Isotretinoin Administration Improves Sperm Production in Men with Infertility

John K. Amory MD, MPH

A thesis
submitted in partial fulfillment of the
requirements for the degree of

Master of Science

University of Washington
2017

Committee:
Nina Isoherranen
Kenneth Thummel
Edward Kelly

Program Authorized to Offer Degree:
Pharmaceutics

John K. Amory MD, MPH

University of Washington

Abstract

Isotretinoin Administration Improves Sperm Production in Men with Infertility

John K. Amory MD, MPH
Chair of the Supervisory Committee:
Dr. Nina Isoherranen, Associate Professor
Department of Pharmaceutics

Objective: There is currently no effective medical therapy for men with infertility due to oligoasthenozoospermia (OA). As men with OA have lower concentrations of 13-*cis*-retinoic acid in their testes, we hypothesized that men with infertility from OA might have improved sperm counts when treated with isotretinoin (13-*cis*-retinoic acid).
Design and Setting: We conducted a single-site, single-arm, pilot study to determine the impact of therapy with isotretinoin on sperm indices in 19 infertile men with OA.
Subjects: Subjects were men between 21 and 60 years of age with infertility of more than 12 months associated with sperm concentrations below 15 million sperm/ml.
Intervention: All men received isotretinoin 20 mg by mouth twice daily for 20 weeks.
Outcome Measures: Subjects had semen analyses, physical examinations, routine blood counts, and chemistries every four weeks during treatment.
Results: Nineteen men enrolled in the study. Median (25^{th}, 75^{th}) sperm concentration increased from 2.5 (0.1, 5.9) million/ml at baseline to 3.8 (2.1, 13.0) million/ml at the end of treatment (p=0.006). No significant changes in sperm motility were observed. There was a trend towards improved sperm morphology (p=0.056). Six pregnancies (three spontaneous and three from ICSI) and five births occurred during the study. Four of the births, including all three of the spontaneous pregnancies, were observed in men with improvements in sperm counts with isotretinoin therapy.
Conclusions: Isotretinoin therapy of men with OA is associated with improvements in sperm production. Additional studies of isotretinoin therapy for men with infertility from OA are warranted.

Table of Contents

List of Figures **Page**

List of Tables **Page**

List of Abbreviations

d	deuterium
HDL-C	high density lipoprotein-cholesterol
FSH	follicle-stimulating hormone
ICSI	intracytoplasmic sperm injection
IU	international unit
LH	luteinizing hormone
OA	oligoasthenozoospermia
PHQ-9	Patient health questionnaire-no. 9
WHO	World Health Organization

Acknowledgements

I would like to express my deep gratitude to Dr. Nina Isoherranen for her years of collaboration and guidance.

I would also like to thank Dr. Kenneth Thummel and the faculty of the Department of Pharmaceutics for allowing me to participate in the Masters of Science program, which has been of great benefit to my personal and professional development.

Lastly, I would like to thank the study subjects and their wives for participating in this study. Hopefully, this work will improve the ability of couples struggling with infertility to conceive.

Dedication

To my mother, Margaret Anne Malo Amory, an incredible parent, an indefatigable supporter and an all-around lovely human being. Descansa con los ángeles mi maravillosa madre.

Chapter 1: Introduction

1.1 Male Infertility from Oligoasthenozoospermia

Infertility affects 15% of all couples, with roughly one million couples seeking medical assistance for infertility yearly in the US (1). Infertility attributable to the male partner accounts for 40% of all infertility, and the most common forms of male infertility involve impairments in sperm production (2). However, a causal factor for impaired sperm production cannot be identified in most men (Figure 1).

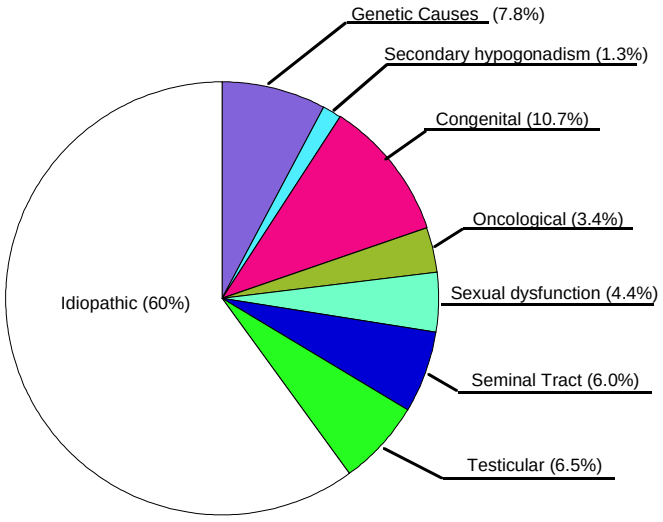

Figure 1. Causes of infertility in men. Derived from a cohort of 1737 consecutive men presenting with male infertility (data from reference #3).

In addition, effective treatments, such as surgery or gonadotropin therapy, are only useful for 10-20% of men. In men with some sperm production, *in vitro* fertilization coupled with intra-cytoplasmic sperm injection can lead to fertility (4); however, these procedures are expensive and unsuccessful in some cases. Therefore, new approaches to the treatment of infertility in men are sorely needed.

1.2 Retinoids and Spermatogenesis

Since 1925, it has been known that vitamin A deficiency causes infertility in laboratory animals (5). Vitamin A is converted to its active form, retinoic acid, in the testes via the activity of retinol and retinal dehydrogenases (See Figure 2) (6).

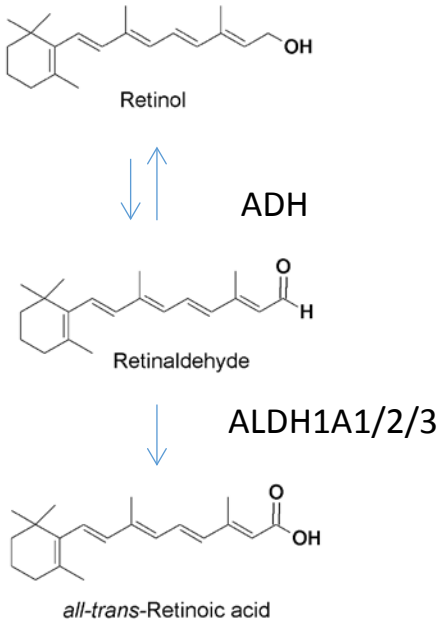

Figure 2. Retinoic acid biosynthesis. Retinol is first oxidized to retinaldehyde by alcohol dehydrogenases (ADH), and then oxidized to all-trans-retinaldehyde by three related aldehyde dehydrogenases (ALDH1/2/3).

In vitamin A/retinoic acid deficient rodents, the conversion of undifferentiated to differentiating spermatogonia is arrested, and re-supplementation with vitamin A or retinoic acid restores fertility (7, 8). The effects of vitamin A on spermatogenesis are dependent on retinoic acid receptors (9-11). Moreover, genetic deletion of retinoic acid receptors in mice results in sterility secondary to impaired sperm production (12-15). Similarly, pharmacological inhibition of retinoic acid biosynthesis (16, 17), or blockade of retinoic acid receptors (18, 19) suppresses sperm production in animal models.

