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Walnuts Improve Semen Quality in Men Consuming a Western-style Diet: Randomized Control Dietary Intervention Trial

Short Title: RCT of Walnut Effects on Sperm

Summary Sentence: In a randomized, parallel two-group, dietary intervention trial, 75 gm of walnuts/day added to a Western-style diet improved sperm vitality, motility and morphology in healthy men ages 21–35 years.

Key Words: Semen Quality, Randomized Dietary Intervention, Sperm Aneuploidy

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Abstract

Purpose: We tested the hypothesis that 75 gm of whole-shelled walnuts/day added to a Western-style diet of healthy young men would beneficially affect semen quality.
Methods: A randomized, parallel two-group, dietary intervention trial with single-blind masking of outcome assessors, was conducted with 117 healthy men, age 21 – 35 years, who routinely consumed a Western-style diet. Primary outcome evaluated was improvement from baseline to 12 weeks in conventional semen parameters and sperm aneuploidy. Secondary endpoints included blood serum and sperm fatty acid (FA) profiles, sex hormones, and serum folate.
Conclusions: The group consuming walnuts (n=59) experienced improvement in sperm vitality, motility, and morphology and the group continuing their usual diet but avoiding tree nuts (n=58) saw no change. Comparing differences from baseline between the groups, significance was found for vitality p=0.003, motility p=0.009, and morphology (normal forms) p=0.04. Serum FA profiles improved in the walnut group with increases in omega-6 (p=0.0004) and omega-3

(p=0.0007) but not the control group. Only the plant source of omega-3, α-linolenic acid (ALA), increased (p=0.0001). Sperm aneuploidy was inversely correlated with sperm ALA, particularly sex chromosome nullisomy (-0.41, p=0.002). Findings demonstrated that walnuts added to a Western-style diet improved sperm vitality, motility and morphology.

Introduction

Food has been linked with human reproductive success throughout history [1]. Dietary habits and essential nutrients to promote successful reproductive outcomes have been identified for the maternal peri-conceptional and peri-natal period [2-7], but healthy dietary habits and essential nutrients for paternal reproductive fitness are less clear. Selenium in the form of selenoproteins protects sperm from oxidative damage and defines sperm morphology in the epididymis [8-10]; zinc and vitamins C and E have anti-oxidant properties believed important to male fertility [9] [11-15]; low folate intake has been associated with sperm aneuploidy [13], and polyunsaturated fatty acids (PUFAs) have been shown to play critical roles in sperm maturation and membrane function in animal and human laboratory studies [16-18] as well as some, but not all, clinical investigations of male infertility [19-28]. Although interest in establishing dietary recommendations to optimize male fertility is growing, evidence on which to base recommendations is still being defined and further research is needed.

Evidence is particularly limited for men who routinely consume Western-style diets that may lack optimal nutrients and PUFA profiles needed for healthy sperm and fertility. Best known sources of dietary PUFAs for persons consuming a Western-style diet include fish and fish oil supplements, flax seed, and tree nuts [29]. Nuts provide a rich source of α-linolenic acid (ALA), a natural plant source of omega-3. Of popular tree nuts, walnuts are particularly rich in ALA, omega-6 fatty acids, anti-oxidants and micronutrients including folic acid.

In the current study we tested the hypothesis that 75 gm of whole-shelled walnuts/day added to a Western-style diet would beneficially affect semen quality. The primary endpoint evaluated with respect to efficacy of the walnuts was improvement from baseline to 12 weeks in conventional semen parameters (sperm concentration, vitality, motility, morphology) and sperm aneuploidy. These semen quality parameters provide clinical markers for male sub-fertility [30]. Secondary endpoints included blood serum and sperm fatty acid (FA) profiles and sex hormones as potential underlying factors associated with conventional semen parameters, and serum folate as a potential underlying factor associated with sperm aneuploidy.

Materials and Methods

This was a parallel two-group, randomized dietary intervention trial, with single-blind masking of outcome assessors, conducted at a major university. The research was approved by the Medical IRB, Human Research Protection Program, University of California, Los Angeles and all research participants gave written, informed consent.

