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ORIGINAL ARTICLE

Male Endocrinology

The effectiveness of zinc supplementation in men with isolated hypogonadotropic hypogonadism

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on behalf of Hypogonadotropic Hypogonadism Intervention Study (HHIS) Group

A multicenter, open-label, randomized, controlled superiority trial with 18 months of follow-up was conducted to investigate whether oral zinc supplementation could further promote spermatogenesis in males with isolated hypogonadotropic hypogonadism (IHH) receiving sequential purified urinary follicular-stimulating hormone/human chorionic gonadotropin (uFSH/hCG) replacement. Sixty-seven Chinese male IHH patients were recruited from the Departments of Endocrinology in eight tertiary hospitals and randomly allocated into the sequential uFSH/hCG group (Group A, $n = 34$) or the sequential uFSH plus zinc supplementation group (Group B, $n = 33$). In Group A, patients received sequential uFSH (75 U, three times a week every other 3 months) and hCG (2000 U, twice a week) treatments. In Group B, patients received oral zinc supplementation (40 mg day⁻¹) in addition to the sequential uFSH/hCG treatment given to patients in Group A. The primary outcome was the proportion of patients with a sperm concentration $\geq 1.0 \times 10^6$ ml⁻¹ during the 18 months. The comparison of efficacy between Groups A and B was analyzed. Nineteen of 34 (55.9%) patients receiving sequential uFSH/hCG and 20 of 33 (60.6%) patients receiving sequential uFSH/hCG plus zinc supplementation achieved sperm concentrations $\geq 1.0 \times 10^6$ ml⁻¹ by intention to treat analyses. No differences between Group A and Group B were observed as far as the efficacy of inducing spermatogenesis ($P = 0.69$). We concluded that the sequential uFSH/hCG plus zinc supplementation regimen had a similar efficacy to the sequential uFSH/hCG treatment alone. The additional improvement of 40 mg day⁻¹ oral zinc supplementation on spermatogenesis and masculinization in male IHH patients is very subtle. *Asian Journal of Andrology* (2017) 19, 280–285; doi: 10.4103/1008-682X.189621; published online: 21 October 2016

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INTRODUCTION

Congenital isolated hypogonadotropic hypogonadism (IHH) is characterized by absent or incomplete sexual maturation and infertility due to isolated defects in gonadotropin-releasing hormone (GnRH) release or action. IHH can be classified as Kallmann syndrome (KS, with anosmia or hyposmia) or normosmic isolated hypogonadotropic hypogonadism (nIHH, with intact sense of smell).^{1–4} The American Association of Clinical Endocrinologist Medical Guidelines recommended the regimen of gonadotropin replacement for male IHH patients wishing to conceive includes the initial use of human chorionic gonadotropin (hCG) for 6–12 months with the later addition of follicular-stimulating hormone (FSH) or human menopausal gonadotropin (hMG) until pregnancy is achieved.⁵ In a previous study, we reported that the efficacy of sequential use of urinary FSH (uFSH; 75 U, three times per week, every other 3 months) in spermatogenesis was not inferior to the continual use of uFSH (75 U, three times per week). Of note, the sequential regimen could markedly reduce

medical costs and provide a more preferable treatment option for IHH patients.⁶ However, <30% of IHH patients using the sequential uFSH/hCG regimen achieve normal sperm concentration (>15 million ml⁻¹) ranges, according to the WHO.⁷ Thus, auxiliary treatment measures are necessary to further increase spermatogenesis in IHH patients.

Zinc has been implicated in many aspects of male reproduction, such as testicular development, testosterone synthesis, and sperm quality. Zinc serves as a cofactor for numerous metalloenzymes involved in DNA and protein synthesis, which are critically involved in germ cell development.^{8–10} Clinical studies with zinc-deprived adult males showed that testosterone synthesis and spermatogenesis were dependent on adequate dietary zinc supplementation.^{11,12} Therefore, oral zinc supplementation could improve sperm concentrations in subfertile males with asthenozoospermia or oligozoospermia.¹³ We hypothesized that oral zinc supplementation may further improve spermatogenesis in male IHH patients and could be successful in optimizing the sequential uFSH/hCG regimen as previously reported.⁶

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SUBJECTS AND METHODS

Study design and subjects

This multicenter, open-label, randomized, controlled superiority trial was conducted between October 2009 and December 2012 in accordance with the ethical principles of the Declaration of Helsinki and the principles of current Good Clinical Practices. The study protocol was reviewed and approved by the Institutional Review Board of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from each participant. This study was conducted in eight tertiary hospitals in China. This trial is registered with ClinicalTrials.gov, number NCT 01403532.

