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Melphalan Alone or Conjugated to a Follicle Stimulating Hormone- β Peptide Kills Murine Testicular Cells *in vitro* and Transiently Suppresses Murine Spermatogenesis *in vivo*

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Abstract

New approaches to sterilizing male animals are needed to control captive and wild animal populations. We sought to develop a non-surgical method of permanent sterilization for male animals by administering the gonadotoxicant melphalan conjugated to peptides derived from the β -chain of follicle stimulating hormone (FSH β). We hypothesized that conjugating melphalan to FSH β peptides would magnify the gonadotoxic effects of melphalan while minimizing systemic toxicity. The ability of conjugates of melphalan and FSH β peptides to kill murine testicular cells was first tested *in vitro* in a three-dimensional testicular cell co-culture system. In this system, melphalan caused considerable cell death as measured both by increases in LDH concentrations in the culture supernatant and direct visualization of the cultures. Of the conjugates tested, melphalan conjugated to a 20 amino acid peptide derived from human FSH β consisting of amino acids 33-53 (FSH β (33-53)-melphalan) was very potent, with cell cytotoxicity and LDH release roughly one-half that of melphalan. The effects of melphalan and FSH β (33-53)-melphalan on spermatogenesis were then tested *in vivo* in mature C56Bl/6 male mice. Four weeks after intraperitoneal injection, all mice treated with either FSH β (33-53)-melphalan or melphalan had ~75% reductions in testicular spermatid counts compared with control animals. Testicular histology revealed significant reduction in mature spermatids and spermatocytes in most tubules. However, 12 weeks

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after the injection, testicular spermatid counts and histology were similar to controls, except in one animal receiving FSH β (33-53)-melphalan that had no apparent spermatogenesis. We conclude that melphalan and FSH β (33-53)-melphalan are potent gonadotoxicants in male mice resulting in marked suppression of spermatogenesis 4 weeks after a single intraperitoneal injection. However, this effect is transient in most mice as spermatogenesis is similar to control animals 12 weeks after drug administration. Melphalan or FSH β (33-53)-melphalan may be useful for the temporary control of fertility in male animals, but additional research will be needed to develop a single dose method of permanent sterilization for male animals.

Keywords

Gonadotoxicants; Sterilization; Male Animal Contraception; Spermatogenesis

Introduction

The most common method of sterilizing male animals relies on removal of the testes, which requires skilled personnel and can cause significant post-procedural pain (1). Removal of the testes also deprives the animal of testosterone, which may lead to health and behavioral problems (2), and increase the risk of prostate cancer (3-5) or osteoporosis (2, 6) later in life. An alternative approach to male sterilization is the injection of a solution of calcium chloride (7) or zinc gluconate (8) directly into the testes. These injections are very effective from a contraceptive standpoint; however, severe injection site reactions requiring surgery can occur in up to 4% of recipients (9), with others experiencing scrotal ulceration or dermatitis (10). Therefore, there is a need for novel methods of permanent sterilization for male animals. Ideally, such a method would be inexpensive and administered with a single injection.

Several investigators have examined the potential of gonadotoxicants for the purposes of male sterilization. For example, ketoconazole (11), alpha-chlorohydrin (12) and emblein (13) have been tested in animals. However, the suppression of spermatogenesis was incomplete and systemic toxicity was limiting (14). We sought to test a novel approach to male sterilization using the potent gonadotoxicant melphalan. Melphalan, an alkylating agent, is widely used in the treatment of lymphomas and multiple myeloma in both humans and animals. Infertility is frequently seen after administration of melphalan (15, 16), and melphalan is fairly unique among gonadotoxicants in that it kills both spermatogonial stem cells and dividing germ cells *in vivo* (17). Melphalan has been used extensively in animals for the treatment of malignancies (18, 19), and its pharmacokinetics and marrow toxicity are well described (20, 21). Despite its widespread use in animals, the effect of melphalan on spermatogenesis in animals has not been well studied. We sought to: 1) determine if melphalan could be used to induce sterility, and 2) investigate if we could facilitate melphalan's effect and minimize any toxicity by targeting the melphalan specifically to the testes via conjugation to peptides derived from the β -chain of human follicle stimulating hormone (FSH- β).