Study Subjects and Dietary Intervention

Eligible participants were males, age 21 through 35, who reported routinely eating a Western-style diet. Men were ineligible for inclusion if they had a known food allergy, history of reproductive disorders or vasectomy, were current smokers, were currently taking anti-oxidant supplements or medications for chronic illness, or were using illicit drugs. Recruitment was

through flyers posted on campus. A total of 120 men were enrolled October 2009 to September 2010. Three men completed a pilot trial of the walnut intervention. Subsequently the remaining 117 participants were randomly assigned to one of two parallel groups: group one continued to consume their usual diet but added 75 gram of whole-shelled English walnuts per day (n=59); and group two continued to consume their usual diet avoiding consumption of tree nuts (n=58). Walnuts were supplied in pre-weighed, 1 ounce snack packs provided by the California Walnut Commission, Folsom, California (http://www.walnuts.org).

Participants were randomized in a 1:1 manner to either walnut intervention or control using a computerized random proportion model in permuted blocks of 10 [31]. Randomization was performed at the Center for Human Nutrition with each allocation assignment placed into a sealed envelope and transported to the Robbins' lab where subjects were enrolled. Participants opened the envelope after informed consent at the baseline visit. Subsequent to the baseline visit, allocation was concealed from the researchers. The dietary trial lasted 12 weeks during which participants attended two research clinic visits, one at baseline and one at the 12 week interval. Prior to completing the study, three men were withdrawn by the researchers due to possible nut allergy, two left the geographic area, and three were otherwise lost to follow-up (Figure 1.) [32] There were no significant differences in baseline demographics, hormone and FA profiles, and semen parameters between the group of eight men who did not complete the study and the 109 men who did ($p>0.05$).

Dietary Measures

Usual diet at baseline was determined using a self-administered 2007 NCI diet history questionnaire [33], and three-day food record [31]. The food frequency questionnaire assessed underlying dietary patterns while the three-day food record provided measurement of recent dietary intake. To evaluate compliance, 24-hour dietary recalls were conducted over the telephone by trained interviewers twice each month on randomly chosen days throughout the 12 week study. The Automated Self-Administered 24-hour Dietary Recall (ASA24) software template was used for data collection and entry. [34] Researchers were blinded as to intervention or control group status when collecting and entering ASA24 data.

Height and body weight were measured and body mass index (BMI) calculated as kg/m^2. Men reported usual exercise level which was recorded as to specific activity, intensity, and duration. Total physical activity level was calculated and recorded in units of MET-minutes per week (MET-min/wk) according to the IPAQ scoring protocol [35]. One MET equals the energy expended during rest (3.5 ml O_2 x kg (-1) x min (-1).

Semen Analysis

All men were instructed to abstain for 2 to 3 days prior to providing the study specimen with abstinence period verified by self-report on day of sample collection. Semen samples were collected in a private clinical examination room located adjacent to the research laboratory allowing analysis within 60 minutes of sample production. A single researcher, who was blinded as to intervention versus control group status, performed the conventional semen analyses. Each sample was allowed to liquefy at room temperature and routine semen analysis performed according to standard procedures described in the WHO laboratory manual for examination and processing of human semen [30]. Hamilton-Thorne Biosciences IVOS

(http://www.hamiltonthorne.com) was utilized for count and motility parameters according to version 12.1 protocols. Samples with high concentrations were diluted using autologous sperm-free seminal plasma to achieve concentrations below ~50 x 10^6 per mL for IVOS motility assessments. Value for motile cells was set at Path Velocity (VAP) ≥5.0 μ/s. Minimum values for progressively motile cells were set at VAP 25 μ/s and Straightness (STR) 80%. Standards were purchased from Fertility Solutions (http://fertilitysolutions.com). Vitality was determined by membrane exclusion of eosin vital dye (http://www.fishersci.com). For sperm morphology, slides were scored by single technician who was blinded as to intervention or control group status, who had completed an American Society of Andrology Sperm Morphology Laboratory Training. Test slides for quality control validation were from Fertility Solutions. Strict criteria [36] were used at x100 magnification. For sperm aneuploidy, 10μL of semen was smeared onto slides, air dried, and evaluated for aneuploidy of chromosomes X, Y, and 18 using sperm Fluorescence *In Situ* Hybridization (FISH) adapted from Robbins et al. [37]. A single technician, who was blinded as to walnut intervention versus control group, systematically analyzed 5000 sperm per sample according to strict scoring criteria [38].