Ninety-eight male IHH patients were initially recruited and screened using GnRH and hCG stimulation tests. Of these, 67 met the inclusion criteria and were randomly allocated into two groups: sequential uFSH (Group A, $n = 34$) and sequential uFSH plus zinc (Group B, $n = 33$) (Figure 1). The patients were followed up at 3, 6, 9, 12, 15, and 18 months after the treatment was initiated. Information about physical examination, semen analysis, and hormone measurements was collected at each visit. All of the follow-up appointments were completed by December 2012.

Eligibility criteria included men (1) between ages of 18- and 45-year-old, (2) without spontaneous puberty, (3) with serum testosterone levels <100 ng dl⁻¹ (3.5 nmol l⁻¹) in the presence of low or normal gonadotropins, and (4) with otherwise normal testing of the anterior pituitary gland. Exclusion criteria included (1) previous exposure to pulsatile GnRH or FSH-containing preparations, (2) receiving other preparations containing zinc before or during this study, (3) sperm concentrations $\geq 1.0 \times 10^6$ ml⁻¹, and (4) moderate or severe liver and renal dysfunction.

Randomization and masking

Participants were randomly assigned to Groups A and B (1:1). The randomization was conducted independently at a central office using a computer-generated random allocation sequence table with permuted blocks of six and with stratification by centers. Allocation concealment was performed by enclosing assignments in sequentially numbered, opaque, closed envelopes. Patients, investigators, trial staff, and statisticians were masked to the treatment allocations.

Interventions

Patients in both Groups A and B received sequential uFSH/hCG regimen as reported previously: 2000 U human chorionic gonadotropin (hCG)

twice a week for all 18 months and 75 U uFSH three times a week (every other 3 months) for 6–18 months.⁶ In Group B, the patients received the same regimen as those in Group A with an additional oral supplementation of 40 mg day⁻¹ of zinc as zinc gluconate (280 mg day⁻¹) for all 18 months, which was provided by the trial centers. Both hCG and uFSH were injected intramuscularly either by the nurses or by the patients themselves.

Outcomes

A sperm concentration of $\geq 1.0 \times 10^6$ ml⁻¹ during the 18 months of treatment was defined as the primary outcome. The value of 1.0×10^6 ml⁻¹ has commonly been used in clinical trials as it is representative of sperm concentrations capable of achieving pregnancy.^{14–16} The testicular volume, serum testosterone concentration, sperm activity, sperm count per ejaculate, and time of spermatogenesis were defined as the secondary outcomes. Self-reported pregnancy was also recorded.

Clinical measurements

All of physical examinations were performed by senior endocrinologists. Body weight, height, body mass index (BMI), Tanner stage, and penis length were evaluated during each visit, as described previously.⁶ Testicular volumes were also calculated from ultrasound examinations of scrotal content (Linear Array Transducer, 10–12 MHz; GE LOGIQ E9, GE LOGIQ 9, USA) using the formula of length \times width \times depth \times 0.71.¹⁷

Serum biochemical measurements

As described previously, blood samples were collected in the morning 24–72 h after the last hCG and uFSH injections and immediately centrifuged. Serum was frozen at -80°C until assayed. Serum LH, FSH, and testosterone were measured by chemiluminescence immunoassays (CLIA) (Abbott, USA). Serum zinc concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS, Agilent, USA).

Semen analysis

Semen analysis was conducted according to the World Health Organization guidelines, as described previously.^{6,7}

