonal male contraceptives, that is, if the candidate drug exerts its effects behind the BTB and affects germ cell development, germ cell adhesion, spermiogenesis and/or meiosis in the adluminal (apical) compartment [25,29,32]. For instance, a study involving the oral administration of [^{3}H]-adjudin to adult rats has shown that less than 5% of the administered adjudin was detected in the testis [10], illustrating the BTB poses a major barrier to block the entry of adjudin to the testis. Thus, the search of a novel approach, bypassing the barrier imposed by the BTB, remains a priority in the field.

Studies have shown that there is an autocrine-based functional axis in the testis. Here, laminin-γ3 expressed by elongated spermatids [19,21], which forms a bona-fide adhesion protein complex with α6ß1-integrin expressed by Sertoli cells [20,33–35], is cleaved by MMP-2 and transiently expressed at the apical ES in stage VIII tubules [20]. This, in turn, generates a biologically active fragment designated the F5-peptide [15] that is released from domain IV of the laminin-γ3 chain [14,15,18]. Interestingly, the F5-peptide is capable of inducing BTB remodeling [14,15], thereby facilitating the transport of preleptotene spermatocytes across the immunological barrier. On the other hand, the F5-peptide also potentiates breakdown of apical ES [14] to facilitate sperm release during spermiation with both cellular events taking place in stage VIII tubules [36]. In short, these dual actions of the F5-peptide in potentiating the breakdown of the apical ES and in inducing restructuring of the basal ES/BTB thus coordinate the cellular events of spermiation and BTB remodeling that take place simultaneously at the opposite ends of the seminiferous epithelium at stage VIII of the epithelial cycle. In brief, the F5-peptide is exceedingly effective in making the Sertoli cell TJ-barrier "leaky" based on studies both in vitro and in vivo [14,15]. Also, F5-peptide *per se* was able to induce defects in spermatogenesis but due to the limited transfection efficiency of the current transfection reagent, we did not assess its antifertility effects in male rats [14,15]. However, we have examined herein if the F5-peptide is used in conjunction with adjudin, this approach can considerably reduce the effective dose of adjudin, thus providing a novel approach to improve the bioavailability of any non-hormonal male contraceptive drug that exerts its anti-fertility effects behind the BTB. More importantly, the disruptive effects of the F5-peptide on the Sertoli cell BTB in vitro and in vivo are reversible, and its long-term health risks should be minimal since this is an endogenously produced biomolecule.

Indeed, as noted herein, while the use of adjudin at 10–20 mg/kg b.w via oral gavage at doses that were ineffective at inducing infertility or extensive germ cell exfoliation in particular at 10 mg/kg b.w. [10], the use of the F5-peptide via overexpression was found to potentiate the disruptive effects of adjudin. Results of the fertility test and histological analysis reported herein have shown that using this approach, the amount of adjudin needed to achieve transient male infertility in rats can be lowered from three doses of 50 mg/kg b.w. (by oral gavage) [10] *vs.* three doses of 10 or 20 mg/kg b.w. (also by oral gavage), illustrating a considerably reduction in effective dosing. This proof-of-concept study thus supports the notion that the effects of adjudin at a low dose (e.g., 10 mg/kg b.w.) but coupled with overexpression of the F5-peptide is mediated by changes in BTB integrity induced by F5-peptide (i.e., making the immunological barrier "leaky" as earlier reported [14,15]). This, in turn, improves the bioavailability of adjudin and

enhances its access to the adluminal compartment behind the BTB in the seminiferous epithelium to effectively induce germ cell exfoliation.

Based on the fertility data reported herein, rats regained their fertility quickly at Day 82 when these rats were infertility at Day 36, illustrating the suppression of spermatogenesis is very transient. This is likely due to the presence of some undifferentiated spermatogonia as noted in the seminiferous epithelium based on histological analysis. As such, ~40 days were sufficient to bring back the fertility, at least partially, since 10% of the spermatogenesis outputs in rodents is sufficient to maintain some capacity of fertility [22]. Additional discussion can be found in Supplemental Material section.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.contraception.2019.01.007.

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