Blood Collection

Blood samples were collected into two 16 x 100 mm x 8.5 mL BD Vacutainer® Plus plastic serum collection tubes (http://paswhitepapers.bd.com). Within 30 minutes of collection, samples were centrifuged at 3000 rpm for 15 min, serum pipetted into 2 mL polypropylene tubes, then stored at −80°C until analysis. Frozen serum was allowed to thaw at room temperature prior to respective assays.

Blood Serum and Sperm Fatty Acid (FA) Analysis

Blood serum FAs were converted to methyl esters (FAME) using a methanol/benzene mix and acetylchloride according to the method by Bagga et al. [39]. After heat treatment for 60 min at 100ºC, sodium carbonate was added and samples centrifuged at 913xg for 10 min at 4ºC. Benzene supernatant was used to separate and quantify the FAMEs by use of an Agilent Technologies (http://www.home.agilent.com) 5890A series II gas chromatograph fitted with a model 7673 automatic split-injection system and flame ionization detector and SP2380 stabilized phase fused silica capillary column (30m x 0.32 mm i.d., 0.25 um film thickness, Supelco, Inc, http://www.manta.com). Quantification was based on recovery of a known quantity of internal standard (heptadecanoic acid, NuChek Preparation Inc., http://www.nu-chekprep.com) and on the response ratio of FA standards purchased from NuChek Preparation Inc. For quality control a pooled serum sample was used with each batch of serum samples. Using this pooled serum sample the following coefficient of variation was established: C16:0 1.6%, C16:1 2.7%, C18:0 2.6%, C18:1 3.4%, C18:2 0.7%, C18:3 0.7%, C20:2 8.7%, C20:4 0.7%, C20:5 3.2%, kC22:5 4.2% and C22:6 2.2%. Semen was centrifuged at 600xg.for 6 min at 4ºC to separate semen plasma and sperm. The sperm pellet was dispersed in 50 μl PBS and mixed with methanol/benzene mix and treated like the serum. Methylated FAs were separated and quantified using the same gas chromatography conditions as described for serum FA analysis.

Blood Hormone Assays

Total testosterone (T), estradiol (E2), follicle stimulating hormone (FSH), luteinizing hormone (LH) and sex hormone binding globulin (SHBG) were determined using chemiluminescent immunometric assays with an Immulite 1000 automatic analyzer (Siemens Medical Solutions

Diagnostics, http://www.usa.siemens.com). Hormone controls, standards (Siemens Medical Solutions Diagnostics) and serum pools generated in our laboratory were analyzed for quality control. The coefficient of variation for T was 6.8-13%, E2 6.3-15%, FSH 2.3-3.7%, LH 4.8-6.5% and 4.1-7.7% for SHBG. Duplicate samples with >10% coefficient of variation were re-analyzed. Sensitivity of the assays reported by the manufacturer are 0.5 nmol/L for T, 55 pmol/L for E2, 0.1 mIU/mL for FSH and LH, and 0.2 nmol/L for SHBG.

Serum Folate Assay

Serum folate was determined by a chemiluminescent, competitive binding technique using natural folate binding protein (Elecsys Folate III Assay, Cobas, Roche Diagnostics, https://www.cobas-roche.co.uk). Controls and standards (Cobas, Roche Diagnostics) and serum pools generated in the laboratory were analyzed for quality control. The intra-assay coefficient of variation ranged from 4.7 to 7.0%. Limit of detection ranged from 3.41 to 45.4 nmol/L.

Statistical Analyses

Summary statistics are reported as percents for nominal data or mean ± standard deviation (std) for continuous data. Fatty acids are expressed as a percent of total FAs. Unpaired student's t-test was used to test for differences between control and intervention group and paired student's t-test to test for differences within group from baseline to 12 weeks for variables that followed a normal (Gaussian) distribution or, if the distribution was non-normal, variables were transformed to approximate normal distribution. Mann-Whitney U test and Wilcoxon matched-pairs signed-rank were used for comparisons of non-normally distributed data. Spearman correlation coefficient or Pearson correlation coefficient were used to calculate pair-wise correlations. Two-tailed significance level was defined as $p<0.05$. Excluding samples with abstinence intervals outside a range of 1.5 to 3 days did not change study conclusions thus these samples were retained in the final analyses. Sample size of 50 men per group for sperm parameters and 40 for sperm aneuploidy was estimated for detecting a modest difference of the difference between the two groups from baseline to 12-weeks, 80% power and α-0.05. Statistical software package STATA 12 (http://stata.com) was used for statistical analyses.