Statistical analysis

The present study was designed as a superiority trial to explore whether zinc supplementation could improve spermatogenesis in IHH patients preliminarily; 67 patients were recruited and randomly allocated at a ratio of 1:1 into two groups. For sample size calculation, we assumed a superiority margin of 25% in the zinc supplementation group versus control group, and expected an efficacy rate of 65% in the control group, as indicated in previous studies.^{18–20} Assuming a 5% dropout rate, 33 patients in each group are needed to yield 80% power for superiority with $\alpha = 0.05$. All efficacy analyses were performed based on intention-to-treat (ITT). The primary outcome was also analyzed based on per-protocol (PP) principle, which included the patients who completed the whole process of the 18-month treatment. *T*-tests and nonparametric Wilcoxon rank-sum tests were utilized for normally distributed and skewed variables, respectively. Kaplan–Meier plots were used to analyze the time for the primary outcome and initiation of spermatogenesis, which were compared between groups with the use of the log-rank test. Chi-square or Fisher's exact tests were used for comparing binary outcomes. Statistical analysis was performed using SAS version 8.1 software (SAS Institute Inc., Cary, NC, USA). The results are presented as mean \pm s.d. or median (inter quartile range). $P < 0.05$ was considered statistically significant.

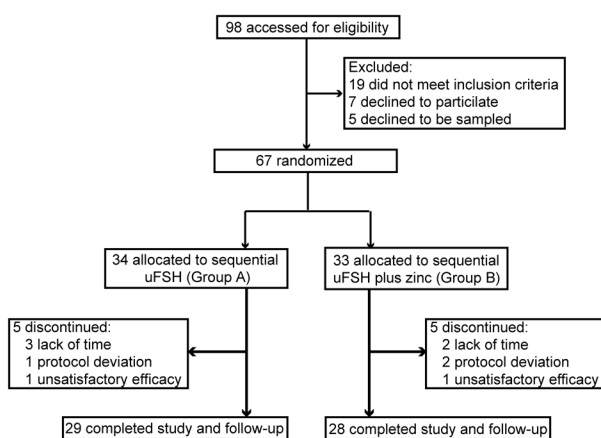


Figure 1: Flowchart of patients' randomization and follow-up. uFSH: urinary follicular-stimulating hormone.

RESULTS

A total of 98 patients were initially screened. Of those, 19 patients did not meet the inclusion criteria, 7 declined to participate, and 5 declined blood sampling. In addition, ten patients did not complete the study (five from each group). The reasons for these dropouts are indicated in **Figure 1**.

Baseline characteristics

Clinical characteristics and the hormonal profiles of the patients at baseline are shown in **Table 1**. The mean age of the IHH patients was 24.1 ± 4.9 years in Group A and 22.3 ± 4.0 years in Group B. Sixteen of 34 (47.1%) participants in Group A and 11 of 33 (33.3%) participants in Group B were diagnosed as Kallmann syndrome for presenting with complete anosmia or hyposmia. Low levels of serum testosterone, LH, FSH, and no obvious secondary sexual characteristics were observed in all the patients at baseline. Small testes, short penises, and azoospermia were also recorded in each IHH patient.

Primary outcome

Intention to treat analysis showed that 19 of 34 (55.9%; 95% CI, 37.9%–72.8%) patients in Group A and 20 of 33 (60.6%; 95% CI, 42.1%–77.1%) patients in Group B achieved the primary outcome (sperm density $\geq 1.0 \times 10^6 \text{ ml}^{-1}$) ($P = 0.69$; **Figure 2**). Per-protocol principal analysis showed 19 of 29 (65.5%; 95% CI, 45.7%–82.1%) patients in Group A and 19 of 28 (67.9%; 95% CI, 47.7%–84.1%) patients in Group B achieved the primary outcome (sperm density $\geq 1.0 \times 10^6 \text{ ml}^{-1}$) ($P = 0.55$; **Figure 2**).

Table 1: Baseline characteristics of the study participants

	Group A (n=34)	Group B (n=33)	P
Age (year)	24.1±4.9	22.3±4.0	0.12
Height (cm)	172.6±6.4	173.8±7.5	0.43
BMI (kg m ⁻²)	22.2±6.6	20.8±3.3	0.61
Penis length (cm)	4.3±1.0	4.6±1.2	0.26
Testicular volume (ml)	1.5 (1.0–3.0)	1.4 (1.0–2.3)	0.90
Pubic hair stage	2 (2–2)	2 (2–3)	0.88
Genital Tanner stage	2 (1–2)	2 (1–2)	0.83
Serum LH (IU l ⁻¹)	0.3 (0.1–0.8)	0.2 (0.1–0.4)	0.25
Serum FSH (IU l ⁻¹)	1.0 (0.5–1.4)	0.8 (0.4–1.0)	0.10
Serum testosterone (nmol l ⁻¹)	1.4 (1.0–1.7)	1.4 (0.9–1.8)	0.97
Serum zinc (mg l ⁻¹)	1.2 (1.1–1.5)	1.3 (1.1–1.6)	0.58