1.3 Retinoic Acid and Male Infertility-Prior Work

Because of the known role of Vitamin A in spermatogenesis, Vitamin A was studied as a treatment for male infertility in the 1950s in two studies, with disappointing results (20, 21). However, these results are only partially informative as Vitamin A requires conversion to retinoic acid within the testes to mediate its effects. Therefore, Vitamin A therapy would not improve spermatogenesis in men whose intratesticular retinoic acid concentrations are low due to either impaired biosynthesis or increased metabolism of retinoic acid in the testes. Therefore, we have focused our investigations on treatment of infertile men with retinoic acid rather than Vitamin A, which bypasses any defect in the biosynthesis of retinoic acid from Vitamin A. To understand the relationship between intratesticular retinoic acid and sperm production in man, we measured tissue concentrations of all-trans and 13-cis-retinoic acid in testicular tissue from 24 men undergoing scrotal surgery, and found that concentrations of 13-cis-retinoic acid were significantly reduced in the men with abnormal sperm production (22) (Figure 3) .

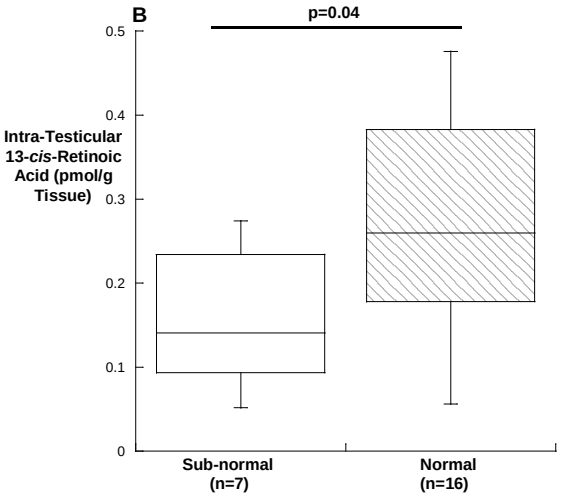

Figure 3. Intratesticular retinoic acid in men. Intratesticular 13-*cis* retinoic acid in 24 men expressed as median and interquartile ranges. 13-*cis*-retinoic acid is significantly lower in men with sub-normal sperm quality as compared to men with normal sperm analyses (p=0.04)

This finding suggested that some men with infertility might have reduced concentrations of retinoic acid in their testes, possibly due to deficient biosynthesis.

Follow-up work demonstrated that men presenting with infertility had significantly lower concentrations of ALDH1A2, an enzyme that produces retinoic acid, in their testes compared to fertile men (23). (figure 4)

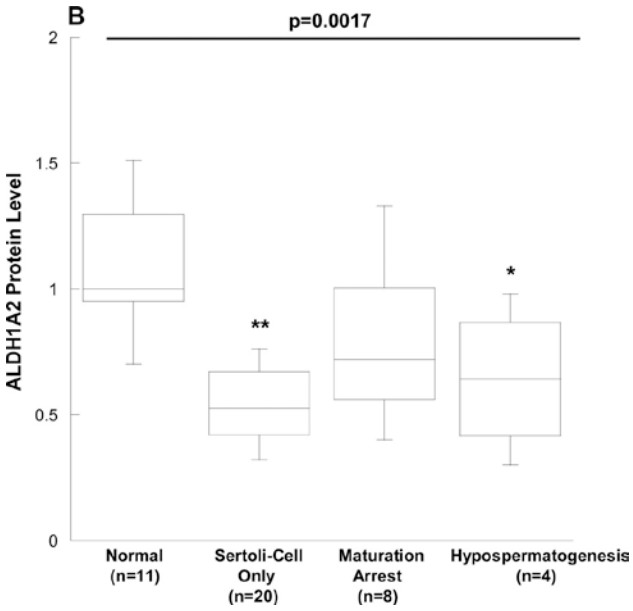

Figure 4. ALDH1A2 levels from testicular tissue of normal men and men with infertility, stratified by histological subtype. Values are expressed relative to the mean of the group of normal men. **p<0.001 compared with normal men; *p<0.05 compared with normal men

If some men with infertility have reduced concentrations of intratesticular retinoic acid, treatment with retinoic acid could potentially improve their sperm output and their fertility. The medical literature supports the notion that the administration of 13-*cis*-retinoic acid is beneficial for sperm production. Four cohort studies, enrolling a total of 126 normal men being treated with isotretinoin for acne have examined the effects of 13-*cis*-retinoic acid/isotretinoin on sperm production (24-27). Unexpectedly, men in the three of the studies exhibited significant improvements in their sperm counts during treatment. Unfortunately, these observations were not widely recognized, and isotretinoin was never tested as a possible treatment for male infertility.

1.4. Pharmacokinetics of Retinoic Acid Administration

Oral 13-*cis*-retinoic acid in the form of isotretinoin is currently approved for the treatment of cystic acne and other dermatological conditions, and its pharmacokinetics and side effect profile are well known (28-31). We focused on the use of isotretinoin (13-*cis* retinoic acid) instead of all-*trans* retinoic acid as this isomer of retinoic acid is available in a generic formulation (32), has fewer side effects and more predictable pharmacokinetics. In addition, 13-*cis* retinoic acid is not a good CYP26 substrate (33) and is therefore more likely to cross the blood-testes barrier, which expresses high levels of CYP26 in the peritubular myoid cells (34). Oral isotretinoin is commonly dosed between 0.25-1 mg/kg for the treatment of acne. (35). Taking the potential for adverse effects into account in our pilot study we opted to study a relatively low dose of 20 mg twice daily, which corresponds to a daily dose of ~0.5 mg/kg in the average young man with infertility. The usual isotretinoin treatment course for an individual with cystic acne is 20 weeks, so for our pilot study we used this treatment duration in accordance with the FDA's guidance. Therefore, to determine the impact of isotretinoin therapy on sperm production in men with infertility, we conducted a single-arm pilot study of isotretinoin in nineteen men with infertility from oligoasthenozoospermia (OA) not due to identifiable hormonal, structural or genetic causes.

Chapter 2: Isotretinoin for Male Infertility

Chapter 2 has been submitted for publication in Andrology

2.1 Introduction

There is currently no effective medical therapy for men with infertility due to oligoasthenozoospermia (OA). As men with abnormal sperm production have lower concentrations of 13-cis-retinoic acid in their testes, we hypothesized that men with infertility from OA might have improved sperm counts when treated with isotretinoin (13-cis-retinoic acid). We felt that a pilot population of 19 men would be sufficient to determine if some men would respond to isotretinoin therapy, and was long enough to gain some insight into the kinetics of this response. We felt this pilot data of sperm production would be valuable in the design of future studies of isotretinoin therapy for the treatment of men with infertility from reduced sperm production aimed at determining the impact of isotretinoin therapy on pregnancy rate.

