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Original Article

Effects of micronutrients on the reproduction of infertility rat model induced by adenine

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Abstract: Male infertility is a serious global medical and social issue demanding more specific and effective treatments. In this study, we generated a male infertility rat model using adenine induction to study the effects of certain micronutrients on reproduction. Fifty male SD rats were used in the study, and thirty of them received daily intragastric administration of 300 mg/kg adenine for four weeks. The thirty adenine treated mice were evenly divided into 3 groups to receive intra-gastric administration of micronutrient mixture of vitamin A, vitamin C, vitamin E, Zinc, and selenium (micronutrient group), normal saline (model control group), and methyl testosterone solution (androgen group). The other twenty rats used were normal male SD rats that were evenly divided into two groups to receive intra-gastric administration of normal saline (normal control group) and micronutrient mixture first then adenine 40 min later (micronutrient prevention group). After four weeks of micronutrient and other treatments, all rats were sacrificed for analyses. Compared with those in the model control group, the rats in the micronutrient group showed significantly improved physical signs, significantly increased body weights, significantly increased testis index, significantly increased sperm counts and motility, significantly decreased sperm malformation, and significantly repaired testis tissue. Compared with those in the model control group, the rats in the micronutrient group showed significantly decreased FSH levels and recovered LH and testosterone levels. The rats in the micronutrient prevention group did not show significant differences in sperm counts, sperm motility, sperm malformation, and hormonal levels from those in the normal control group. The findings from this study provide evidence for the potential application of micronutrients in male infertility treatments.

Keywords: Micronutrients, infertile rats, reproduction, adenine

Introduction

Infertility is a serious global medical and social issue. According to The World Health Organization, about 8% of couples of reproductive age suffer from infertility, about 20% of which are caused by male factors. The incidence of male infertility is 10-25% in developed countries but up to 30% in developing countries. The cases of male infertility also seem to be gradually increasing [1].

Male infertility may be caused by disorders in sperm production, epididymal maturation, sperm transport and accessory sex gland function. However, sperm abnormalities are considered the main reason of male infertility as sperm production and maturation are critical indicators of male reproductive ability. Spermatogenic oligozoospermia and asthenospermia because of testis sperm production disorders are the top causes of sperm abnormalities. Oligospermia refers to the condition in which lower than 20 million/mL density or smaller than 1 mL semen volume was determined by multiple semen tests. Asthenospermia is diagnosed when sperm motility is < 40% and sperm forward motility is < grade 4.

Unfortunately, specific and effective methods are still lacking to treat oligospermia and asthenozoospermia. One of the most important topics in male infertility research is how to improve spermatogenesis. In recent years, many micronutrients, especially vitamin A, vitamin C, vitamin E, zinc, and selenium, have been reported to be associated with spermatogenesis and androgen synthesis and secretion [2-5]. However, studies on the synergy among these micronutrients are still very limited. Therefore, in

this study, we applied adenine to develop rat infertility model, which was used to determine the effects of the micronutrient mixture of vitamin A, vitamin C, vitamin E, zinc, and selenium on male reproduction by observing the changes in spermatogenesis of the damaged rat testis. The findings from this study provide evidence for the potential application of micronutrients in male infertility treatments.

Materials

Animals

The experimental animals were 3-month-old male SD rats with 300 ± 10 g of weights, provided by the Experimental Animal Center of Zhejiang Province (license number: SCXK-Zhejiang-2003-0001). The rat food was also provided by The Experimental Animal Center of Zhejiang Province, and the rearing conditions met the requirements of the No. 2001001 Zhejiang Facility standard.

Main instruments and reagents

Electronic scale (division value 0.5 g) and electronic analytical balance (model FA1104N) (division value 0.1 mg) (Shanghai Precision & Scientific Instrument Co., Ltd.); Constant temperature water bath (Shanghai Yuejin Medical Instrument); Ultra-speed freezing centrifuge (BiofugeStratos, Heraeus, Germany); Optical Microscope model CH20BIMF200 (Nissan Olympus).

Zinc sulfate ($ZnSO_4 \cdot 7H_2O$) (Hangzhou Connaught Food Limited); Vitamin C (Zhejiang RuiXin Pharmaceutical Co., Ltd.); Vitamin A (Xiamen Fish Oil Factory); Vitamin E (Zhejiang Medicine Co., Ltd.); Selenite (1% Na_2SeO_3) (Hangzhou Connaught Food Limited); Methyl testosterone (Shanghai Hualian Pharmaceutical Co., Ltd); Adenine (Shanghai Bo'ao Biotech Limited); Serum FSH, LH, and T radioimmunoassay kit (Hangzhou Aidikang Medical Laboratory Center).