Data are presented as mean±s.d. or median (inter quartile range). BMI: body mass index; LH: luteinizing hormone; FSH: follicular stimulating hormone; s.d.: standard deviation

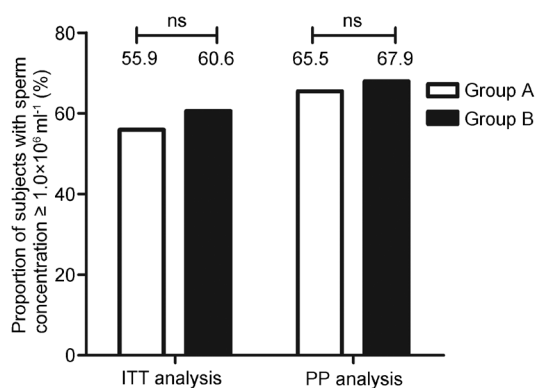


Figure 2: Proportion of patients with sperm concentration $\geq 1.0 \times 10^6 \text{ ml}^{-1}$ by intention-to-treat and per-protocol analysis after 18 months of treatment. ITT: intention-to-treat; PP: per-protocol; ns: nonsignificant.

Testis volume and penis length

No statistical differences were observed in testis volume and penis length between Group B and Group A after 18 months of treatment (**Supplementary Table 1**). The median testicular volume increased from 1.5 ml to 5.3 ml in Group A, and from 1.2 ml to 4.2 ml in Group B after 18 months of treatment (**Supplementary Table 1** and **Figure 3a**). Median penis length increased from 4.6 cm to 6.9 cm in Group A and from 4.2 cm to 6.2 cm in Group B (**Supplementary Table 1**).

Serum hormone concentrations and secondary sex characteristic

Serum testosterone concentrations were markedly increased during the first 3 months in both groups (**Figure 3b**) and were maintained in the normal adult male range during the entire 18 months of treatment. No statistical differences were observed in serum testosterone concentrations between Groups B and A (**Supplementary Table 1** and **Figure 3b**). The Tanner stage for pubic hair was increased from 2.1 at baseline to 4.6 at 18 months in Group A and from 2.1 at baseline to 4.4 at 18 months in Group B ($P = 0.86$, **Supplementary Table 1**). The genital Tanner stage was increased from 1.7 to 4.3 in Group A and from 1.8 to 4.3 in Group B ($P = 0.60$; **Supplementary Table 1**). Obvious development of secondary sex characteristics, such as beard growth and voice changes, was observed in all patients in both groups.

Serum zinc concentrations

The median serum zinc concentration was 1.2 mg l^{-1} ($18.1 \mu\text{mol l}^{-1}$) in Group A and 1.3 mg l^{-1} ($20.2 \mu\text{mol l}^{-1}$) in Group B at baseline. Levels

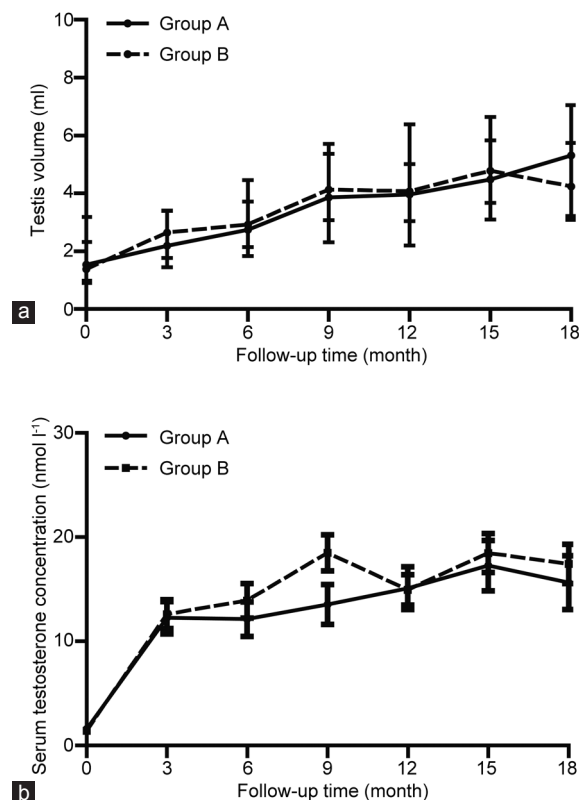


Figure 3: The changes of testicular volume and serum testosterone concentration throughout the period of the 18-month follow-up. Single testicular volume (a) and serum testosterone concentration (b) in sequential uFSH group and zinc supplementation group through the 18-month treatment period. The data are presented as mean ± standard deviation.