Results

Characteristics of the study population (age, education level, race, BMI, body weight, METs) did not differ between the walnut intervention and control group at baseline indicating that the randomization procedure had been successful. No significant changes in BMI, body weight, or METs were noted in either group at the 12-week post-dietary intervention visit (Table 1). Days of abstinence prior to collecting the semen study sample did not differ between the walnut intervention and control group at baseline (3.2 [std 4.2] and 3.3 [std 4.2] days, respectively) or at the post-dietary intervention visit (3.4 [std 1.7] and 3.2 [std 2.1] days, respectively.

Fatty acid profiles in blood serum did not differ between the walnut intervention and control group at baseline (Table 2). Grouped omega-6 and omega-3 FAs in serum showed significant increases at the 12-week follow-up visit within the walnut group (p=0.0004, 0.0007, respectively), but not the control group. When comparing the 12-week change in the walnut group to the change in the control group, the difference was significant for omega-6 (p=0.004) and omega-3 (p=0.003). Among the omega-3 fatty acids, only ALA showed a significant change and this was an increase seen only in the walnut intervention group (p=0.0001). Fatty acid

profiles in sperm were not significantly different between the walnut intervention and control group at baseline or at the 12-week post-intervention visit except for eicosadienoic acid where within group differences in sperm between baseline and 12-weeks were not significant for either group ($p>0.05$), however, comparing change in walnut group (an increase) to change in control group (a decrease), the difference became significant ($p= 0.02$).

Sperm concentration, vitality, motility and morphology (normal forms) were not different between the walnut intervention and control groups at baseline (Table 3). Post-dietary intervention measures showed improvement within the walnut group for sperm vitality ($p = 0.0001$), motility ($p=0.004$), and morphology normal forms ($p=0.003$) but not within the control group ($p>0.05$). When comparing change from baseline in the walnut group to change from baseline in the control group, the following were significant: vitality ($p=0.003$), motility ($p=0.009$), and morphology normal forms ($p=0.03$). Progressive motility increased within the walnut group (mean increase 3.1% (std 8.0), $p=0.001$) but not within the control group. When comparing change from baseline in progressive motility in the walnut group to the change in the control group, $p = 0.02$. Sex hormones were not different between the groups at baseline or at 12-weeks except for FSH. The walnut group started with a lower FSH level than controls ($p=0.04$) and remained lower at post-intervention . Other than two subjects who were outliers, the FSH values were within normal limits for healthy men of this age (data not shown) [40].

Sperm aneuploidy for chromosomes X, Y and 18 (reported as percent of sperm with numerical abnormality out of total sperm scored) was not different between the walnut (n=54) and control group (n=49) at baseline or at the 12-week post-intervention visit (Table 3). However, within the walnut group sperm aneuploidy was decreased at 12 weeks (Paired t-test $p=0.003$). Specifically, decreases were seen in sex chromosome disomy and sperm missing a sex chromosome (Paired t-test, $p=0.002$ and $p= 0.01$, respectively). No significant changes from baseline occurred within the control group. At the 12-week visit, sperm ALA was inversely correlated with the proportion of sperm missing a sex chromosome (Spearman correlation -0.41, $p=0.002$) and percent of sperm with any numerical chromosomal abnormality (Spearman correlation -0.34, $p=0.01$). No significant correlations were found between sperm aneuploidy measures and other omega-3s.

Serum folate levels were not significantly different between the walnut and control group at baseline with mean values for the walnut group of 18.6 (std 6.3) range 7.1 – 44.2 nmol/L and control group 19.6 (std 5.5) range 9.6 – 40.1 nmol/L. Change from baseline to 12 weeks when compared between the groups was not significant ($p>0.05$). Serum folate was not correlated with sperm aneuploidy measures at baseline or at the 12-week follow-up visit ($p>0.05$).