2.2 Experimental

2.2.1. Subjects and Study Design

As isotretinoin had not previously been studied for the treatment of male infertility due to impaired sperm production, we opted for a simple, single-arm pilot study design with the subject's baseline semen analyses serving as their own controls. The subjects were infertile men between 21-60 years of age with a sperm concentration of less than 15 million/ml on two baseline samples and more than twelve months of infertility. Men were recruited from Seattle area infertility clinics using informational flyers and were reimbursed for expenses related to study participation. Exclusion criteria included: genetic causes of infertility (e.g. Klinefelter's or Y-chromosome microdeletions), hypogonadotropic hypogonadism, the use of anabolic steroids or illicit drugs within the last 12 months,

consumption of more than 4 alcoholic beverages daily, therapy with isotretinoin or all-*trans*-retinoic acid in the last 12 months, severe mental health problems requiring medications, a score of greater than 15 on the Patient Health Questionnaire-9 (PHQ-9), abnormal serum chemistry values according to our laboratory normal values indicative of liver or kidney dysfunction, current therapy with tetracycline, phenytoin, rifampin, phenobarbital, highly-active anti-retroviral therapy or other inducers of CYP enzymes, infection with HIV, a history of inflammatory bowel disease, or fasting serum triglycerides of greater than 500 mg/dl. This study was approved by the Institutional Review Board of the University of Washington and conducted under IND#120703 from the US FDA and registered on clinicaltrials.gov as trial #NCT02061384: "A Pilot Trial of 13-*cis*-retinoic acid (Isotretinoin) for the Treatment of Men With Oligoasthenoteratozoospermia" prior to any study procedures.

2.2.2 Study Procedures

Prior to participation, subjects signed an Institutional Review Board approved informed consent document. At each study visit, a physical examination and vital signs were performed. In addition, a blood sample was obtained for the blood counts, serum chemistries and fasting lipids, testosterone, LH, FSH and retinoic acid after an overnight fast. In addition, subjects completed the PHQ-9 depression questionnaire at each visit. For assessment of spermatogenesis, subjects provided two baseline semen samples after 48 hours of abstinence and at least one week apart to confirm the diagnosis of idiopathic oligoasthenozoospermia (OA). They also provided a third semen sample prior to starting therapy and every four weeks during the twenty week treatment phase,

as well as 4,12 and 24 weeks after completing therapy. During the treatment phase, subjects were prescribed isotretinoin at a dose of 20 mg twice daily for 20 weeks for self-administration. Subjects eleven through nineteen also received calcitriol at a dose of 0.25 micrograms twice daily. Medication compliance was monitored by self-reported subject logs and pill counts of returned medication. In addition, subjects were assessed for side effects at each clinic visit. The semen samples were collected after at least 48 hours of abstinence from ejaculation. Serum samples for measurements of retinoic acid were collected before the morning dose. Blood was collected into vacutainers wrapped in aluminum foil to prevent light exposure that could degrade retinoic acid, and allowed to clot for 30 minutes at room temperature before centrifugation at 1000-2000g for 15 minutes. Following centrifugation, the serum samples were decanted and frozen in light-protected vials at –80^{o}C until analysis. Semen samples and seminal plasma samples were similarly light protected.

2.2.3 Measurements

Semen samples were allowed to liquefy for 30 minutes at 37^{o}C and then analyzed for sperm concentration, count and motility within sixty minutes of liquefaction using the WHO protocol (36). Sperm morphology was assessed using the “strict” WHO criteria (36). Blood counts and serum chemistry and hormone tests were performed by the clinical laboratory at the University of Washington.

Serum 13-*cis,* all-*trans* and 4-oxo-13-*cis*-retinoic acid concentrations were measured using an AB Sciex (Framingham, MA) qTrap 5500 mass spectrometer equipped with an Agilent Technologies (Santa Clara, CA) 1290 Infinity ultrahigh

pressure liquid chromatography system as described previously (37). Deuterated (d) compounds were used as internal standards. To prepare samples for analysis, 80 µL of 250 nM 13-*cis*-retinoic acid-d5, 500 nM 4-oxo-all-*trans*-retinoic acid-d3, and 100 nM all-*trans*-retinoic acid-d5 were added as internal standards in acetonitrile to 80 µL of the samples. Samples were then centrifuged twice at 3,000 x g for 10 min at 4°C and the supernatant was collected for quantification. Retinoic acid concentrations in seminal plasma were measured using an AB Sciex (Framingham, MA) qTrap 6500 mass spectrometer coupled to a Shimadzu (Kyoto, Japan) LC-20AD liquid chromatography system using the approach described above. Retinoic acid species were separated with an Ascentis® Express RP-Amide 15 cm x 2.1 mm, 2.7 µm column (Sigma-Aldrich, St. Louis, MO) with a mobile phase flow of 500 µL/min and solvents A: 60:40 water:methanol and B: 60:40 acetonitrile:methanol with 0.1% formic acid in A and B. The gradient was 0→2 min 40% B, 2 → 10 min increase to 55% B, 10→17 min further increase to 90% B, then 95% B hold for 3 min before return to initial conditions and column equilibration for 4 min. For detection, positive mode atmospheric pressure chemical ionization was used and m/z *trans*itions of: 301→205 for 13-*cis*-retinoic acid and all-trans-retinoic acid, 315→159 for 4-oxo-13-*cis*-retinoic acid, 306→116 for 13-*cis*-retinoic acid-d5 and all-*trans*-retinoic acid-d5, and 300→226 for 4-oxo-all-*trans*-retinoic acid-d3 were monitored for quantification. All data was analyzed using Analyst software (AB Sciex, Foster City, CA). The intra and inter-assay coefficients of variation were between 2-8%, and the lower limit of quantitation was

2 nM for 13-*cis*-retinoic acid and all-*trans*-retinoic acid and 8nM for 4-oxo-13-*cis*-retinoic acid.

2.2.4 Statistical Analysis

Due to the variability of single measurements of sperm parameters, the co-primary endpoints of change in concentration and change in total motile sperm count, the three pre-treatment semen samples were averaged for the "baseline" value and the three peak semen samples were averaged for the "end-of treatment" value. For the three men who discontinued isotretinoin therapy early, data from the last three available sperm samples were carried forward and used in the analysis. Secondary endpoints included sperm motility, sperm morphology and total motile sperm count, as well as changes in laboratory measures, serum and semen retinoic acid concentrations and mood effects and other side effects.

Baseline and end of treatment sperm parameters were compared using a Wilcoxon sign-rank test due to non-normality. For analysis of laboratory assessments, retinoic acid concentrations and questionnaire data, a paired t-test was used, using an adjusted p-value of 0.01 to correct for multiple comparisons. The administration of calcitriol to subjects eleven through nineteen had no apparent effect on any sperm parameter or laboratory measure, so this variable was not included further in the analysis. In exploratory analyses, univariate and multivariate linear and logistic regression were performed to determine if significant relationships between any patient characteristic or measure and changes in sperm parameters or the likelihood of response were present. All analyses were performed using STATA Version 10.0

(College Park, TX, USA). For all unadjusted comparisons, an alpha of 0.05 was considered significant.

