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Original Article

Preliminary Report on the Safety of a New Herbal Formula and Its Effect on Sperm Quality

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Purpose: Male infertility is a serious problem, and its prevalence has been increasing. Therefore, we investigated the safety of a new herbal formula and its effects on sperm quality.

Materials and Methods: An *in vitro* cytotoxicity test in TM3 Leydig cells was performed to evaluate cell viability after administration of five types of herbs separately and of a new herbal formula containing these five. An *in vivo* test in male mice was performed to evaluate the influence of the new herbal formula on the reproductive organs and sperm quality. After the 8- and 28-day oral administration of the new herbal formula, the weights of the reproductive organs were measured and the sperm count and motility were evaluated.

Results: In the *in vitro* cytotoxicity test, less than 80% cell viability at concentrations of 500 mg/L and 1,000 mg/L of *Rubus coreanus* Miquel and *Cuscuta chinensis* Lam was observed. However, more than 80% cell viability was observed at all the tested concentrations of the new herbal formula. After the 8- and 28-day oral administration, there were no considerable changes in body weight. The weights of the testes, epididymis, and seminal vesicles after the 8- and 28-day oral administration were similar to those of the control. The sperm count and activity were significantly improved compared with those of the control group at 8 and 28 days after 100, 200, and 400 mg of oral administration.

Conclusions: The safety of the new formula and its positive effect on the sperm quality were observed after the oral administration of the formula.

Key Words: Infertility, male; Phytotherapy; Safety; Spermatozoa

INTRODUCTION

An investigation reported that approximately 15% of sexually active couples suffer from infertility and that the prevalence of infertility is increasing [1]. Further, 20% of infertility was related to only male factors and 30% to 40%

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of the couples suffered from infertility because of both male and female factors [2,3]. Approximately 42.6% of male infertility was caused by known etiologies such as varicocele, maldescended testes, or hypogonadism, and the cause of male infertility was idiopathic in 30% to 45% of the cases [4]. A considerable number of infertile and subfertile men showed abnormal semen parameters without specific etiologies. The male infertility of idiopathic causes may be associated with genetic or environmental factors; however, there were a few identified causes of these risk factors for inducing idiopathic male infertility. Moreover, there was no satisfactory management of the male infertility of idiopathic causes.

Oxidative stress is regarded as one of the underlying mechanisms of idiopathic male infertility. Reactive oxygen species (ROS) cause infertility by decreasing sperm motility to break the sperm membrane and directly damage the sperm DNA [5,6]. Antioxidant supplementation is a type of management of oxidative stress-related infertility. Previously, there were studies reporting the improvement of sperm morphology and motility after oral antioxidant administration such as carnitine, selenium, and astaxanthin [7-9]. Moreover, these studies demonstrated that the semen quality might be increased after reducing ROS by the scavenging ability of an oral antioxidant agent.

Several herbs that were used in traditional medicine demonstrated antioxidant or free radical scavenging activities [10,11]. Further, some of the herbal supplements with antioxidant properties exhibited a beneficial effect on the sperm function. *Cornus officinalis* Sieb. Et Zucc, *Schizandra chinensis* Baillon, *Rubus coreanus* Miquel, *Cuscuta chinensis* Lam, and *Lycium chinense* Mill are herbs that have been used popularly in Korean oriental medicine, and each of them showed antioxidant activities in various diseases except male infertility causing decreased sperm quality [12-17]. Therefore, a study on the effects of these five types of herbs on sperm quality is required. Traditionally, Korean oriental medicine has used a mixture of herbs because the mixture has sometimes exhibited an enhanced therapeutic effect compared to the use of a single herb or extract. However, the exact mechanism was not identified.

Thus, we performed a preliminary study on the toxicity of each herb (*Cornus officinalis* Sieb. Et Zucc, *Schizandra chinensis* Baillon, *Rubus coreanus* Miquel, *Cuscuta chinensis* Lam, and *Lycium chinense* Mill) and an herbal compound made with these herbs. Further, we evaluated the effect of this herbal compound on normal reproductive organ and sperm function.