Discussion

We found that 75gm of walnuts per day added to a Western-style diet improved sperm vitality, motility, and morphology (normal forms) in a group of healthy young men when compared to a control group of men consuming usual diet but avoiding tree nuts. Improved semen quality was associated with increases in blood serum omega-6 FA and in the plant source of omega-3 (ALA) but not with other omega-3s. These findings are consistent with a literature showing a distinct change in FA profiles during sperm maturation and differentiation that are key to cellular functions such as phagocytosis of residual bodies by Sertoli cells affecting sperm morphology and provision of fluidity to sperm membrane for motility [16][41-44].

In clinical populations, Attaman et al., [19] reported dietary intake of omega-3 FA correlated positively with normal sperm morphology, Chavarro et al., [45] reported higher *trans*-fatty acid levels in sperm were associated with lower sperm concentration, Gulaya et al., [26] reported lower levels of sperm DHA in oligoasthenozoospermic compared with normospermic men, Aksoy et al., [23] and Safarinejad et al., [21] reported higher omega-6 to omega-3 ratios in infertile versus fertile men. Other researchers have reported no difference in PUFAs across fertile and infertile men [22][25]. Safarinejad [46] conducted a double-blind control trial of fish oil supplements in an infertility clinic population located in Tehran, Iran. Participants were men with idiopathic oligoasthenoteratospermia randomized to 1.12 gm eicosapentaenoic acid (EPA)/day plus 0.72 gm of DHA/day or a corn-oil placebo. The group taking omega-3 supplements showed statistically significant increases in EPA and DHA in RBCs, sperm cells, and seminal plasma with concomitant improvement in sperm concentration, count, motility, and morphology.

Both the Safarinejad [46] RCT with EPA and DHA omega-3 supplements and our RCT that enriched usual diet with ALA omega-3 through eating walnuts found improved sperm motility and morphology (normal forms). In our study, the walnut dietary intervention resulted in a changed serum FA profile for ALA but not EPA and DHA suggesting the beneficial omega-3 effects on sperm from walnuts were related to the plant source of omega-3, ALA. Safarinejad [46] reported changes in sperm DHA and EPA associated with DHA/EPA supplements, however, in our study a significant increase in serum ALA for the walnut group ($p=0.0001$) was not reflected as a significant change in sperm ALA ($p>0.05$). Differences in the study findings may reflect that walnuts, as a natural food, contain multiple nutrients that act synergistically with dietary ALA [47] for cellular-level changes of benefit to sperm, or possibly, our 12-week intervention was not adequate to effect changes in sperm FA (the Safarinejad trial of EPA/DHA supplements lasted 32 weeks) [46]. In spite of this, in the present study, percent of sperm with abnormalities in chromosome number were inversely correlated with sperm ALA as was proportion of sperm missing a sex chromosome. No significant correlations were found with other omega-3s.

Important differences between the Safarinejad [46] EPA/DHA supplement trial and our walnut intervention dietary trial include the study populations. The EPA/DHA supplement trial was conducted in Iran among men (mean age ~32) attending an infertility clinic and diagnosed with idiopathic oligoasthenoteratospermia whereas the walnut intervention included healthy young men (mean age ~25) without known history of infertility, consuming a typical Western-style diet. Both studies generate important findings relevant to the different populations. For men diagnosed with idiopathic oligoasthenoteratospermia, the DHA/EPA intervention improved count, motility and morphology (normal forms) on a background of abnormal sperm parameters associated with known infertility. In the case of healthy young men eating walnuts, the intervention improved sperm parameters of vitality, motility, morphology (normal forms), and sperm aneuploidy on a heterogeneous background of semen parameters for men of unproven fertility. For example, at baseline 22% of study participants fell below the WHO lower reference limit (5^{th} centile, 95% CI 38-42) for total motile percent [30, p.238]. In the walnut intervention group, but not the control group, the greatest proportion of men experiencing improvement in total motile percent at 12 weeks was seen for men who had been in the lowest quintiles of

motility (both total motile percent and progressive motility percent) at baseline.