Reagents preparation

The required doses for rats were determined based on the conversion ratio of 0.018 (rat, 200 g to human, 70 kg) [6] and the human micronutrient intake UL values: vitamin A, 0.27 μg/g; vitamin E, 0.072 mg/g; vitamin C: 0.09 mg/g; $ZnSO_4 \cdot 7H_2O$, 0.02 mg/g; 1% Na_2SeO_3, 5.78 μg/g; and methyl testosterone, 0.014 mg/g. The required dose of adenine was 300 mg/kg.

0.432 g vitamin E and 1.62 mg vitamin A were mixed completely with 2 drops of Tween -80 and a small amount of distilled water, before being evenly mixed with 0.54 g vitamin C, 0.12 mg $ZnSO_4 \cdot 7H_2O$, 34.68 mg Na_2SeO_3, and distilled water in a total volume of 30 mL mixed with distilled water volume to 30 mL. The mixture was placed at 4°C for further experiments.

Three grams adenine was dissolved completely in 100 mL normal saline and placed at 4°C for further experiments.

42 mg methyl testosterone was milled and fully dissolved in 30 mL, and placed at 4°C for further experiments.

Methods

Infertility animal model development and treatments

Forty 3-month-old SD rats were randomly divided into two groups based on weights, with 10 in the normal control group and 30 in the infertility model group. The model group rats received daily intra-gastric administration of adenine at 300 mg/kg in a volume of 0.5 mL/100 g for 30 days, while the normal control rats were given normal saline. All rats had full access to regular foods and water and weighed once weekly. Changes in physical symptoms were monitored, and the levels of serum testosterone, FSH, and LH were measured to determine the success of model development.

Fifty rats were used in this study, with 10 rats included in one of the following five groups: normal control group, model control group, micronutrient group, androgen group, and micronutrient prevention group.

The 30 infertility model rats were randomly divided into 3 groups with 10 rats in each one, which were the model control group receiving daily intra-gastric administration of normal saline, the micronutrient group receiving daily intra-gastric administration of micronutrient mixture, and the androgen group receiving daily intra-gastric administration of methyl testosterone solution. All administration volumes were 0.5 mL/100 g.

Twenty more normal male SD rats were randomly divided into two groups with 10 rats in each one, which were the normal control group receiving daily intra-gastric administration of normal saline, and the micronutrient preven-

Table 1. Body weights of rats in the normal control group and the infertility model group ($\bar{x} \pm S$)

Group	n	Pre-treatment (g)	4-week later (g)
Infertility model	30	338 ± 10	275 ± 27**
Normal control	10	359 ± 21	417 ± 18

Note: **$P < 0.01$, compared with the normal control group; n, number of rats.

tion group receiving daily intra-gastric administration of micronutrient mixture first then adenine 40 min later.

All rats had free access to regular foods and water and were weighed weekly for 4 weeks. Then the rats were sacrificed for the measurements of serum testosterone, FSH, and LH, along with testis index, epididymis index, sperm count, sperm motility, and sperm malformation rate, and pathological morphology.

Observation criteria and methods

Rat general activity, eating, drinking, and urination were monitored, along with weekly measurements of body weights.

The testis and epididymis on the same side were dissected, washed with cold saline rinse, dried with filter paper, and weighed for the calculation of testis and epididymis coefficients. The testis and epididymis coefficients were determined with the formula: organ coefficient = (organ weight/body weight) * 100, and the procedure:

Sperm suspension preparation and observation

One epididymis from each rat was weighed and cut into pieces in saline at 37°C and filtered with 4-layer paper filters. One out of 5 mL of the mixture was further mixed with 9 mL saline at 37°C to obtain the final sperm suspension solution.

Sperm counts: 20 μL sperm suspension was slowly added to the CBC board and let stand for 30 s, and the sperms were counted as R following the red blood cell counting method. The sperm number was calculated with the formula, sperm count/L = R * 50 * dilution factor * 10^6.