Follicle-stimulating hormone (FSH) is a protein hormone produced in the anterior pituitary. FSH binds to the FSH receptor (FSHr) on Sertoli cells within the seminiferous tubules of the testes (22), stimulating them to nurture the developing germ cells. The FSH α chain is not required for binding to the FSHr, but the FSH β chain specifically binds to the FSHr with high affinity and is required for receptor activation (23). Due to its high expression in the gonads and very low levels of expression in other tissues, FSHr provides an elegant targeting mechanism for drug delivery to the gonads. Indeed, FSH β has been shown to be an effective gonad-specific drug delivery vehicle for experimental forms of reversible contraception in mice. In one such study, the contraceptive efficacy of adjuvin was increased 10,000-fold by conjugation to FSH β (24). In another example, FSH β conjugated to a peptide that interrupted the integrity of the blood-testes barrier caused significant loss of germ cells and a decrease in fertility (25).

Use of the entire FSH β molecule for targeting, however, is impractical for widespread use due to the expense of either the chemical synthesis or recombinant production of the entire FSH β chain, which is 112 amino acids in length. Fortunately, FSH β -derived peptides that bind to the FSH receptor with high affinity have been described (26-30). The most attractive candidates for drug delivery are peptides based on the amino acids in positions 33-53 and 81-95 of FSH β (27, 28). Each of these peptide sequences has good *in vitro* FSH receptor binding inhibition and demonstrated *in vivo* efficacy. Furthermore, there is good recognition of these conserved peptides by the FSHr across species (27, 31). Peptides with terminal modifications and substitutions of serine for cysteine increase FSHr binding, as measured by competitive inhibition studies (26, 30). These peptides have been used to target drugs to the gonadal tissue. Both peptides specifically targeted ovarian cancer cells with nanoparticles (32, 33). Therefore, we hypothesized that the conjugation of FSH β -derived peptides to the gonadotoxicant melphalan would allow for sterilization of male animals while minimizing the side effects induced by high-dose melphalan administered alone. We tested melphalan and FSH β -melphalan conjugates *in vitro* and *in vivo* for their ability to kill mouse testicular cells and thereby induce sterility.

2. Material and Methods

2.1 Melphalan and FSH β -melphalan Conjugate Preparation

Melphalan was obtained commercially (Sigma, St. Louis, MO). Two different FSH β -derived peptide sequences residues β 33-53 and β 81-95 were employed as the carriers for melphalan. The melphalan was conjugated to either the amino or carboxy terminus of each peptide, with or without the presence of terminal modifications and addition or deletion of terminal amino acids. The molecular weights and FSH β -amino acid composition of the sixteen tested are listed in Table 1. FSH β -melphalan conjugates were obtained from RS synthesis (Louisville, KY), Biomatik (Wilmington, DE) or Synbiosci (Livermore, CA) and were prepared using standard Fmoc-based solid phase peptide synthesis followed by HPLC purification until greater than 95% purity. Preparations were free of detectable unconjugated melphalan.