MATERIALS AND METHODS

1. Preparation of herbal formula (KH-204)

The main ingredients of the herbal formula, which were considered in this study, were obtained from five plants: 32% *Cornus officinalis* Sieb. Et Zucc, 4% *Schizandra chinensis* Baillon, 16% *Rubus coreanus* Miquel, 16% *Cuscuta chinensis* Lam, and 32% *Lycium chinense* Mill.

2. *In vitro* cytotoxicity test in TM3 mouse Leydig cells

1) Cell culture

TM3 mouse Leydig cells were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich Co. LLC.) at 37°C in 5% CO_2, 95% O_2 in a humidified cell incubator, and the medium was changed every two days.

2) MTT cytotoxicity assay

Cell viability was determined using the MTT assay kit according to the manufacturer's protocol. We evaluated the cytotoxicity of each of the five types of herbs (*Cornus officinalis* Sieb. Et Zucc, *Schizandra chinensis* Baillon, *Rubus coreanus* Miquel, *Cuscuta chinensis* Lam, and *Lycium chinense* Mill) and of the new herbal formula that contained these five types of herbs. In order to determine the cytotoxicity of each of these five herbs and the new herbal formula, the cells were treated with the five herbs separately and the new herbal formula at concentrations of 62.5 mg/L, 125 mg/L, 250 mg/L, 500 mg/L, and 1,000 mg/L for 24 hours. Cultures of the control group were treated with phosphate buffered saline. Twenty micriliter of the MTT labeling reagent was added to each well, and the plates were incubated for 3 hours. Then, 200 μL of dimethyl sulfoxide was added to each well, and the cells were incubated for another 20 hours. The absorbance was then measured with a microtiter plate reader (VersaMax;

Molecular Device, Sunnyvale, CA, USA) at a test wavelength of 570 nm. Optical density (O.D.) was calculated as the difference between the absorbance at the wavelength of the control group and that at the test wavelength. The percentage viability was calculated as (O.D. of treated sample/control O.D.) ×100. A percentage viability of more than 80% was considered to denote very high viability [18-20].

3. *In vivo* evaluation of the effect of the new herbal formula on the reproductive organs in male mice

1) Animals

Thirty 8-week-old male imprinting control region mice were purchased from Orientbio Inc. (Seongnam, Korea). The