Sperm motility and viability: the motility of 200 sperms from 20 μL sperm suspension were observed under microscope and categorized into 4 grades according to the WHO standards: grade 0, no activity and no forward movement; grade I, bad activity and faint forward movement; grade II, general activity and moderate forward movement; and grade III, good motility and active forward movement.

Normal sperm activity: 50% or more sperms with forward movements and 25% or more sperms with fast forward movements.

The sperm motility rate is the ratio of active sperm count (total number of grades I, II, and III sperms) to the total sperm count (total number sperms at all 4 grades).

Sperm deformity rate: sperm smear was prepared from the sperm suspension, dried, fixed with methanol, and stained with 2% aqueous solution of eosin for 1-2 hours. The morphology of 100 sperms was observed for each rat for the calculation of sperm deformity rate. Normal sperms have oval heads with lengths at 4.0-5.5 μm and widths at 2.5-3.0 μm, and the acrosomes account for 40-70% of the heads; no any defects should be found in the neck, middle, or tail regions. The sperm abnormalities mainly occur as defects in head shapes and sizes, which include without hooks, large heads, small heads, conical heads, banana shapes, pear heads, and double heads. Defects in the neck and middle regions include missing tails and dissociated heads, and defects in the tail region include short tails, multiple tails, and broken tails.

Testicular histological examination

One testicle from each rat was dissected and immediately fixed with 10% neutral formalin solution for 24 h, dehydrated in gradient ethanol, embedded in paraffin, sectioned at 5 gym with 5-10 intermittent uniform slices, and stained with hematoxylin-eosin (HE) for the morphological observation of seminiferous tubules.

Detection of serum hormonal levels

Serum levels of sex hormones are important indicators to evaluate spermatogenesis and testicular tissue structures. Among them, the levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone are important indicators for male infertility diagnosis.

Ten mL tail blood was taken from each rat and centrifuged at 4,000 rpm for 10 minutes after coagulation to separate the serum and store it

Table 2. Hormone levels of rats in the normal control group and the infertility model group ($\bar{x} \pm S$)

Group	n	T (ng/mL)	FSH (mIU/mL)	LH (mIU/mL)
Infertility model	30	1.02 ± 0.24**	3.29 ± 0.52**	0.98 ± 0.33**
Normal control	10	2.64 ± 0.43	1.85 ± 0.23	1.38 ± 0.27

Note: **P < 0.01, compared with the normal control group; n, number of rats.

Table 3. Comparison of weight changes of rats in different groups ($\bar{x} \pm S$)

Group	n	Week 1	4 weeks later
Normal control	10	332 ± 16	420 ± 17
Model control	10	269 ± 27	272 ± 31
Micronutrient	10	287 ± 41*	396 ± 26★
Androgen	10	275 ± 36*	377 ± 19★
Micronutrient prevention	10	341 ± 23	400 ± 41★

Notes: *P < 0.05 vs normal control group; ★P < 0.05 vs model control group.

at 4°C. Radioimmunoassay (RIA) was used to measure FSH, LH, and testosterone levels.

Statistical analysis

The SPSS11.0 statistical software was used for the statistical analysis. The measurement data were analyzed with normal distribution test and homogeneity of variance test. Differences with P < 0.05 values were considered statistically significant, and all data are presented as $\bar{x} \pm S$.

Results

Infertility rat model development

The rats in the model group received 4 weeks of daily intra-gastric administration of 300 mg/kg adenine. From day 5, the rats became apathetic and tired of movements, along with dim eyes, dry and messy hair, and significantly increased water intake and urination. Their body weights also stopped increasing after 30 days. The rats in the normal control group showed generally good conditions, including quick reaction, bright eyes, vigorous appetites, smooth and dense hair, normal water intake and urination, and normal gradual body weight increase (**Table 1**).

After the 4-week treatment, rat tail blood samples were collected for the measurements of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone to determine whether the model had been successfully created. As shown in **Table 2**, the model group showed significantly higher levels of testosterone, LH, and FSH than the normal control group, indicating the successful creation of the model.

Effects of micronutrients on the reproduction of the infertility model rats

Effects of micronutrients on the general condition of rats: The rats in the micronutrient group started to show increase activity from day 5, along with increase food intake and decreased urination. Their body weights showed apparent increases, significantly higher than those of model control group (P < 0.05). Compared to the rats in the normal control group, the rats in the micronutrient group showed similar body weights 4 weeks later, indicating that micronutrient treatment recovered the body weights (P > 0.05). These results were similar to those of the positive control, the androgen group rats. The rats in the model control group, however, failed to show any improvements in general activity and body weights, demonstrating that the damage caused by adenine did not self-recover, i.e. the modeling effects were stable.