2.2 *In Vitro* Cytotoxicity Assay and Imaging

Melphalan and FSH β -melphalan conjugates were evaluated for cytotoxicity using a lactate dehydrogenase release assay in a three-dimensional *in vitro* testicular co-culture model (34). In brief, the testes of male Sprague–Dawley rat pups (Harlan, Hayward, CA) were dissected from 5-day-old pups, decapsulated under a dissection microscope and the seminiferous cords/tubules were pooled and digested in a solution of 0.1% collagenase (Worthington Biochemical Corporation, Lakewood, NJ), 0.1% hyaluronidase (Sigma, St. Louis, MO), DNase I (0.001 mg/ml, Sigma, St. Louis, MO), and Eagle's minimal essential medium (MEM Life Technologies Inc., Gaithersburg, MD) for 20 min at 37°C (35, 36). Cells mixtures underwent two rounds of further digestion with 0.1% collagenase and DNase I for 20 min at 37°C, before a final digestion performed by the addition of 0.05% trypsin-ethylene diamine tetraacetic acid for 3 min. Cells were filtered through a 100 μ m nylon mesh cell strainer (BD Biosciences, Franklin Lake, NJ) to yield single cell suspension, which consists primarily of Sertoli cells, Leydig cells and type A spermatogonia. Cells were re-suspended in hormone-free and serum-media and plated in 35-mm Primaria (BD Biosciences, Franklin Lake, NJ) dishes at a density of 0.8×10^6 cells/ml in serum-free medium. An extracellular matrix Matrigel overlay (BD Biosciences, Franklin Lake, NJ) was applied directly to the medium 30 min after seeding at a final concentration of 150 μ g/ml (30 μ l of Matrigel/35 mm dish) medium. Cells were then placed into a 37°C atmosphere in a humidified chamber with 95%/5% air/CO₂, and allowed to acclimate for 48 hours prior to chemical treatment. Melphalan and FSH β -melphalan conjugates were tested at final cell culture concentrations of 1, 5, and 20 μ mol. For the treatment, culture media was mixed with the stock solution for each of the constructs or melphalan. The constructs were prepared in phosphate buffered saline. For the solution of melphalan, 3.3 mg of melphalan was suspended in 1 ml ethanol, followed by an addition of 750 μ l DMSO (with vortexing), and then diluted with 750 μ l normal saline to a final volume of 2.5 ml. DMSO was added to untreated control cultures. At each timepoint, 50 μ l of media was harvested from the culture plates into 96 well plates and used for released lactate dehydrogenase (LDH) cytotoxicity assay. LDH is normally a cytosolic enzyme, but it is released upon cell lysis, and is therefore a useful marker of cytotoxicity. LDH was measured 24, 48, and 72 hours after treatment using CytoTox 96® non-radioactive cytotoxicity assay (Promega Corporation, Madison, WI, USA). The assay results were calculated relative to the percentage of the maximum LDH release data, which was generated by a set of three maximum LDH release controls for each respective timepoint. In addition, photomicrographs of the cultures were obtained after 24, 48 and 72 hours of culture. Lastly, cultures were stained for Anti-Mullerian hormone, a marker of Sertoli cells using anti-AMH primary goat antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and an Alexa Fluoro 488 rabbit anti-goat IgG (Life Technologies, Grand Island, NY, USA) and visualized using fluorescent microscope (Nikon Instruments, Melville, NY and Nuance Imaging Systems, PerkinElmer, Waltham, MA, USA) after 24, 48 and 72 hours of culture.

2.3 The Effect of Melphalan and FSH β (33-53)-melphalan Conjugate on Spermatogenesis

Based on the results from the *in vitro* testing, the FSH β (33-53)-melphalan conjugate (“conjugate F”) was selected for testing in male C57/Bl6 mice. Twenty-four male mice,

aged 16 weeks, were used for this experiment. Six mice each were injected intraperitoneally with: Group 1) 7.5 mg/kg melphalan once on day 0; Group 2) 71 mg/kg FSH β (33-53)-melphalan conjugate (F) on day 0, 3, 5 and 8 (four injections); Group 3) FSH β (33-53)-melphalan conjugate (F) 284 mg/kg once on day 0, or Group 4) sterile saline (negative control) once on day 0. The dose was based on an earlier pilot study. The repeated dose group (group #2) was included to determine if a longer exposure might be superior to the single large dose (group #3). Three mice from each group were sacrificed 4 weeks after the injection(s) for documentation of an acute effect on spermatid counts and testicular histology and the remaining three mice in each group were sacrificed 12 weeks after the injection(s) to determine if the effects observed at 4 weeks persisted. Blood was sent for complete blood counts and routine chemistries. For testicular spermatid counts, one testes was individually weighed and homogenized in 0.1M sodium phosphate buffer, pH 7.4, containing 0.1% Triton X-100, using an all-glass Kontes 15 mL homogenizer (8 strokes). Ten microliters of the homogenate was counted using a Neubauer phase contrast hemocytometer. Total homogenization-resistant spermatids per gram and per organ were calculated by correcting for the number of squares counted, dilution, volume, and weight as described previously (37, 38). In addition, a portion of one testis was fixed in Bouin's solution and stained with Hematoxylin-Periodic acid Schiff's reagent for morphological analysis. All procedures involving the use of animals and animal tissues were approved by the University of Washington's Institutional Animal Use and Care Committee prior to being performed.