2.3 Results

2.3.1 *Study Enrollment*

Twenty-seven subjects were screened and nineteen were enrolled. Six men were ineligible due to sperm criteria, and two subjects elected not to participate for personal reasons. Nineteen men enrolled and started treatment. The mean (±SD) age was 35 ± 4.6 years of age, and the mean body-mass index was 28 ± 4.9 kg/m^2. Eighteen of these men were White, and one was Asian. Sixteen men completed all study procedures and three men discontinued treatment early, all after twelve weeks of treatment. Two of these men discontinued due to a pregnancy in their partner, one man discontinued due to a perceived lack of efficacy.

2.3.2 Effect of Isotretinoin on Semen Parameters

Overall, the median (25^{th}, 75^{th}) sperm concentration increased from 2.5 (0.1, 5.9) million/ml at baseline to 3.8 (2.1, 13.0) million/ml at the end of treatment (p=0.006). Four subjects had a sperm concentration of greater than 15 million/ml at some point between week 16 of treatment and week four of follow-up, compared with no subject at baseline (p<0.001). Sperm concentrations before, during and after treatment for the 12 subjects who had a baseline total sperm count of greater than one million are depicted in Figure 5A and for those with a baseline count of less than one million in Figure 5B.

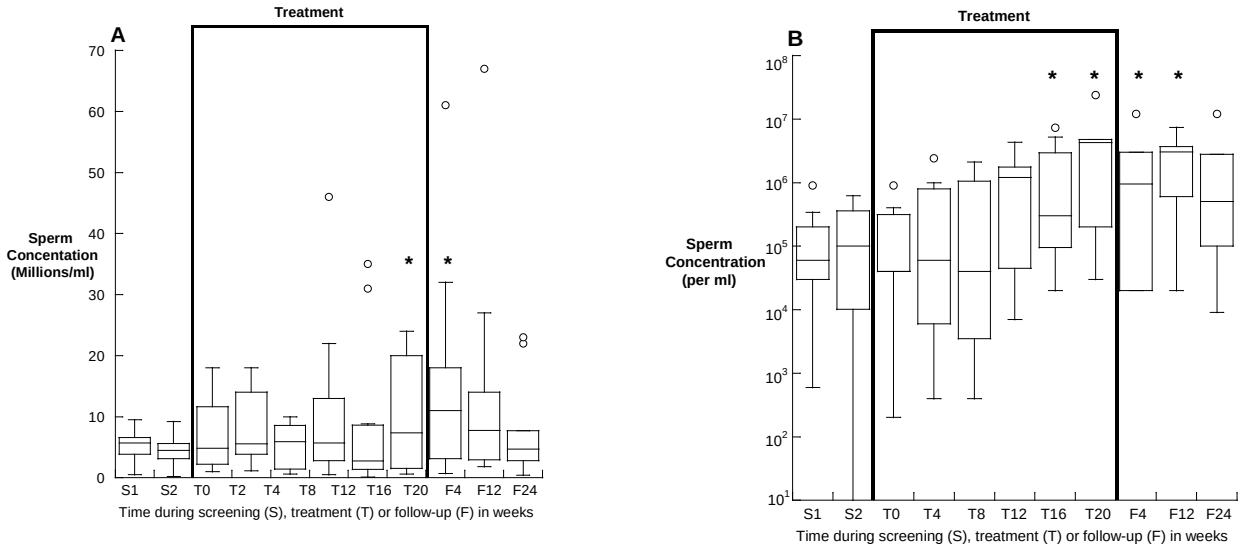

Figure 5. Sperm concentation (medians, interquartile ranges) before, during and after treatment with isotretinoin for twenty weeks. Panel A depicts the twelve men with a baseline total motile sperm count of greater than one million, and panel B depicts the seven men with fewer than 1 million total, motile sperm at baseline. Note the log scale of the y-Axis of panel B. *p<0.05 compared with baseline.

Median sperm motility did not significantly differ between baseline and treatment at any timepoint. Due to low sperm counts in some subjects, sperm morphology was only available on ten subjects at baseline and thirteen subjects at week 20. Among these men, there was a trend towards improved sperm morphology with 0.5 (0, 3) percent strict normal sperm at baseline compared with 1 (0, 5) percent at the end of treatment (p=0.056) (Figure 6).

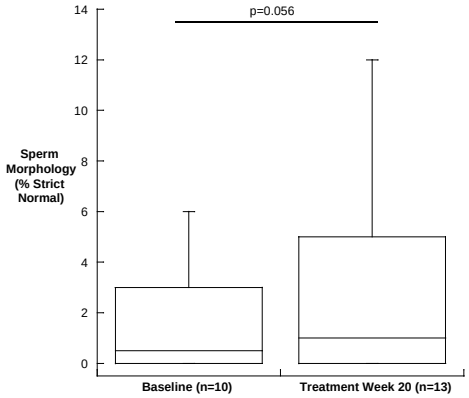

Figure 6. Sperm morphology (% strict normal) before and after treatment with isotretinoin for twenty weeks.

The numbers of total motile sperm before, during and after treatment for all 19 men are depicted individually in Figure 7A and 7B.

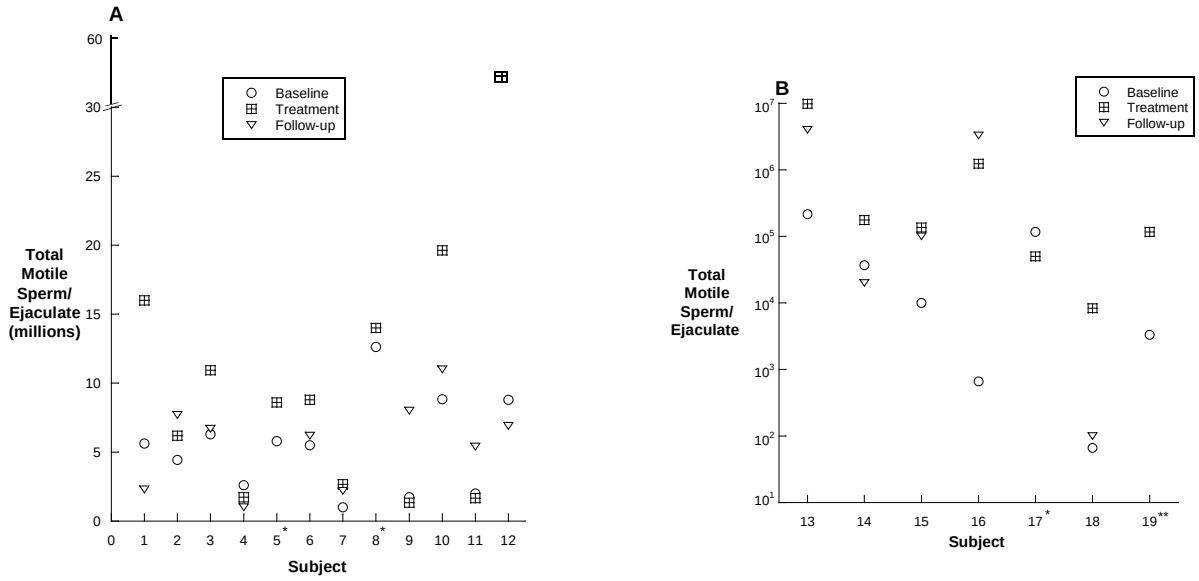

Figure 7. Total motile sperm counts for each subject. Panel A depicts the twelve men with a baseline total motile sperm count of greater than one million, and panel B depicts the seven men with fewer than 1 million total, motile sperm at baseline (B). Note the log scale of the y-axis of panel B. * no follow-up sample; ** follow-up sample with total, motile sperm count of zero.