were 1.2 mg l^{-1} ($18.4 \text{ } \mu\text{mol l}^{-1}$) in Group A and 1.2 mg l^{-1} ($18.3 \text{ } \mu\text{mol l}^{-1}$) in Group B after 18 months of therapy (**Supplementary Table 1**). There were no statistical differences between the two groups ($P = 0.67$; **Supplementary Table 1**).

Sperm count and motility

Twenty-two of 34 (64.7%; 95% CI, 46.5%–80.3%) patients in Group A and 23 of 33 (69.7%; 95% CI, 51.3%–84.4%) patients in Group B exhibited spermatogenesis (sperm density $\geq 0 \times 10^6 \text{ ml}^{-1}$) during 18 months of treatment by intention to treat analysis ($P = 0.66$; **Figure 4**). Median sperm concentrations were $1.5 \times 10^6 \text{ ml}^{-1}$ in Group A and $2.5 \times 10^6 \text{ ml}^{-1}$ in Group B, which was not statistically different between the two groups ($P = 0.91$, **Supplementary Table 1**). Ten of 34 (29.4%; 95% CI, 15.1%–47.5%) patients in Group A and 9 of 33 (27.3%; 95% CI, 13.3%–45.5%) patients in Group B achieved sperm concentrations of $>15 \times 10^6 \text{ ml}^{-1}$ (**Figure 4**), which is defined as a normal count according to the WHO Laboratory Manual for the Examination and Processing of Human Semen (5th edition).⁷ Median sperm activity was 44.7% and 36.1% in Group A and in Group B, respectively ($P = 0.79$, **Supplementary Table 1**).

Timing of spermatogenesis

The median time for initial spermatogenesis was 12 months in Group A and 9 months in Group B (**Figure 5a**), and the median time to achieve the primary outcome was 15 months in Group A and 12 months in Group B (**Figure 5b**) by Kaplan–Meier survival analysis. There were no statistical differences between Group A and Group B in the timing of initial spermatogenesis and achievement of the primary outcome.

DISCUSSION

In our previous study, the sequential uFSH/hCG regimen was effective in inducing spermatogenesis in male IHH patients.⁶ However, the proportion of the patients whose sperm concentrations reached normal levels after receiving 18 months of gonadotropins remained relatively small. Both clinical and animal studies show that zinc is essential to spermatogenesis.^{8–13,21} In the present study, we found that zinc supplementation did not further improve spermatogenesis in combination with the standard uFSH/hCG regimen given to IHH patients.

We cannot exclude that zinc might have a weak effect on spermatogenesis, which could not be detected in our current study. Azoospermia in IHH patients is mainly the result of GnRH and LH/FSH deficiency; without administration of LH/FSH preparations, the induction of spermatogenesis is almost impossible. Thus, the drastic

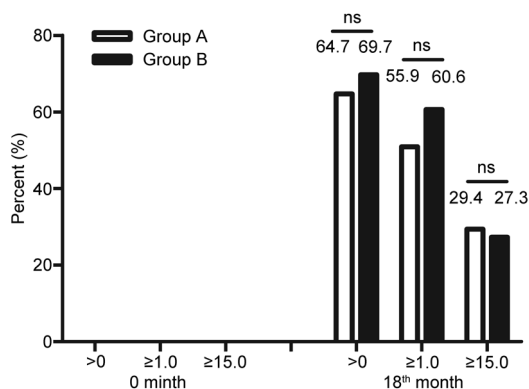


Figure 4: Proportion of patients with sperm concentration $>0 \times 10^6 \text{ ml}^{-1}$, $\geq 1.0 \times 10^6 \text{ ml}^{-1}$ and $\geq 15.0 \times 10^6 \text{ ml}^{-1}$ by intention-to-treat at 0 and 18th month. ns: nonsignificant.

recovery of spermatogenesis due to gonadotropin treatment for both groups may have resulted in a ceiling effect that masked any subtle difference caused by additional zinc treatment.