2.4 Statistical Analysis

The results of the cytotoxicity assay are presented in a descriptive fashion. Comparisons of the testicular weights, spermatid counts and daily sperm production in between treatments were performed by a Kruskal-Wallis ANOVA with a Wilcoxon rank-sum post-hoc test. Analyses were performed using STATA version 10 (College Station, TX, USA). An alpha of <0.05 was considered significant.

3. Results

3.1 *In vitro* Cytotoxicity Testing of Melphalan and FSH β -melphalan Conjugates

The results of the cytotoxicity testing of melphalan and the FSH β -melphalan conjugates (Table 1), as demonstrated by the release of LDH over time, are summarized in Figure 1. Good cytotoxicity was observed when the N-terminal acetate was included with the β 33-53 peptide C-terminal conjugate F. The β 33-53 peptide lacking an N-terminal acetate was less effective (C, D) than conjugate F. There was also less cytotoxicity when the melphalan was placed on the N-terminus of the β 33-53 peptide (conjugate E). We also found that the FSH β peptides based on the FSH residues β 81-95 (conjugates G, H, I) were less cytotoxic than conjugate F regardless of melphalan position and/or the presence of N-terminal acetate or C-terminal amides. Two different lots of construct (F), the FSH β (33-53)-melphalan conjugate, prepared months apart yielded similar results.

We then studied amino acid substitution of this core motif, Ac33-53X-amide (F), which proved sensitive to further peptide alterations. Deletion of six (M), three (N) or even one (Q)

C-terminal amino acid from the core sequence greatly diminished cytotoxicity compared to conjugate F. In contrast, deletion of two (R) or four (S) amino acids from the N-terminus resulted in more mild decreases in cytotoxicity compared with conjugate F. Addition of a C-terminal lysine, the next amino acid in the FSH β peptide, and then conjugation of melphalan to its side chain (K), also has reduced toxicity compared to conjugate F. Other approaches, such as the conjugation of two or three melphalan molecules to the C-terminus (O, T) did not improve cytotoxicity compared to conjugate F. Lastly, a construct containing the reported minimal sequence necessary for receptor binding (39) (residues β 34-37 attached to α 92-96) was less cytotoxic than 33-53 FSH β -melphalan (data not shown). Construct F was then tested in combination with the FSH α chain. This combination resulted in slightly improved cytotoxicity as compared with FSH β (33-53)-melphalan conjugate alone (Figure 1).

Examples of the appearance of the cultures over time are shown in Figure 2. The testicular cell cultures with melphalan exhibit marked cell death over 72 hours compared to control cultures (Figure 2). Similarly, FSH β (33-53)-melphalan cultures exhibited significant cytotoxicity compared to control cultures, but not as much as was observed in the melphalan cultures. To insure that the FSH β (33-53)-melphalan was specific for gonadal cells, a cytotoxicity experiment using mouse fibroblasts was performed. At 72 hours, the melphalan cultures showed 20% of maximal LDH release. In contrast the 33-53 FSH β -melphalan cultures exhibited less than 5% maximal levels of LDH release ($P < 0.01$) suggesting the cytotoxic capacity of FSH β (33-53)-melphalan was specific for gonadal cells compared to melphalan alone. These cultures were stained for Anti-Mullerian Hormone (AMH) expression, a marker of Sertoli cells and examined using immunofluorescopy (Supplementary Figure 1). Bright staining for AMH is observed in the control cultures after 24 and 48 hours of incubation, but very little is observed in either the melphalan or FSH β (33-53)-melphalan cultures, implying that Sertoli cells may be an early target of these compounds.