Overall, the median (25^{th}, 75^{th}) total, motile sperm count per ejaculate increased from a baseline of 2 (0.04, 5.8) million sperm to 2.7 (0.18, 10.9) million sperm after 20 weeks of treatment (p=0.004). In the twelve men who began with an average total motile count between 1 and 10 million, the median baseline total motile sperm count increased from 5.6 (2.3, 7.5) million sperm to 8.7 (2.2, 15) million sperm (p<0.001). In the seven men who began the study with a total motile count of less than one million, the total motile sperm count increased from 0.01 (0.0006, 0.12) million sperm to 0.13 (0.05, 1.2) million sperm (p=0.04) at the end of treatment.

The percent change in total motile sperm count with treatment is depicted in Figure 8. Overall, the median (25^{th}, 75^{th}) percent change in all 19 men was a 122 (10, 1266) percent increase.

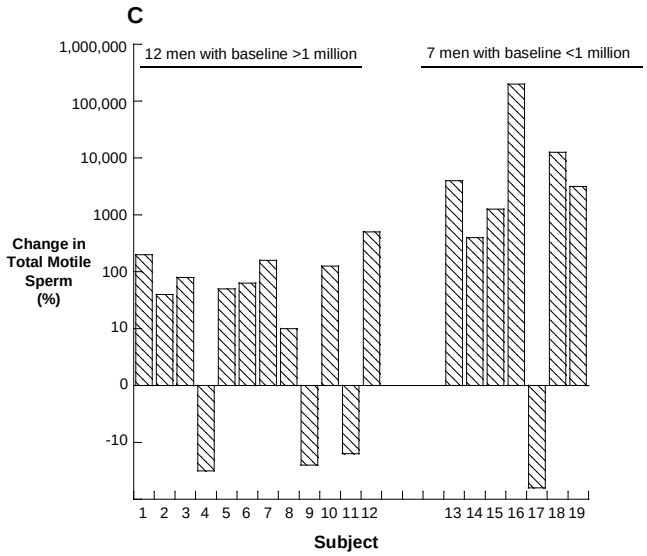

Figure 8. The percent change in total motile sperm between baseline and the end of treatment.

2.3.3. *Pregnancies Observed*

Six pregnancies and five births occurred in the female partners of men enrolled in the study. Three of the six pregnancies were spontaneous, and three were the product of ICSI. Four of the six pregnancies, including all three spontaneous pregnancies, were observed in men with improvements in sperm concentrations with isotretinoin therapy. Two of the three men (subjects #5 & #8) who fathered spontaneous pregnancies discontinued isotretinoin use when pregnancy was identified after 12 weeks of treatment. The third subject with a spontaneous pregnancy (subject #1*2*) fathered twins after approximately 16 weeks of therapy. There was one miscarriage in the partner of a subject who had become pregnant via ICSI. All six offspring from the five pregnancies appeared normal at birth without identifiable birth defects and continue to do well.

2.3.4 Serum and Semen 13-cis-Retinoic Acid

The average serum 13-*cis*-retinoic acid (isotretinoin) and all-*trans*-retinoic acid concentrations before, during and following treatment are shown in Figure 9A and 9B. The mean (± SD) serum 13-*cis*-retinoic acid concentrations during treatment was 693 ± 201 nM, which was more than one hundred times higher than the baseline measurement of 6.7 ± 2.3 nM ($p<0.0001$). In contrast, the average all-*trans*-retinoic acid concentration of 10.2 ± 2.1 nM was only 30-40% higher than the mean baseline values of 6.7 ± 2.3 nM ($p<0.001$). Subjects also had marked increases in the 13-*cis*-retinoic acid metabolite serum 13-oxo-*cis*-retinoic acid during treatment which increased from 28 ± 7.5 nM at baseline to an average of 5418 ± 1710 nM during treatment ($p<0.0001$).

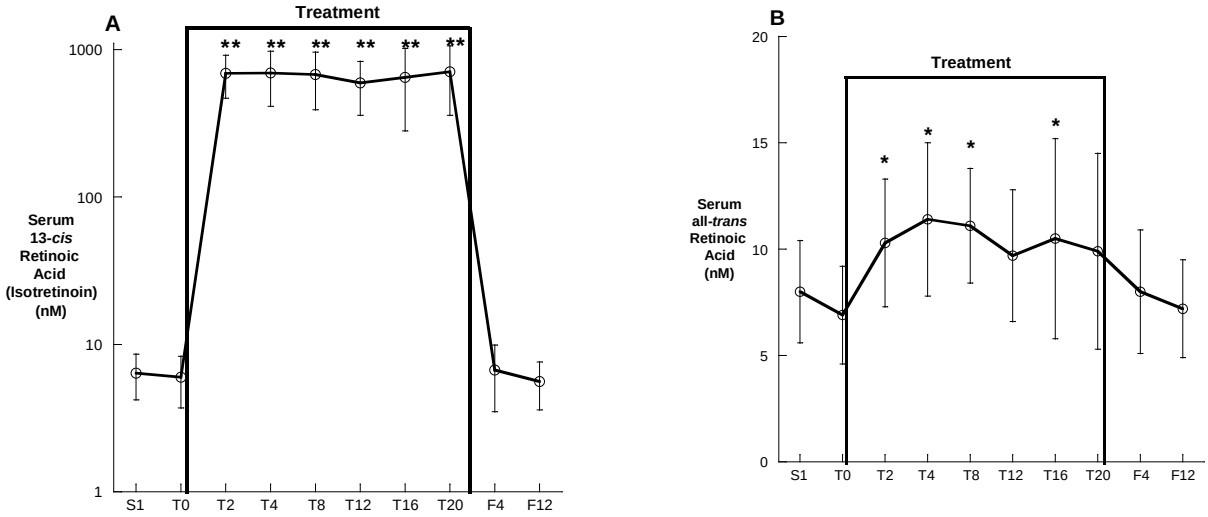

Figure 9. Serum 13-*cis*-retinoic acid (A) and all-*trans*-retinoic acid (B) concentrations before, during and after treatment. All values are means ± SD. *p<0.05, **p<0.01 compared with baseline.

Seminal plasma 13-*cis*-retinoic acid concentrations were undetectable at baseline and after treatment. During treatment, the average seminal 13-*cis* retinoic acid concentration was 9.6 ± 2.4 nM. The highest measureable value in any subject at any timepoint was 22 nM. Seminal all-*trans*-retinoic acid concentrations were undetectable in any subject at baseline and after treatment. During treatment, only seven of 122 semen samples had a detectable concentration of all-trans-retinoic acid. These concentrations ranged from a 2.4 to 3.1 nM.