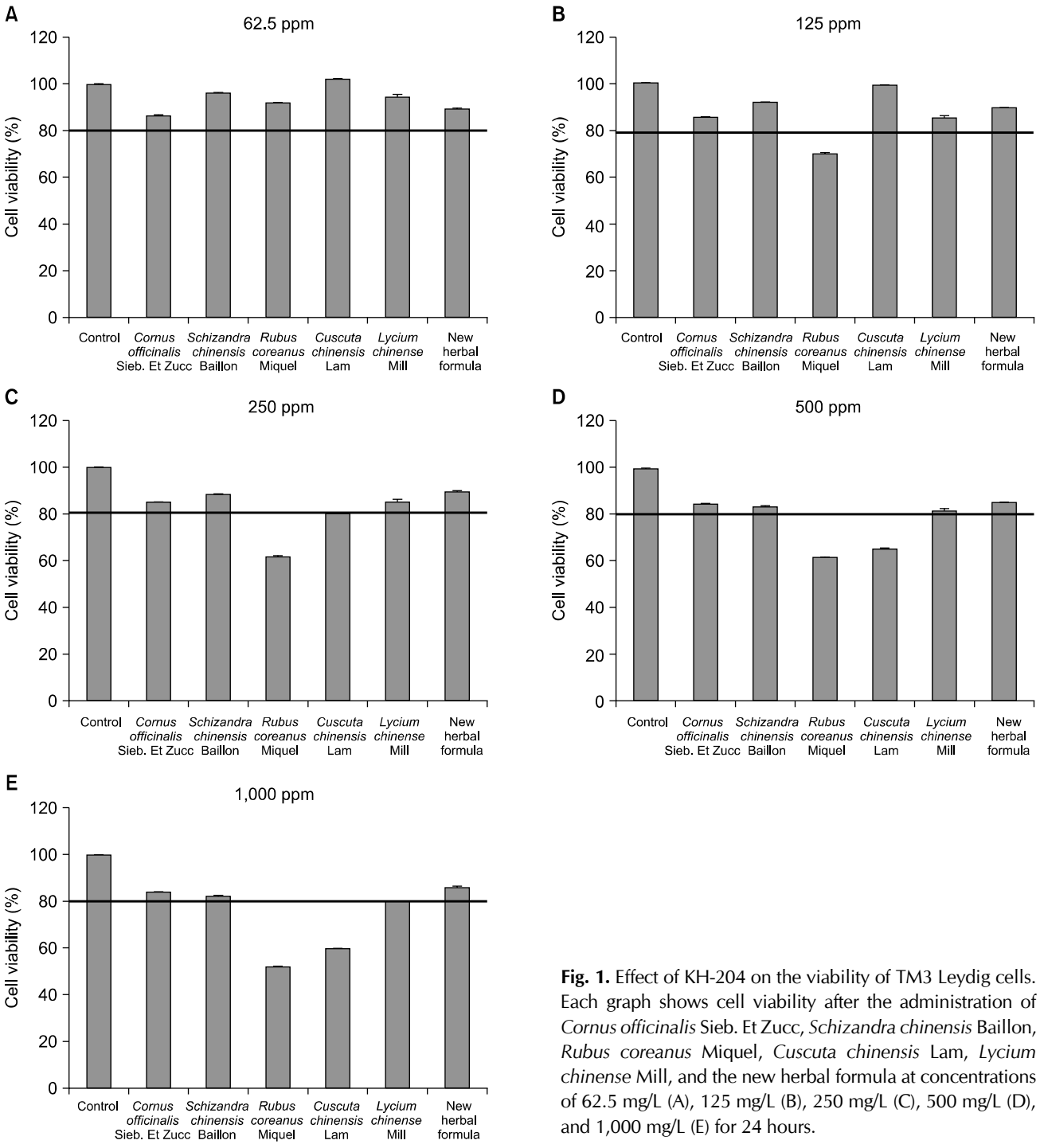

Fig. 1. Effect of KH-204 on the viability of TM3 Leydig cells. Each graph shows cell viability after the administration of *Cornus officinalis* Sieb. Et Zucc, *Schizandra chinensis* Baillon, *Rubus coreanus* Miquel, *Cuscuta chinensis* Lam, *Lycium chinense* Mill, and the new herbal formula at concentrations of 62.5 mg/L (A), 125 mg/L (B), 250 mg/L (C), 500 mg/L (D), and 1,000 mg/L (E) for 24 hours.

mice were divided into five groups: the control group (oral administration with sterile water, n=6), 50 mg/kg/d KH-204 oral administration group (n=6), 100 mg/kg/d KH-204 oral administration group (n=6), 200 mg/kg/d KH-204 oral administration group (n=6), and 400 mg/kg/d KH-204 oral administration group (n=6). After 8-day and 28-day oral administration with KH-204, the mice from all the groups were sacrificed and their testes, epididymides, and seminal vesicles were excised. The experimental protocol was approved by the Catholic University Animal Ethics Committee (CUMC-2013-0030-01).

2) Evaluation of sperm count and activity in the control and oral administration with the new herbal formula groups

Cauda epididymal sperm were collected from the control group and the group that was orally administered the new herbal formula. Each sperm sample was analyzed in duplicate for sperm count and activity. The sperm count represents the number of sperm in 1 mL of the medium. Sperm motility is expressed as the percentage of sperm that showed any movement (×200 magnification).

4. Data analysis

The data were expressed as the mean±standard deviation. The data of each group were compared and analyzed statistically by using the Wilcoxon signed rank test. The significance was set at $p<0.05$.