3.2 Effects of Melphalan and 33-53 FSH β -melphalan on Spermatogenesis *in vivo*

Given the demonstrated cytotoxicity of FSH β (33-53)-melphalan and its specificity *in vitro*, it was selected for testing *in vivo*. After 4 week of treatment, all animals dosed with either melphalan or FSH β (33-53)-melphalan had >75% reduction in testicular spermatid counts compared with the placebo group ($P < 0.01$ for all comparisons) (Table 2 and Figure 3). Unfortunately, 12 weeks after treatment, quantitatively and histologically normal spermatogenesis had returned in all treatment groups (Table 2 and Figure 3). Notably, however, one animal who received the single high-dose of FSH β (33-53)-melphalan had no apparent spermatogenesis 12 weeks after the injection, suggesting that this animal might have been sterilized by treatment. Histological study of the testes of this animal showed that spermatocytes, but no round or elongating spermatids were present at this time. In terms of systemic toxicity, complete blood counts and serum chemistries remained normal in all groups, except for a slight, non-significant reduction in lymphocytes count at 4 weeks in the group treated with melphalan alone. Lymphocyte count was normal at 12 weeks in this group (data not shown).

4. Discussion

Herein, we report on our efforts to develop a method of permanently sterilizing male animals by the use of a gonadotoxicant, melphalan, alone and conjugated to a peptide derived from FSH β intended to specifically target the melphalan to the testes. Our extensive *in vitro* work resulted in the identification of a conjugate consisting of a 20-amino acid peptide and melphalan (conjugate F) that exhibited significant cytotoxicity *in vitro* that appeared to be more specific for testicular cells as opposed to fibroblasts. This FSH β (33-53)-melphalan conjugate was very sensitive to changes in the amino acid sequence, the presence or absence of C-terminal modifications and the position (amino or carboxy terminus) and number of melphalan molecules. Placing the melphalan at the carboxy terminus of the 33-53 β peptide with the terminal amide modification resulted in the best cytotoxicity. Whether the cytotoxicity of the other conjugates *in vitro* was due to the melphalan interfering with peptide binding to the FSH receptor or conformational shifts in the 3-dimensional structure of the peptides introduced by binding to melphalan is unknown. It does appear that the FSH β (33-53)-melphalan conjugate was more specific for testicular cells than melphalan, as it had little toxicity to fibroblasts, in contrast to the melphalan alone, which resulted in significant cytotoxicity to fibroblasts.

When tested *in vivo* the FSH β (33-53)-melphalan conjugate, and well as melphalan markedly suppressed spermatogenesis by 4 weeks. Histological analysis from testes of animals treated with either melphalan or FSH β (33-53)-melphalan suggest that damage to spermatogenesis was induced early in the process, probably at the level of spermatogonia that are undergoing mitosis prior to the meiotic divisions necessary to make mature spermatozoa. This would make sense given the known mechanisms of action of nitrogen mustard alkylating agents that target cells undergoing mitosis (40). Likely the post-mitotic germ cells are unaffected and complete spermatogenesis, leaving only Sertoli cells and undifferentiating spermatogonia in many of the tubules 4 weeks after treatment. These unaffected spermatogonia then begin dividing, and appear to successfully re-populate many of the tubules 12 weeks after treatment. However, some areas remain depleted even after 12 weeks, with only Sertoli cells present on histological examination, implying that more severe injury to the spermatogonia may have occurred in these tubules. However, most of the tubules appear to have recovered 12 weeks after the injection of melphalan or the FSH β (33-53)-melphalan. This suggests that the majority of the undifferentiated spermatogonia seem to be resistant to the effects of the melphalan, and these cells form a “reservoir” of stem cells that can repopulate the seminiferous tubules after injury. This finding has significant implications for efforts to develop a non-surgical method of sterilizing animals, as it suggests that all spermatogonia must be destroyed to effectively sterilize an animal and prevent recovery of spermatogenesis. Whether a larger dose of the FSH β (33-53)-melphalan would accomplish this as observed in one of the mice will be the subject of future study. To date, only certain types of chemotherapy (e.g. busulfan), pesticides and ionizing radiation have been described to have this effect. Busulfan is of some interest as it is one of the most potent gonadotoxicants known and is routinely used to sterilize mice (41). Unfortunately, busulfan’s chemical structure did not allow for linkage to FSH β (33-53), and it is too toxic when administered alone in terms of bone marrow and neurological toxicity to be considered

for sterilizing larger captive and wild animals (42-44). Indeed, melphalan is unique among gonadotoxicants in its ability to be conjugated to FSH β (33-53), likely due to the fact that it is an amino acid derivative. These approaches make them impractical for non-surgical sterilization. A better understanding of the mechanisms of sperm stem cell self-renewal may be required before a non-surgical approach to male sterilization can be safe and practical.