At the same time, other sperm parameters or testosterone synthesis showed no statistical differences between the two groups. In general, the effects of zinc on sperm motility remain controversial. Some studies found that high serum zinc could enhance sperm motility while others reported that it suppressed sperm progressive motility.^{22–24} In our study, the median motile sperm percentages (Grade A, B, and C) were 44.7% in Group A and 36.1% in Group B, and no obvious enhanced or suppressed effect of zinc on sperm motility could be observed. It had been reported that zinc is helpful in testosterone synthesis. Zinc deficiency was associated with hypogonadism and dysplasia of secondary sex characteristics in humans.¹¹ In this study, the serum testosterone concentrations and subsequent secondary sex characteristic development (e.g., penis length, testicular volume, pubic hair stage, and genital tanner stage) were markedly improved in both groups. However, no statistical differences were observed between Groups A and B.

The sequential use of uFSH plus zinc supplementation had a trend for an earlier induction of spermatogenesis. However, neither the median time to induce spermatogenesis nor the median time of achieving the primary outcome showed a statistical difference

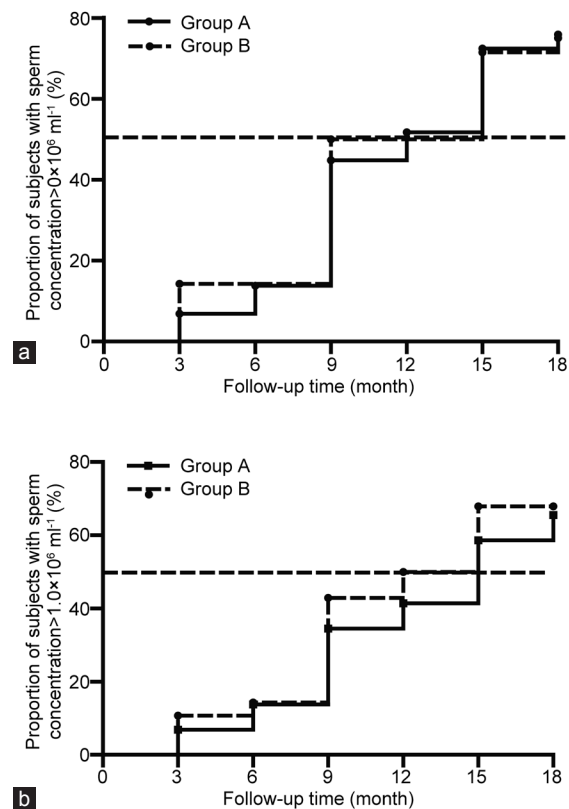


Figure 5: Kaplan–Meier survival analysis for the median time of spermatogenesis (a) and achievement of sperm concentration $\geq 1.0 \times 10^6 \text{ ml}^{-1}$. (b) The median time of spermatogenesis was 12 months and achievement of the primary outcome 15 months in the sequential uFSH group. The median time of spermatogenesis was 9 months and achievement of the primary outcome was 12 months in the zinc supplementation group. There were no statistical differences between these two groups in the median time of spermatogenesis and achievement of the primary outcome. uFSH: urinary follicular-stimulating hormone.

between the two groups after therapy was initiated. The impregnate condition was recorded in 14.7% (5/34) patients in the sequential uFSH treatment group and 12.1% (4/33) patients in the sequential uFSH plus zinc supplementation group 6 months after the end of the trial. Most patients (80%) in our study were not yet married during this study.

One factor to take into consideration could be the absence of zinc deficiency in the patients in these two groups; serum zinc concentrations at baseline or simple dietary surveys did not suggest that they had decreased zinc levels. Although the patients in Group B took additional zinc supplements, there was no statistical difference in serum zinc concentrations between the groups, which also had been observed in previous studies.^{25,26} The lower limit of normal fasting serum zinc has been set as 0.7–0.75 mg l⁻¹.²⁴ In this study, the serum zinc concentrations in Groups A and B were above the normal lower limit. The adult human body contains 1–3 g of zinc, and only about 0.1% of it is replenished daily in agreement with zinc's biological half-life of about 280 days.^{27,28} The body can regulate zinc excretion and absorption to maintain zinc homeostasis. Additional zinc intake was not associated with improved semen quality in healthy men.²⁹ Therefore, it is possible that zinc in the IHH patients in Group B may have been promptly excreted rather than accumulated in the body.