RESULTS

1. Effect of the new herbal formula on viability of TM3 Leydig cells

The viability of cells incubated with each of the five herbs and with the new herbal formula at concentrations of 62.5 mg/L, 125 mg/L, 250 mg/L, 500 mg/L, and 1,000 mg/L for 24 hours is shown in Fig. 1. Cell viability was more than 80% after the administration of the five types of herbs and the new herbal formula at the concentration of 62.5 mg/L. At the concentrations of 125 and 250 mg/L, the cell viability in the case of *Rubus coreanus* Miquel (69.9% and 61.9%, respectively) was less than 80%. The cell viability in the case of the other four herbs and the new herbal formula was more than 80%. At the concentrations of 500 and 1,000 mg/L, the cell viability in the case of *Rubus coreanus* Miquel (62.1% and 52.2%, respectively) and *Cuscuta chinensis* Lam (65.6% and 59.9%, respectively) was less than 80%. The cell viability in the case of the other three types of herbs and the new herbal formula was more than 80%.

Table 1. The weights of reproductive organs after 8-day oral administration with the new herbal formula

Variable	Control	New herbal formula (50 mg/kg)	New herbal formula (100 mg/kg)	New herbal formula (200 mg/kg)	New herbal formula (400 mg/kg)
Body weight (g)	37.0±1.7	36.5±1.1[a]	36.7±1.1[a]	34.6±1.9[a]	29.3±1.1[a]
Testis (mg)	110±10	110±20[a]	110±10[a]	130±10[a]	120±10[a]
Epididymis (mg)	40±0	41±2[a]	41±4[a]	41±2[a]	37±2[a]
Seminal vesicle (mg)	80±20	80±10[a]	80±20[a]	90±20[a]	90±10[a]

Values are presented as mean±standard deviation. [a]$p>0.05$ compared with the control group.

Table 2. The weights of reproductive organs after 28-day oral administration with the new herbal formula

Variable	Control	New herbal formula (50 mg/kg)	New herbal formula (100 mg/kg)	New herbal formula (200 mg/kg)	New herbal formula (400 mg/kg)
Body weight (g)	37.0±1.5	36.6±0.9[a]	39.3±1.7[a]	37.6±1.3[a]	33.7±1.2[a]
Testis (mg)	110±10	120±10[a]	160±10[a]	120±10[a]	120±20[a]
Epididymis (mg)	40±10	40±10[a]	40±10[a]	50±10[a]	40±10[a]
Seminal vesicle (mg)	120±30	120±30[a]	160±120[a]	150±20[a]	120±20[a]

Values are presented as mean±standard deviation. [a]$p>0.05$ compared with the control group.

2. Body weights and reproductive organ weights after oral administration with the new herbal formula

Body weights were similar to those of the control group except in the case of mice that were administered with 400 mg of the new herbal formula after the 8- and 28-day oral administration. After the 8-day oral administration with 400 mg of the new herbal formula, a minimal decrease in the body weight was observed; however, the difference was not statistically significant. The body weights were similar in all of the groups after the 28-day oral administration with the new herbal formula. In addition, the weights of the testis, epididymis, and seminal vesicles did not differ depending on the administration dose per day after the 8- and 28-day oral administration with the new herbal formula (Table 1, 2).

3. Effect of the new herbal formula on sperm count and activity

The sperm count was significantly increased compared with the control group after the oral administration of 100, 200, and 400 mg of the new herbal formula ($p<0.05$) (Fig. 2, 3). Moreover, significantly increased sperm motility was observed compared with the control group after the oral administration of 50, 100, 200, and 400 mg of the new herbal formula ($p<0.05$) (Fig. 2).

DISCUSSION

In the *in vitro* cytotoxicity test conducted as part of this study, we observed *Rubus coreanus* Miquel to be toxic at the concentration of 125 mg/L. Further, both *Rubus coreanus* Miquel and *Cuscuta chinensis* Lam were observed