2.3.5 Adverse Events

A significant increase in mean fasting serum triglycerides and a significant decrease in mean serum HDL were noted during treatment (**Table 1**). There were no symptoms or clinical events attributable to these changes in serum lipids.

Table 1. Vital signs, laboratory assessments and mood assessments before during and after treatment with Isotretinoin 20 mg twice daily for twenty weeks (n=19). All data are expressed as means ± SD. *p<0.05 compared with baseline.

		Treatment			**Follow-up**	
	Baseline	Week 4	Week 12	Week 20	Week 12	Week 24
Vital Signs						
Weight (kg)	93 ± 16	92 ± 16	92 ± 15	91 ± 17	92 ±16	93 ± 18
Systolic blood pressure (mmHg)	128 ± 33	129 ± 14	130 ± 11	125 ± 14	124 ± 10	129 ± 17
Diastolic blood pressure (mmHg)	80 ± 11	81 ±10	81 ± 9	77 ± 10	76 ± 8	81 ± 10
Pulse (beats/min)	72 ± 12	75 ± 13	74 ± 11	73 ± 9	72 ± 9	73 ± 6
Blood Counts						
White Blood Cells (1000s/μl)	6.3 ± 1.8	6.3 ± 1.4	6.2 ± 1.1	6.1 ± 1.0	5.9 ± 0.9	6.1 ± 1.4
Hematocrit (%)	43 ± 2.7	43 ± 2.9	43 ± 2.9	43 ± 2.7	42 ± 2.6	43 ± 3
Platelets (1000s/μl)	219 ± 51	232 ± 61	224 ± 46	219 ± 41	215 ± 51	224 ± 57
Lipids						
Cholesterol (mg/dl)	187 ± 33	200 ± 40	200 ± 30	197 ± 34	177 ± 19	184 ± 27
HDL-C (mg/dl)	53 ± 7.7	51 ± 8.4	48 ± 8*	48 ± 7.3*	51 ± 6.4	51 ± 8
LDL-C (mg/dl)	115 ± 30	124 ± 34	124 ± 23	122 ± 30	103 ± 20	107 ± 28
Triglycerides (mg/dl)	95 ±40	130 ±66*	136 ±87*	139 ± 52*	108 ± 52	127 ± 61
Hormones						
Testosterone (ng/dl)	3.9 ± 1.8	3.9 ± 1.8	3.9 ± 1.6	3.9 ± 1.9	3.9 ± 2.0	3.9 ± 1.8
LH (IU/L)	5.6 ± 2.6	6.2 ± 2.9	5.8 ± 2.2	5.4 ± 2.1	5.4 ± 2.1	5.4 ±2.5
FSH (IU/L)	11 ± 8.1	10.5 ± 8	9.8 ± 7.0	9.0 ± 7.1	10.2 ± 7	9.2 ± 9.4
Inhibin B (pg/ml)	49 ± 20	ND	ND	54 ± 18	ND	45 ± 19
Mood Questionnaire						
PDQ-9 Score	1.3 ± 0.4	1.0 ± 0.4	1.8 ± 0.7	1.2 ± 0.6	1.0 ± 0.4	1.1 ± 0.3

ND=Not done

There were 59 adverse effects reported by fifteen different subjects during treatment (Table 2). Importantly, no subject complained of worsening mood during treatment, which is a known side effect of isotretinoin. There were no serious adverse events.

Table 2: Adverse Events during the Study (n=59)

Adverse Event	Number of Instances	Possibly, Probably or Definitely Related	Unlikely or Not Related	% of all Adverse Events
Dry Facial Skin	17	17	0	29
Angular Cheilitis/Chapped Lips	16	16	0	27
Headache	4	4	0	7
Epistaxis	3	3	0	5
Rash	3	3	0	5
Upper Respiratory Infection	3	0	3	5
Pharyngitis	2	0	2	3
Cold sore	1	0	1	2
Dental Infection	1	0	1	2
Sprained Ankle	1	0	1	2
Tick bite	1	0	1	2
Conjunctivitis	1	0	1	2
Dysuria	1	0	1	2
Cellulitis	1	0	1	2
Chikungunya*	1	0	1	2
Norovirus	1	0	1	2
Dacyrocystitis	1	0	1	2
Onychomycosis	1	0	1	2
Total	59	43 (73%)	16 (26%)	

Adverse Event Severity			
Grade	Number	Percent (%)	Details
1 (Mild)	55	93	No interventions for most of these events other than facial moisturizers and lip balm
2 (Moderate)	3	5	Dental infection, cellulitis and pharyngitis treated with antibiotics in consultation with subject's provider.
3 (Severe)	1	2	*Chikungunya infection was diagnosed in India during a subject's visit there. Subject had a full recovery with supportive care.
4 (Life Threatening)	0	0	None

2.3.6 Factors Associated with Isotretinoin Response

Using univariate and multivariate linear and logistic regression, no demographic, hormonal, sperm or pharmacokinetic parameter could be identified that was associated with a significantly greater likelihood of responding to isotretinoin therapy, either in terms of improvements in sperm production or the chance of pregnancy.

2.4 Discussion

2.4.1 Overview of Results

Herein, we present data from the first study to test the hypothesis that men with infertility from OA will exhibit increased sperm counts from treatment with 13-*cis*-retinoic acid (isotretinoin). There are two notable features of the data from this study. Firstly, there is similarity between our results and those seen in normal men receiving isotretinoin therapy for the treatment of acne. Both populations experienced increased sperm counts, but no changes in sperm motility (24-27). Secondly, significant increases in sperm concentrations in men receiving isotretinoin treatment are not apparent until after twelve weeks of treatment. This observation is consistent with the known role of retinoic acid in spermatogonial differentiation and the 9-12 week maturation period for sperm in humans (38, 39). As a result, longer periods of isotretinoin treatment may be of benefit to men with infertility. Lastly, the elevations in sperm concentrations appear to persist for several weeks after drug discontinuation, presumably as the germ cells stimulated to differentiate by retinoic acid complete maturation within the seminiferous tubules.

2.4.2 Mechanism of Isotretinoin Action

During spermatogenesis, pulses of retinoic acid have been shown to move along the seminiferous tubules coincident with the spermatogenic wave (40). In mice, during stages VII to IX, the retinoic acid pulse plays a role in several crucial processes, including: spermiation, tight junction remodeling, and the transition of undifferentiated spermatogonia into differentiating spermatogonia, possibly via SALL4A (41). In

addition, retinoic acid is essential for the transcription of the genes *Stra8* and *Rec8*, which are necessary for meiotic initiation (42).