Another factor to take into consideration that might account for the lack of differences may be that the doses used were inadequate. To avoid toxicity or gastrointestinal side effects, we followed the Nordic Nutrition Recommendations, which sets the upper limit of selective zinc intake at 45 mg day⁻¹, and a dose of <45 mg was a commonly used dose.³⁰ However, in a previous study, infertile men were given a much higher dose of 500 mg day⁻¹ of zinc sulfate (201 mg day⁻¹ of zinc) alone, and a marked improvement was observed in sperm count and progressive motility.³¹ It is possible that the dosage we used was too low to induce detectable effects; a higher safe dose can be attempted in the future study.

Moreover, a possible caveat should be noted that records of zinc administration were kept by self-report. Each patient in Group B received a card to record the administration of zinc. More than half of the patients in Group B complained about the bad taste of the zinc preparation. While records showed all of the IHH patients had taken more than 90% of the zinc gluconate, there is a possibility that some patients did not disclose that they missed or skipped a significant number of zinc doses. Different preparations and patterns of zinc administration that are easily accepted by patients should be studied in the future.

We overestimated the efficacy of zinc supplementation in the trial design by choosing a superiority margin of 25%, according to the efficacy range (46%–89%) reported in previous studies.^{18–20} However, *post hoc* analysis showed that the improvement in the primary outcome was only 4.7% (55.9% vs 60.6%) for ITT and 2.4% (65.5% vs 67.9%) for PP analysis. A *post hoc* power analysis shows that 3456 patients are required to detect existing differences (4.7% for ITT) at 80% power. IHH is such a rare disease (1/8000–10 000 in males) that this large sample size is not able to be achieved. Thus, this is a preliminary study to explore the effectiveness of oral zinc supplementation on spermatogenesis in male IHH patients and provides some evidence for clinicians who are concerned with this issue.

CONCLUSION

Our results indicate that sequential use of uFSH/hCG plus zinc supplementation regimen has a similar efficacy to the sequential use of uFSH/hCG alone. The additional improvement of 40 mg day⁻¹ of oral zinc supplementation on spermatogenesis and masculinization in male IHH patients is very subtle.

AUTHOR CONTRIBUTIONS

DLZ and XYL designed the trial; YLL, MNZ, GYT, SYS, YHZ, YC, JZ, HH, BN, HL, QHG, and YG recruited and followed up the patients, collected the data; YLL and MNZ performed and interpreted the data analysis, prepared the figures, and drafted the manuscript; DLZ and XYL revised the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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Supplementary information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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Supplementary Table 1: The secondary outcomes before and after therapy

Items	Group A (n=34)		Group B (n=33)		P
	Pretherapy (0 month)	After-therapy (18 months)	Pretherapy (0 month)	After-therapy (18 months)	
Sperm concentration ($\times 10^6$ ml ⁻¹)	0	1.5 (0–24.3)*	0	2.5 (0–18.1)*	0.91
Sperm motility (a + b + c) (%)	0	44.7 (0–61.1)*	0	36.1 (0–63.3)*	0.79
Testicular volume (ml)	1.5 (1.0–3.0)	5.3 (3.2–7.0)*	1.4 (1.0–2.3)	4.2 (3.2–5.3)*	0.54
Serum testosterone (nmol l ⁻¹)	1.4 (1.0–1.7)	14.6 (12.1–23.9)*	1.4 (0.9–1.8)	13.7 (9.3–16.4)*	0.28
Serum zinc (mg l ⁻¹)	1.2 (1.1–1.5)	1.2 (1.1–1.4)	1.3 (1.1–1.6)	1.2 (0.9–1.4)	0.67
Penis length (cm)	4.3 \pm 1.0	6.9 \pm 1.3*	4.6 \pm 1.2	6.9 \pm 1.7*	0.88
Pubic hair stage	2 (2–2)	5 (4–5)*	2 (2–3)	5 (4–5)*	0.86
Genital Tanner stage	2 (1–2)	4 (4–5)*	2 (1–2)	4 (4–5)*	0.60

Data are presented as mean \pm s.d. or median (inter quartile range). *There are significant differences between before and after therapy in the Group A and Group B ($P < 0.001$); P: the difference between the two groups after the therapy. s.d.: standard deviation