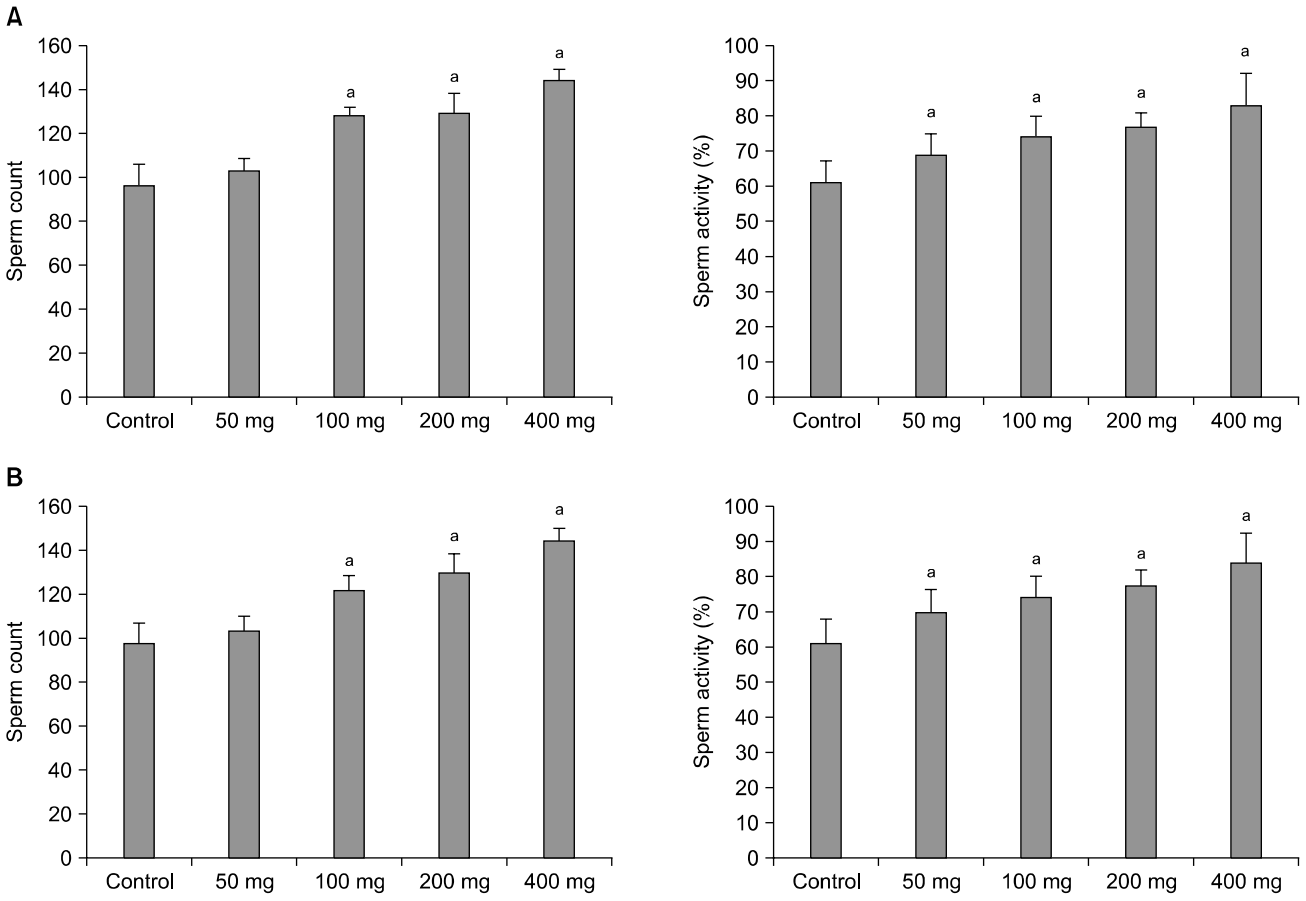

Fig. 2. Effect of the new herbal formula on sperm count and activity. (A) Sperm count and activity after 8-day oral administration of the new herbal formula. (B) Sperm count and activity after 28-day oral administration of the new herbal formula. [a]$p<0.05$ compared with that of the control group.