Meanwhile, the rats in the micronutrient prevention group were as active and healthy as those in the normal control group (P > 0.05), indicating that that micronutrients were able to effectively prevent adenine-induced body weight loss (**Table 3**).

Effects of micronutrients on testis and epididymis weights and testis coefficients

No significant differences in testis weights were observed among different experimental groups (P > 0.05). However, significantly higher epididymis weights were observed in the micronutrient group, the androgen group, and the micronutrient prevention group, compared with those in the model control group (P < 0.01 or P < 0.05); the testis coefficients of micronutrient group and androgen group were significantly different from those of the model control group (P < 0.05), indicating that body weight recovery affected the organ coefficients in the micronutrient group and the androgen group.

As shown in **Table 4**, the testis coefficient of the micronutrient group was similar to that of the model control group but significantly different from that of the normal control group (P < 0.05).

Table 4. Rat testis weights, epididymis weights, and testis coefficient of varied groups ($\bar{x} \pm S$)

Group	n	Testis weight (g)	Epididymis weight (g)	Testis coefficient (g/kg)
Normal control	10	3.021 ± 0.165	1.166 ± 0.178	6.80 ± 1.90
Model control	10	2.620 ± 0.784	0.960 ± 1.119	9.16 ± 0.909
Micronutrients	10	2.799 ± 0.602	1.139 ± 0.100**	6.96 ± 1.091*
Androgen	10	2.972 ± 0.418	1.114 ± 0.054*	7.66 ± 1.065*
Micronutrient prevention	10	3.208 ± 0.267	1.119 ± 0.037*	8.02 ± 1.173*

Notes: *$P < 0.05$ and **$P < 0.01$, vs model control group. *$P < 0.05$ vs. normal control group.

Table 5. Rat sperm counts, motility, and malformation rates ($\bar{x} \pm S$)

Group	n	Motility %	Count ($\times 10^9$/L)	Malformation %
Normal control	10	93.13 ± 2.59	20.13 ± 0.99	8.80 ± 3.49
Model control	10	38.13 ± 5.30	11.00 ± 2.45	77.21 ± 8.75
Micronutrients	10	83.13 ± 3.72**	16.63 ± 1.51**	33.2 ± 7.24**,*
Androgen	10	77.28 ± 6.33**	17.21 ± 0.91**	42.2 ± 7.02**,*
Micronutrient prevention	10	76.88 ± 4.58**	17.88 ± 0.99**	18.83 ± 5.62**

Notes: *$P < 0.05$ vs. normal control group; *$P < 0.05$ and **$P < 0.01$, vs. model control group.

Effects of micronutrients on semen quality: Compared with the model group, the micronutrient group and the androgen group showed not only significantly increased sperm counts and motility but also significantly decreased sperm deformity rates ($P < 0.01$). No differences in sperm counts and motility were observed between these two groups and the normal control group. Taken together, these results indicated that adenine treatment successfully induced the generation of spermatogenesis obstruction model, and that micronutrient treatments significantly improved the semen quality. Furthermore, no significant differences in varied indexes were observed between the micronutrient prevention group and the normal control group, indicating that micronutrients effectively prevented the sperm damages caused by adenine (**Table 5**).

Effects of micronutrients on hormone levels: Compared with the model control group, the micronutrient group, the androgen group, and the micronutrients prevention group all showed significant changes in hormone levels ($P < 0.01$), which were recovered testosterone levels, decreased FSH levels, and increased LH levels. However, no differences in varied indexes were observed between the micronutrient prevention group and the normal control group ($P > 0.05$). Furthermore, significant differences in varied indexes were observed between the model control group and the normal control group, indicating that adenine-induced reproduction damages were not self-recovered after 4 weeks (**Table 6**).