The correlation between the *in vitro* and observed *in vivo* effects on spermatogenesis validates the utility of the three-dimensional testicular co-culture system for detecting compounds that are likely to have deleterious effects on spermatogenesis *in vivo*. This system is relatively inexpensive and sensitive and has been used to determine the toxic effects of a variety of compounds (34, 35). It must be noted, however, that this approach to screening compounds *in vitro* has a limitation in that the behavior of Sertoli cells *in vitro* may differ from their behavior *in vivo*. Sertoli cells in culture can proliferate (41), and therefore may be more sensitive to the effects of melphalan compared to Sertoli cells *in vivo*, which are non-replicating. With these caveats, future work directed at developing novel compounds for male sterility could use this system to screen candidate molecules prior to *in vivo* testing.

One limitation of our study is that we cannot determine if the reduction in spermatogenesis observed in the animals treated with FSH β (33-53)-melphalan was due to specific targeting of the conjugate to the testes as opposed to serving as a “carrier” for the melphalan, which was then cleaved from the peptide after *in vivo* administration. In theory, release of the melphalan prior to binding of the FSH β (33-53)-melphalan to the FSH receptor could account for the testicular toxicity similar to that observed in the melphalan treated animals. However, we believe this is unlikely due to the remarkable resistance of the fibroblasts to FSH β (33-53)-melphalan compared to melphalan alone. Future studies using labeled FSH β (33-53)-melphalan will be required to understand whether its effect on spermatogenesis is secondary to specific targeting. More broadly, it is uncertain if melphalan needs to be released from its peptide carrier to function (42). Prior work on melphalan-cytotoxin conjugates suggests that C-terminal conjugation of a peptide with the melphalan amine does not alter the efficacy of the melphalan implying that release from the FSH β -derived peptide may not be necessary to damage the germ cells (43). In theory, FSH β (33-53)-melphalan should share the same biological fate as FSH, which localizes to the plasma membrane of FSH receptor-bearing cells before accumulating in the endosomal compartment prior to degradation in the lysosomes (44). This should then liberate the melphalan into the cytoplasm of the Sertoli cell. However, germ cell toxicity was more evident histologically than Sertoli cell death. Therefore, either the above mechanism is incorrect, or the Sertoli cells, which are non-dividing in the adult were not susceptible to the effects of the melphalan. In this case, the toxicity of the melphalan to the germ cells could either occur from transfer of the melphalan directly to the germ cells from Sertoli cells, or by passive diffusion within the basal compartment of the testis. Understanding this process will require additional studies using fluorescently labeled FSH β (33-53)-melphalan to better define its cellular trafficking.

Notably, no severe systemic toxicity was observed from either treatment except for a transient non-significant decrease in the white blood cell concentration in the animals

receiving melphalan alone. Bone marrow toxicity with melphalan with larger doses is well described, and is dose limited in the treatment of hematological cancers. Given the small sample size in our study, we likely lacked the power to detect a significant difference in this endpoint. Moreover, repeated dosing of melphalan may have resulted in a larger effect on hematopoiesis.

In summary, we have found that melphalan and FSH β (33-53)-melphalan are potent gonadotoxicants *in vitro* and *in vivo*. Both effectively but not completely suppress spermatogenesis for four weeks after a single administration. However, spermatogenesis mostly recovers within 12 weeks of treatment in most animals. This approach to sterilizing animals requires refinement to become more uniformly effective. In addition, a greater understanding of the biology of sperm stem cell self-renewal would be very helpful in efforts to develop a non-surgical method of animal sterilization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cytotoxicity of melphalan and melphalan-FSH β conjugates against murine testicular cells as measured by release of lactate dehydrogenase (LDH). All cultures were tested in triplicate at 20 μ M of either melphalan or FSH β -melphalan conjugate. Values represent the mean and standard error of LDH.

Figure 2.

Microscopic appearance of cultures of murine testicular cells at 24, 48 and 72 hours after no treatment (control) or co-incubation with 20 μ M melphalan or FSH β (33-53)-melphalan. Notable cell death is observed in both the melphalan and FSH β (33-53)-melphalan cultures over time compared to the control cultures.