Given this information, how does exogenously administered retinoic acid stimulate spermatogenesis in infertile men? Based on our earlier finding that intratesticular 13-*cis*-retinoic acid concentrations are reduced in some men with infertility (22), we hypothesize that the biosynthesis of retinoic acid from vitamin A may be impaired in the men with infertility who responded to treatment with isotretinoin. Interestingly, in contrast to most tissues, almost none of the all-*trans*-retinoic acid in the testes appears to come from circulating pools (43). This finding implies that most intratesticular retinoic acid is biosynthesized within the testes themselves. Previously, our group demonstrated that the biosynthesis of retinoic acid in the human testes correlates strongly with the concentrations of the ALDH1A enzymes (44), which are decreased in the testes of men with infertility (23). It has been postulated that the testes is sequestered from circulating retinoic acid by high levels of expression of CYP26, the enzyme that metabolizes retinoic acid to inactive metabolites, in the peritubular myoid cells (34),and that this localization to the blood-testes barrier serves to prevent the premature initiation of spermatogenesis prior to puberty (45).

Our study, using pharmacological doses of retinoic acid, resulted in 100-fold increases in circulating 13-*cis*-retinoic acid concentrations, which may have overwhelmed the ability of the peritubular myoid cells to prevent exposure of the tubules to circulating retinoic acid. In some men, this addressed their intratesticular retinoic acid deficiency and stimulated sperm production. Importantly, isotretinoin is not as good a substrate of CYP26, the enzyme that metabolizes retinoic acid, as all-*trans*-retinoic acid

(33), possibly making it a better drug for this indication. In any case, the exact mechanism and pharmaceutical means by which retinoic acid supports spermatogenesis will be the subject of future study.

2.4.3 Safety of Isotretinoin Therapy

Isotretinoin is teratogenic (FDA category X), and should not be administered to women of reproductive age without the use of adequate contraception. Importantly, despite many years of use, there is no good evidence in the literature to suggest an increased risk of birth defects in the children of women who conceive while their husbands are taking isotretinoin to treat acne vulgaris. As a result, men using isotretinoin or other retinoids are not required to use condoms for intercourse during therapy. This is likely due to the observation that retinoid exposure via semen to female partners of men taking retinoids appears to so low that it would not be expected to alter endogenous retinoid concentrations (46).

In our study, we found isotretinoin concentrations in the semen during treatment to all be below 30 nM. At this concentration, and assuming 100% absorption across the vaginal mucosa, an ejaculate volume of 5 cc would expose a pregnant female partner to approximately 40 nanograms of 13-*cis*-retinoic acid, which is several orders of magnitude below the chronic 0.5-1.0 mg/kg dose range associated with teratogenicity in humans (47, 48). As a result, exposure of a pregnant female partner to isotretinoin via her sexual partner's semen and the potential for teratogenicity appear to be negligible.

2.4.2 Variability of Response to Isotretinoin

Unfortunately, we were unable to identify a correlate or biomarker of responsiveness to isotretinoin therapy in this pilot study. One hypothesis would be that men with low baseline concentration or 13-*cis*-retinoic acid would be more likely to respond to therapy; however, we did not obtain testicular biopsies at baseline in this study. A biomarker that could predict response would be of significant clinical utility in selecting the most appropriate men with infertility for treatment with isotretinoin. Work is ongoing using metabolomics and proteomic approaches to analyze the specimens from this study in the hopes of finding a biomarker of response for use in future studies.

2.4.5 Conclusions

Because of its widespread use for acne for over 35 years, isotretinoin is widely available. However, before isotretinoin can be incorporated into male infertility treatment algorithms, additional study of its efficacy and safety will be required, and its use outside of controlled, clinical trials is premature. In particular, we chose a dose in the low range of clinical use (approximately 0.5 mg/kg daily) and doses of up to 2 mg/kg daily are sometimes used for the treatment of acne (35). In addition, it is possible longer periods of treatment may result in improved outcomes for men with OA. Lastly, because sperm parameters are an imperfect proxy for fertility, live birth rate should be the primary outcome measure in future studies of isotretinoin therapy for couples with infertility associated with male factor oligoasthenozoospermia. If larger, randomized, placebo-controlled trials demonstrate safety and efficacy, isotretinoin therapy might allow couples in whom the male partner has infertility from OA to conceive

spontaneously or use techniques such as intrauterine insemination. Alternatively, isotretinoin therapy might allow men who don't currently qualify for IVF or ICSI the opportunity to try these procedures to achieve fertility.

In conclusion, our pilot study suggests that a significant subset of men with oligoasthenozoospermia respond to isotretinoin therapy with increased sperm output. Additional study of isotretinoin for the treatment of men with infertility from reduced sperm production is warranted.

Bibliography

1. Chandra A, Copen CE, Stephen ED. Infertility and Impaired Fecundity in the United States, 1982–2010: Data from the National Survey of Family Growth. National Health Statistics Reports 2013;67:1-17.
2. Jequier AM, Holmes SC. Primary testicular disease presenting as azoospermia or oligozoospermia in an infertility clinic. Br J Urol 1993;71:731-735.
3. Punab M, Poolamets O, Paju P, Vihljajev V, Pomm K, Ladva R, et al. Causes of male infertility: a 9-year prospective mono-centre study on 1737 patients with reduced total sperm counts. Hum Repro 2017;32:18-31.
4. Schlegel PN. Non-obstructive azoospermia: a revolutionary surgical approach and results. Semin Reprod Med 2009;27:165-70.
5. Wolbach SB, Howe PR. Tissue changes following deprivation of fat soluble A Vitamin. J Exp Med 1925;42:753-77.
6. Perlman T. Retinoid metabolism: a balancing act. Nat Genetics 2002 31:7-8.
7. Van Pelt AMM, de Rooij DG. Synchronization of the seminiferous epithelium after Vitamin A replacement in Vitamin A deficient mice. Biol Reprod 1990; 43:363-7.
8. Van Pelt AMM, de Rooij DG. Retinoic acid is able to reinitiate spermatogenesis in Vitamin A deficient rats and high replicate doses support the full development of spermatogeneic cells. Endocrinology 1991;128:697-704.
9. Doyle TJ, Braun KW, McLean DJ, Wright RW, Griswold MD, Kim KH. Potential functions of retinoic acid receptor alpha in Sertoli cells and germ cells during spermatogenesis. Ann NY Acad Sci 2007;1120:114-30.
10. Anderson EL, Baltus AE, Roepers-Gajadein HL, Hassold TJ, de Rooij DG, van Pelt AMM, Page DC. Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. Proc Natl Acad Sci, USA 2008;105:14976-80.
11. Chung SS, Wang X, Wolgemuth DJ. Expression of retinoic acid receptor alpha in the germline is essential for proper cellular association and spermiogenesis during spermatogenesis. Development 2009;136:2091-100.