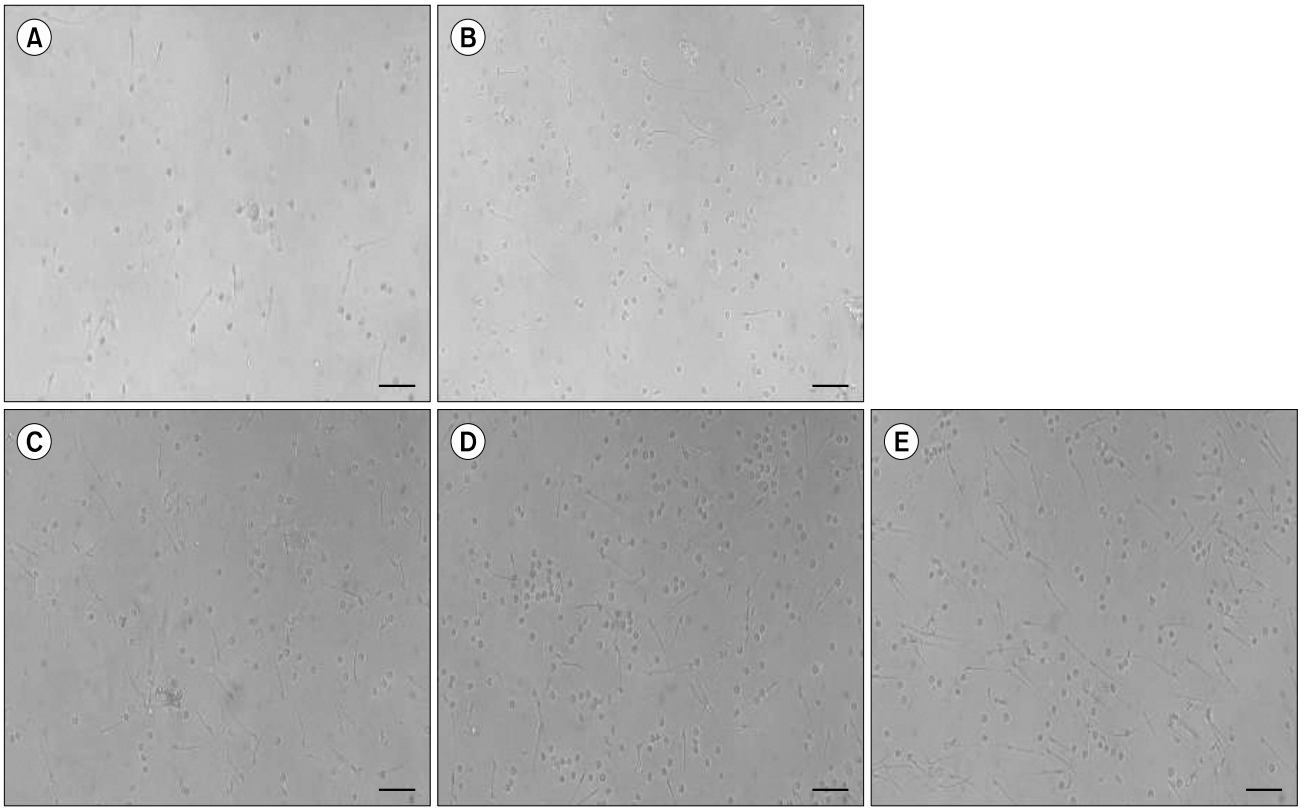

Fig. 3. Appearance of sperm after 28-day oral administration with KH-204. (A) Control, (B) KH-204 50 mg, (C) KH-204 100 mg, (D) KH-204 200 mg, (E) KH-204 400 mg (×200 magnification).

to be toxic at the concentrations of 500 and 1,000 mg/L. However, interestingly, we noted more than 80% cell viability of the new herbal formula at concentrations of 500 and 1,000 mg/L. Moreover, the new herbal formula exhibited more than 80% cell viability at every concentration considered. From these results, we assumed that the toxicity of the new herbal formula might be less than that of the three types of herbs (*Rubus coreanus* Miquel, *Cuscuta chinensis* and *Cuscuta chinensis* Lam) that exhibited toxicity at the concentrations of 500 and 1,000 mg/L. Further, the sperm account and motility were significantly improved compared with those of the control group on the 8th and the 28th day after the oral administration of 100, 200, and 400 mg of the new herbal formula. As a result, the new herbal formula seems to be a safe herbal compound and has a positive effect on sperm quality.