Figure 3.

Murine testis histology. A, no treatment (control); B, treatment with a single dose of 33-53 FSH β -melphalan; C and D, treatment with a single dose of melphalan. Marked loss of spermatocytes and spermatids is observed at four weeks in both the melphalan and 33-53 FSH β -melphalan groups. In B, a tubule section with almost complete absence of germ cells and one with few germ cells are shown; some other tubules had more germ cells including condensed spermatids, similar to panels C and D. In C and D, complete and quantitatively deficient areas of spermatogenesis are seen. Bouin's fixative and Hematoxylin-Periodic acid Schiff's reagent stain. Bars represent 20 microns.

Table 1

Chemical composition of melphalan and FSH β -melphalan conjugates. The X represents the position of a molecule of melphalan in the conjugates relative to the amino acids and the numbers represent the amino acids in the sequence of the human FSH β molecule, shown below using the standard three letter abbreviations.

Conjugate	Peptide with Melphalan (X) placement	N-terminal Modifications	C-terminal Modifications	Molecular Weight (Daltons)
Melphalan	-	-	-	305
C	X33-53	Amine	Acid	2815
D	33-53X	Amine	Acid	2815
E	X33-53	Acetate	Amide	2856
F	33-53X	Acetate	Amide	2856
G	81-95X	Acetate	Amide	1866
H	X81-95	Amine	Acid	1826
I	81-95X	Amine	Acid	1826
K	33-54(X)	Acetate	Amide	2984
M	33-47X	Acetate	Amide	2163
N	33-50X	Acetate	Amide	2521
O	33-53XXX	Acetate	Amide	3431
Q	33-52X	Acetate	Amide	2709
R	35-53X	Acetate	Amide	2592
S	37-53X	Acetate	Amide	2320
T	33-53XX	Acetate	Amide	3143

Amino Acids	Sequence
33-53	Ac-Tyr-Thr-Arg-Asp-Leu-Val-Tyr-Lys-Asp-Pro-Ala-Arg-Pro-Lys-Ile-Gln-Lys-Thr-Ser-Thr-Phe-NH ₂
81-95	Ac-Gln-Ser-His-Ser-Gly-Lys-Ser-Asp-Ser-Asp-Ser-Thr-Asp-Ser-Thr-NH ₂

Table 2

Testicular weight, spermatid counts and daily sperm production of mice with no treatment (control) or treatment with a melphalan or FSH β (33-53)-melphalan four and twelve weeks post-injection.

	Control		Melphalan		FSH β (33-53)-melphalan (Compound F) administered as a single dose		FSH β (33-53)-melphalan (Compound F) administered in multiple doses	
	Week 4	Week 12	Week 4	Week 12	Week 4	Week 12	Week 4	Week 12
Testes Weight (mg)	195 $\pm$ 4.2	209 $\pm$ 10	169 $\pm$ 6.3	169 $\pm$ 6.3	133 $\pm$ 28.6	161.9 $\pm$ 7.8	166.3 $\pm$ 4.4	173 $\pm$ 4.6
Spermatids/mg of Tissue ($\times 10^6$)	0.11 $\pm$ 0.05	0.161 $\pm$ 0.009	0.054 $\pm$ 0.003 *	0.106 $\pm$ 0.10	0.04 $\pm$ 0.02 *	0.08 $\pm$ 0.07	0.04 $\pm$ 0.001 *	0.116 $\pm$ 0.020
Spermatids/Testis ($\times 10^6$)	10.8 $\pm$ 0.3	16.9 $\pm$ 1.5	2.5 $\pm$ 0.06 *	9.2 $\pm$ 0.57	2.7 $\pm$ 2.1 *	7.75 $\pm$ 7.3	1.63 $\pm$ 0.12 *	10.1 $\pm$ 1.3
Daily Sperm Production/testis ($\times 10^6$)	2.3 $\pm$ 0.06	3.48 $\pm$ 0.3	0.515 $\pm$ 0.013 *	1.9 $\pm$ 0.12	0.56 $\pm$ 0.44 *	1.6 $\pm$ 1.5	0.337 $\pm$ 0.025 *	2.1 $\pm$ 0.27

* P<0.01 compared with placebo