12. Lohnes D, Kastner P, Dierich A, Mark M, LeMeur M, Chambon P. Function of retinoic acid receptor gamma in the mouse. Cell 1993;73:643-58.
13. Lufkin T, Lohnes D, Mark M, Dierich A, Gorry P, Gaub MP, et al. High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. Proc Natl Acad Sci USA 1993;90:7225-9.
14. Kastner P, Mark M, Leid M, Gansmuller A, Chin W, Grondona JM, et al. Abnormal spermatogenesis in RAR beta mutant mice. Genes Dev 1996;10:80-92.
15. Ghyselinck NB, Vernet N, Dennefeld C, Giese N, Nau H, Chambon P, et al. Retinoids and spermatogenesis: lessons from mutant mice lacking the plasma retinol binding protein. Dev Dyn 2006;235:1608-22.
16. Amory JK, Muller CH, Shimshoni JA, Isoherranen N, Paik J, Moreb JS, et al. Suppression of spermatogenesis by bisdichloroacetyldiamines is mediated by inhibition of testicular retinoic acid biosynthesis. J Androl 2011; 32:111-9.
17. Paik J, Haenisch M, Muller CH, Goldstein AS, Arnold S, Isoherranen H, et al. Inhibition of retinoic acid biosynthesis by WIN 18,446 markedly suppresses spermatogenesis and alters retinoid metabolism in mice. J Biol Chem 2014; 289:15104-17.
18. Schulze GE, Clay RJ, Mezza LE, Bregman CL, Buroker RA, Frantz JD. BMS-189453, a novel retinoid receptor antagonist, is a potent testicular toxin. Toxicol Sci 2001;59:297-308.
19. Chung SS, Wang X, Roberts SS, Griffey SM, Reczek PR, Wolgemuth DJ. Oral administration of a retinoic acid receptor antagonist reversibly inhibits spermatogenesis in mice. Endocrinology 2011;152:2492-502.
20. Horne HW, Maddock CL. Vitamin A therapy in oligospermia. Fert Steril 1952; 3:245-50.
21. Kapadia RM, Phadke AM. The role of Vitamin A in male infertility. Indian J Med Sci 1959;13:436-43.

22. Nya-Ngatchou JJ, Arnold SLM, Walsh TJ, Muller CH, Page ST, Isoherranen N, Amory JK. Intratesticular 13-*cis*-retinoic acid is reduced in men with abnormal semen analyses. Andrology 2013;1:325-31.
23. Amory JK, Arnold S, Walsh T, Lardone MC, Piottante A, Ebensperger MD, et al. Levels of the retinoic Acid synthesizing enzyme ALDH1A2 are lower in testicular tissue from men with infertility. Fert Steril 2014;101:960-6.
24. Vogt HJ, Ewers R. 13-cis-retinoic acid and spermatogenesis. Der Hautarzt 1985; 36:281-6.
25. Torok L, Kadar L, Kasa M. Spermatological investigations in patients treated with etretinate and isotretinoin. Andrologia 1987;19:629-33.
26. Hoting VE, Schutte B, Schirren C. Isotretinoin and acne conglobata: andrological evaluations. Fortschur Med 1992;23:427-30.
27. Çinar L, Kartal D, Ergin C, Aksay H, Karadag MA, Aydin T, et al. The effect of systemic isotretinoin on male fertility Cutan Ocul Toxicol, 2016; 35: 296–9.
28. Kerr IG, Lippman ME, Jenkins J, Myers CE. Pharmacology of 13-*cis*-retinoic acid in humans. Cancer Res 1982;42:2069-73.
29. Brazzell RK, Vane FM, Ehmann CW, Colburn WA. Pharmacokinetics of isotretinoin during repetitive dosing to patients. Eur J Clin Pharmacol 1983; 24:695-702.
30. Biesalski HK. Comparative assessment of the toxicology of vitamin A and retinoids in man. Toxicology 1989;57:117-27.
31. Muindi JR, Roth MD, Wise RA, Connett JE, O'Connor GT, Ramsdell JW, et al. Pharmacokinetics and metabolism of all-*trans* and 13-*cis*-retinoic acid in pulmonary emphysema patients. J Clin Pharmacol 2008;48:96-107.
32. Zouboulis CC, Bettoli V. Management of severe acne. Br J Dermatol 2015; 172 Suppl 1:27-36.
33. Topletz AR, Thatcher JE, Zelter A, Lutz JD, Tay SC, Nelson W, Isoherranen N. Comparison of the function and expression of the two retinoic acid hydroxylases CYP26A1 and CYP26B1. Biochem Pharm 2011;83:149-163.

34. Wu JW, Wang RY, Guo QS, Xu C. Expression of the retinoic acid metabolizing enzymes RALDH2 and CYP26 during mouse postnatal testis development. Asian J Androl 2008;10:569-77.
35. Del Rosso JQ. Face to face with oral isotretinoin: a closer look at the spectrum of therapeutic outcomes and why some patients need repeated courses. J Clin Aesthet Dermatol 2012;5:17-24.
36. WHO Laboratory Manual for the Examination and Processing of Human Semen 5th Edition, World Health Organization, Geneva, Switzerland, 2010.
37. Arnold SL, Amory JK, Walsh TJ, Isoherranen N. A sensitive and specific method for measurement of multiple retinoids in human serum with UHPLC-MS/MS. J Lipid Res 2012;53:587-98.
38. Heller CG, Clermont Y. Spermatogenesis in man: an estimate of its duration. Science 1963;140:184-6.
39. Misell LM, Holochwost D, Boban D, Santi N, Shefi S, Hellerstein M, Turek PJ. A stable isotope-mass spectrometric method for measuring human spermatogenesis kinetics in vivo. J Urol 2006;175:242-6.
40. Hogarth CA, Arnold S, Kent T, Mitchell D, Isoherranen N, Griswold MD. Processive pulses of retinoic acid propel asynchronous and continuous murine sperm production. Biol Reprod 2015;92:37.
41. Gely-Pernot A, Raverdeau M, Teletin M, Vernet N, Feret B, Klopfenstein M et al. Retinoic acid receptors control spermatogonia cell-fate and induce expression of the SALL4A transcription factor. PLos Genet 2015;11:e100501.
42. Koubova J, Hu YC, Bhattacharyya T, Soh YQ, Gill ME, Goodheart ML, et al. Retinoic acid activates two pathways required for meiosis in mice. PLoS Genet. 2014;10:e1004541.
43. Kurlandsky SB, Gamble MV, Ramakrishnan R, Blaner WS.Plasma delivery of retinoic acid to tissues in the rat. J Biol Chem 1995;270:17850-7.
44. Arnold SL, Kent T, Hogarth CA, Schlatt S, Prasad B, Haenisch M, et al. Importance of ALDH1A enzymes in determining human testicular retinoic acid concentrations. J Lipid Res 2015;56:342-57.

45. Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, et al. Retinoid signaling determines germ cell fate in mice. Science 2006;312:596-600.
46. Schmitt-Hoffaman AH, Roos B, Sauer J, Brown T, Weidekamm E, Meyer I et al. Low levels of alitretinoin in seminal fluids after repeated oral doses in healthy men. Clin Exper Derm 2011 36;(supl 2):12-7.
47. Lammer EJ, Chen DT, Hoar RM, Agnish PJ, Benke PJ, Braun JT, et al. Retinoic acid embryopathy. New Eng J Med 1985;313:837-41.
48. Rosa F. Isotretinoin dose and teratogenicity. Lancet 1987;2:1154.