Strengths of the present study include the twelve-week dietary intervention period that spanned cells differentiating throughout one full cycle of spermatogenesis. Additionally, investigating walnuts as a natural food source allowed evaluation of cumulative effects of multiple nutrients as opposed to individual nutrients in commercial dietary supplements. Finally, in the present study beneficial changes in blood serum FA profiles occurred (increased PUFAs, decreased saturated FAs) that are consistent with a literature showing these same effects of walnuts on blood lipids in studies designed to evaluate cardiovascular risk reduction [48-51]. The choice of 75 gm of walnuts for the current study was derived from review of these and other studies showing doses at which blood lipid levels would be expected to change but not result in weight gain in healthy young males, and be high enough to answer the previously untested research question related to male reproductive health.

A limitation of the present design was collection of blood specimens for hormone analysis throughout the day. This was done to facilitate retention of this busy group of young men. To control for diurnal variability, each man was matched on time of day blood was collected at baseline to same time of day at 12-weeks. Greater than 80% of the study population returned at 12-weeks within a two-hour window of their baseline time and hormone levels at baseline and 12-weeks were correlated ($p<0.001$). Sex hormone and gonadotropin levels were assessed as a measure of reproductive fitness and except for two subjects who were outliers, fell within mean reference intervals commonly reported for this age group of healthy men [40]. The present study did not find an association between folate and sperm aneupolidy whereas Young et al. [13] did. This could reflect differences in exposure assessment and study populations. Young et al., [13] averaged dietary folate intake for men age 20-70 years based on annual consumption patterns assessed by a food frequency questionnaire. The present study of men age 21-35 measured folate in blood serum which reflects recent dietary intake and can fluctuate. Baseline and 12-week measures were found to correlate (Pearson 0.49, $p<0.0001$) but more folate research is needed. Approximately 70 million couples worldwide experience sub-fertility or infertility with 30 – 50% of these cases attributed to the male partner [52]. In the USA, prevalence of men seeking help for fertility is estimated at ~3.3 – 4.7 million [53]. Semen quality is a predictor of male fertility [30] and some studies suggest human semen quality has declined in specific geographic regions of the world, particularly industrialized nations where pollution, lifestyle habits such as smoking, and trends toward a Western-style diet are hypothesized as potential causes [54-56]. Although importance of diet to human reproductive success is clear, existing dietary recommendations primarily focus on women's reproductive health with less attention given to men. Efforts to identify male-specific dietary recommendations that optimize fertility and promote offspring health should be encouraged.

We evaluated walnuts as a whole food contributing not only essential fatty acids but also micro-nutrients important for sperm development and function and found that 75 gm walnuts per day improved sperm vitality, motility, and morphology (normal forms) in a group of healthy, young men eating a Western-style diet. Whether adding walnuts to the diet will go beyond the shifts in sperm parameters as seen in this study to improving birth outcomes for men within fertility clinic populations or in the general population is not yet known and will require further research.

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Figure Legend

Figure 1. Flow diagram of walnut randomized control dietary intervention.

Table 1. Selected characteristics of the study population at baseline with Δ-change at 12-week dietary intervention visit.

	Walnut Group n=59	Control Group n=58	p-value
Age years, mean (std)	25.6 (4.0)	24.8 (3.7)	NS
Education %			
High School	15.8	10.5	
AA or AS degree	22.8	43.9	
BA or BS degree	42.1	31.6	
Masters, MD, PhD	19.3	14.0	
Race %			NS
White	35.6	41.4	
Asian	32.2	29.3	
Hispanic	17.0	20.7	
Black	3.4	1.7	
Mixed & Other	11.8	6.9	
BMI mean (std)			
Baseline	24.98 (4.0)	25.59 (3.5)	NS
Δ at 12-week visit	-0.66 (0.7)	0.12 (0.8)	NS
Weight in lbs mean (std)			
Baseline	174.7 (35.1)	174.1 (20.3)	NS
Δ at 12-week visit	0.14 (5.0)	-0.41 (5.2)	NS
Exercise Metabolic Equivalents (METs) mean (std)			
Baseline	2458 (1935)	3629 (2056)	NS
Δ at 12-week visit	352 (2099)	320 (2294)	NS

- Δ-change values presented for 12-week follow-up are calculated based on 109 men who completed the study; METs (n=103) .

Table 2. Selected fatty acids in blood serum and sperm at baseline with Δ-change at 12-week dietary intervention visit.