Herbs have been used widely in various areas associated with human health, and nowadays, there are numerous attempts to apply herbs to medical use. In line with this trend, several studies have been performed to demonstrate the efficacy of herbs in the treatment of and identify the underlying mechanism of urologic diseases such as erectile dysfunction (ED), infertility, and benign prostate hyperplasia (BPH) [21]. In particular, from the perspective of male infertility, several oral supplements with antioxidant properties have been proposed to improve sperm quality; however, more evidence is necessary before herbs can be used in real clinical practice. Further, infertile couples are apt to try using complementary and alternative medicine including herbs, even though there is no reliable evidence [22]. Thus, our data regarding the effect of the new herbal formula on reproductive organs and sperm quality may provide relevant evidence for the use of the new herbal formula for treating male infertility.

In general, people believe that herbs are harmless plants because they are natural products. There is no doubt that a majority of herbal preparations are safe; however, some of them may show side effects because of the increasing use of herbs in modern society. Further, nowadays, the ad-

verse effects of herbs may also be attributed to the fact that people sometimes use a considerably higher dose of herbs than that used historically in order to show the healing effect of the herbs on a certain disease [23,24]. Therefore, the safety aspects of the use of a certain herb should be investigated thoroughly before considering its medical use. We have evaluated the cell viability of each of five herbs and a new herbal formula containing those five combined according to the remedy prescribed by Korean oriental medicine. In the present study, each of these five herbs and the new herbal formula exhibited more than 80% cell viability at a relatively low concentration. At a higher concentration (500 and 1,000 mg/L), *Rubus coreanus* Miquel and *Cuscuta chinensis* Lam showed less than 80% cell viability. On the basis of these results, we concluded that a high dose of herbs might have a negative effect on the human body. However, the new herbal formula exhibited more than 80% cell viability at every concentration considered. Thus, we concluded that the new herbal formula is a safe herbal compound.

After oral administration of the new herbal formula, the sperm count and motility were improved compared with those of the control group; moreover, the improvement of sperm count and motility showed significance at the higher dose of the new herbal formula. Each of the five types of herbs, which are components of the new herbal formula, exhibits antioxidant properties as mentioned earlier [12-17]. We assumed that the each of five herbs may serve as an antioxidant in the new herbal formula. The balance between ROS and an antioxidant is an important factor that affects sperm quality. During the fertilization process, a low level of free radicals helps sperm transit through the zona pellucida of the ovum by stimulating the acrosome reaction and sperm hyperactivation [25]. However, excessive oxidative stress contributes to a deterioration of sperm quality by breaking the balance between ROS and the antioxidant in men with infertility. Increased ROS production leads to the peroxidation of the sperm acrosomal membrane and decreased acrosin activity; therefore, the fertilization process cannot be completed [26-28]. Further, ROS directly damages the sperm DNA by attacking the purine and pyrimidine bases and the deoxyribose backbone or by initiating apoptosis within the sperm, which induces the caspase-mediated enzymatic degradation of DNA [29]. Therefore, antioxidant administration may help to restore the imbalance of an excessive level of ROS and improve sperm quality.

Although we demonstrated the safety of the new herbal formula and its positive effect on sperm quality, there are several issues that are yet to be solved. We did not evaluate the antioxidant properties of the new herbal formula in this study. Thus, a further study to elucidate the antioxidant mechanism of this formula should be conducted. Further, we investigated the effect of the new herbal formula on sperm quality in normal mice; therefore, the action of the new herbal formula as an antioxidant needs to be evaluated using infertile animal models. Furthermore, studies to find which extracts of the five herbal components contribute to the antioxidant properties of the new formula and how the mixture of each extract demonstrates a consistent effect need to be conducted for determining the medical applications of this new herbal formula.

CONCLUSIONS

To summarize, we demonstrated the preliminary results about the safety of the new herbal formula and its positive effect on sperm quality after the oral administration of this formula. These results may form a fundamental basis for studies on the antioxidant mechanism of the new herbal formula and the formula's antioxidant efficacy in the case of infertile animal models. Further studies are essential for determining whether the new herbal formula can be used as an oral antioxidant in men suffering from infertility.

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