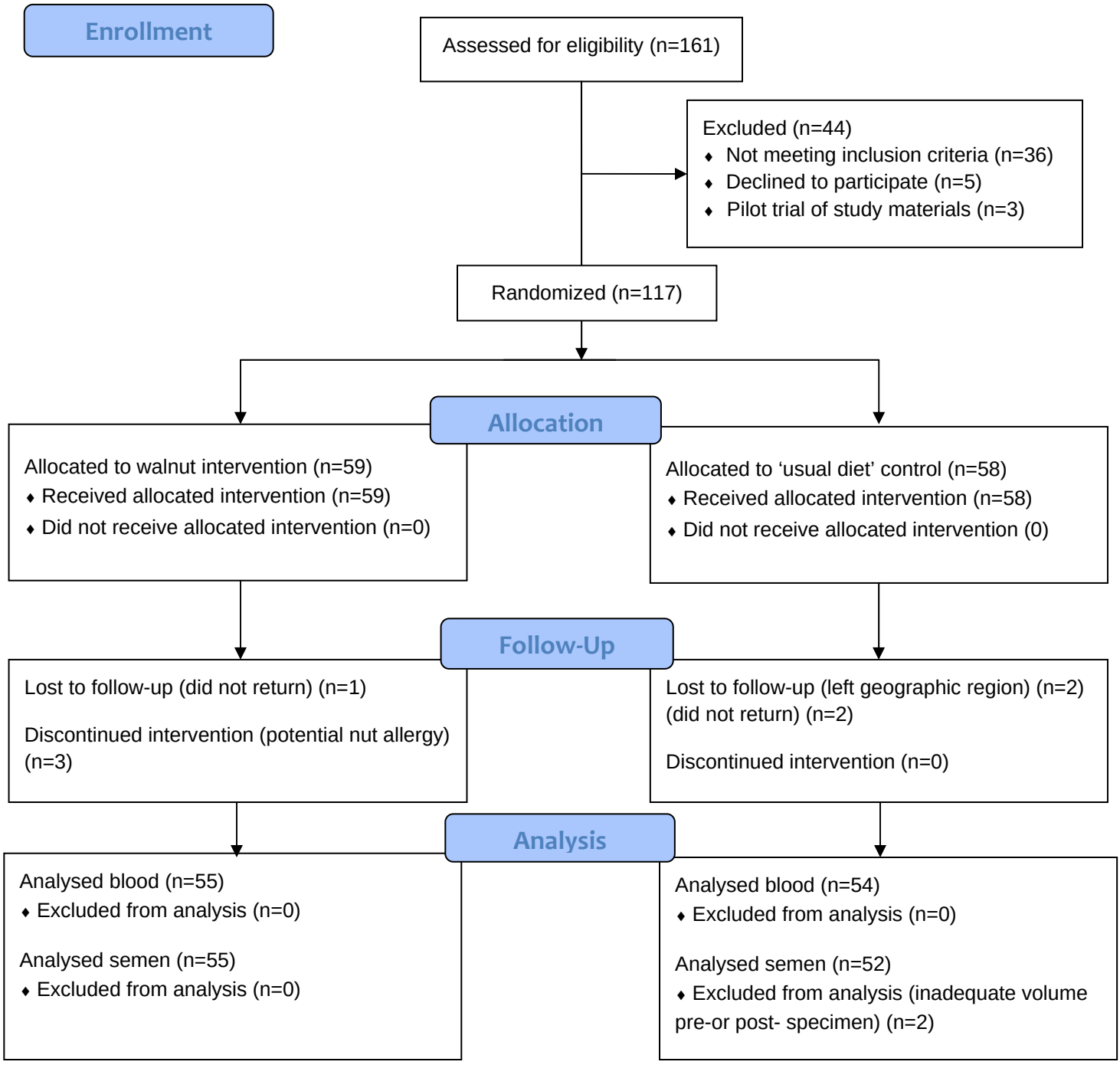
	Walnut Group Mean (std)	Control Group Mean (std)	p-value
Serum α-linolenic acid (ALA)			
Baseline	0.897 (0.37)	0.933 (0.37)	NS
Δ at 12-weeks	0.52 (0.87)	-0.02 (0.39)	0.0001
Sperm ALA			
Baseline	2.1 (0.9)	2.0 (0.7)	NS
Δ at 12-weeks	0.03 (0.6)	0.02 (0.5)	NS
Serum Docosahexaenoic acid (DHA)			
Baseline	2.32 (0.77)	2.05 (0.71)	NS
Δ at 12-weeks	-0.99 (0.51)	0.11 (0.66)	NS
Sperm DHA			
Baseline	28.7 (5.8)	22.8 (5.0)	NS
Δ at 12-weeks	0.89 (4.5)	0.64 (4.4)	NS
Serum Omega-6			
Baseline	41.2 (4.5)	42.5 (3.8)	NS
Δ at 12-weeks	2.8 (4.0)	0.78 (3.4)	0.004
Sperm Omega-6			
Baseline	16.3 (3.3)	15.8 (2.6)	NS
Δ at 12-weeks	1.2 (3.9)	0.4 (2.8)	NS
Serum Omega-3			
Baseline	3.49 (0.84)	3.26 (0.72)	NS
Δ at 12-week visit	0.42 (0.87)	0.09 (0.69)	0.003
Sperm Omega-3			
Baseline	32.9 (5.3)	33.1 (4.6)	NS
Δ at 12-week visit	0.8 (4.3)	0.8 (4.1)	NS

- Values for fatty acids are presented as percent of total fatty acids
- Omega-6 fatty acids = Linoleic + Arachidonic acid; Omega-3 fatty acids= α-linolenic + Eicosapentaenoic + Docosahexaenoic
- Values for blood serum FAs are based on 109 men who contributed pre-post blood samples and sperm FAs are based on a subset of 58 men who contributed pre-post semen samples with adequate cell counts remaining after semen analysis for FA analysis

Table 3. Selected sperm parameters at baseline and change at 12-week dietary intervention visit

	Walnut Group		Control Group		p-value
Sperm Parameters	Mean (std)	Range	Mean (std)	Range	
Concentration x 10^6/mL					
Baseline	71.4 (61.1)	2.9-326.3	71.8 (54.5)	1-316.2	NS
Δ at 12-week visit	2.2 (42.6)		-6.3 (49.6)		NS
Vitality %					
Baseline	76.4 (13.2)	10-93	78.0 (14.4)	0-95	NS
Δ at 12-week visit	5.5 (10.0)		0.51 (7.4)		0.003
Motility % (Progressive + Non-progressive)					
Baseline	51.8 (20.6)	2-90	53.5 (19.6)	0-86	NS
Δ at 12-week visit	5.7 (13.4)		0.53 (10.4)		0.009
Morphology (normal forms) %					
Baseline	7.4 (3.5)	1-18.5	7.1 (2.9)	1-14	NS
Δ at 12-week visit	1.1 (2.7)		0.1 (2.3)		0.03
Sperm X,Y, 18 Chromosome Aneuploidy %					
Baseline	1.2 (0.4)	0.5-2.4	1.3 (0.7)	0.7-4.8	NS
Δ at 12-week visit	0.1 (0.4)		0.04 (0.4)		NS

- Sperm parameters are based on 107 men who provided pre-post semen samples


Enrollment
Assessed for eligibility (n=161)
Excluded (n=44)
♦ Not meeting inclusion criteria (n=36)
♦ Declined to participate (n=5)
♦ Pilot trial of study materials (n=3)
Randomized (n=117)
Allocation
Allocated to walnut intervention (n=59)
♦ Received allocated intervention (n=59)
♦ Did not receive allocated intervention (n=0)
Allocated to 'usual diet' control (n=58)
♦ Received allocated intervention (n=58)
♦ Did not receive allocated intervention (0)
Follow-Up
Lost to follow-up (did not return) (n=1)
Discontinued intervention (potential nut allergy) (n=3)
Lost to follow-up (left geographic region) (n=2) (did not return) (n=2)
Discontinued intervention (n=0)
Analysis
Analysed blood (n=55)
♦ Excluded from analysis (n=0)
Analysed semen (n=55)
♦ Excluded from analysis (n=0)
Analysed blood (n=54)
♦ Excluded from analysis (n=0)
Analysed semen (n=52)
♦ Excluded from analysis (inadequate volume pre-or post- specimen) (n=2)