Effects of micronutrients on testicular morphology: As shown in **Figure 1A**, the rats in the normal control group had normally developed spermatogenic cells in testis seminiferous tubules that were orderly arranged; the lumen was filled with sperms, and the lumen walls were tidy; the base membrane was thin, and the Leydig cells were well-developed. As a contrast, the model control group rats had some degenerated seminiferous tubules, thinned lumen seminiferous epithelia, and reduced sperm counts. Under low magnification microscope, normal and abnormal seminiferous tubules were observed to be staggered into pieces, showing typical "spot-like" changes. Under high magnification microscope, spermatogenesis was observed to be terminated in spermatocyte stage, along with germ cell loss, atrophy of seminiferous tubules, and wall-layer disarrangements, even collapsed seminiferous tubules, decreased Sertoli cells and spermatogonia, interstitial edema, and interstitial cell vacuolar degeneration in some cases (**Figure 1B**). The micronutrient group and androgen group also showed "spot-like" changes, but with significantly less seminiferous tubule abnormalities. Under high magnification microscope, some seminiferous tubular atrophy was observed, along with relatively clear tubular layers, significantly more spermatogonia and Sertoli cells than the model group, and relatively more sperm cells (**Figure 1C**, **1D**). The micronutrient prevention group showed a small amount of "spot-like" changes under low magnification microscope; under high magnification microscope, some atrophy and rare collapse were observed for seminiferous tubules. Spermatogonia and Sertoli cells were decreased, and some sperms' development

Table 6. Rat hormonal levels in varied group ($\bar{x} \pm S$)

Group	n	T (ng/mL)	FSH (mIU/mL)	LH (mIU/mL)
Normal control	10	2.24 ± 0.51	1.58 ± 0.33	1.38 ± 0.31
Model control	10	1.12 ± 0.28**	2.89 ± 0.57**	0.88 ± 0.28**
Micronutrients	10	3.16 ± 0.67★★	2.12 ± 0.40★★	1.30 ± 0.27★★
Androgen	10	2.36 ± 0.41★★	2.24 ± 0.19★★	1.27 ± 0.11★★
Micronutrient prevention	10	2.08 ± 0.49★★	2.01 ± 0.39★★	1.19 ± 0.29★★

Notes: $^{**}P < 0.05$ vs. normal control group; $^{\star}P < 0.05$ and $^{\star\star}P < 0.01$, vs. model control group.

was terminated in the spermatocyte stage (**Figure 1E**).

Discussion

Infertility animal model development

Four major methods have been developed to generate infertility animal models: (1) the castration method with surgical removal of testis and/or epididymis has high success rate, but its clinical relevance is weak; (2) the estrogen method through injection of excessive amounts of estrogen to male animals produces obstruction of spermatogenesis by inhibiting the production of androgen; (3) the causal model method produces obstruction of spermatogenesis through excessive mating activity; (4) the physicochemical damage method, one of the most used methods, generates direct or indirect spermatogenesis disorders though physical or chemical agents, such as high temperature, X-ray, or adenine [7-12].

We used adenine to generate the infertility rat model in this study. Adenine (6-amino-purine) is a nitrogen-containing heterocyclic purine compound. Large doses of adenine may induce renal failure because of the deposition of urate crystal, a purine metabolite, in renal tubules [13, 14]. Therefore, adenine has been widely used to generate chronic renal failure rat models [15]. Only in recent years, adenine was found to induce reproduction damages in rats. However, the mechanisms of these damages are not clear. Adenine may reduce spermatogenesis and testosterone synthesis through the large amount of free radicals generated by the xanthine oxidase (XOD) reaction; it may also damage the gonads by affecting the testicular blood circulation through the renin-angiotensin-aldosterone system [16] or by high blood pressure caused by renal failure [17]. It has also been reported that the adenine-induced rat testicular dysfunction may be related to TGF-β's inhibition of spermatogenesis and promotion of spermatocyte apoptosis [16]. With more and more related research being reported, the adenine method has become more commonly used to generate infertility animal models.

Mechanism of impaired spermatogenesis

Ischemia can cause extensive abscission of spermatocytes or even damage all spermatocytes, since testicular seminiferous epithelia are extremely sensitive to ischemia. The rats in the model control group displayed: reduced testicular weights, indicating ischemia in testis; reduced quality and quantity of sperms, indicating spermatogenesis disorders; thinned and shrunk seminiferous tubules, disarranged epithelia, disassociation of epithelia and basal cells, and disappearance of spermatocytes, indicating spermatogenesis disorders at the spermatocyte levels; atrophy and proliferation of interstitial cells, indicating reduced androgen production and response to interstitial cell-stimulating hormones.

FSH levels are usually significantly increased in male infertile patients with the absence of sperms or low sperm counts, indicating that the testicular seminiferous tubules epithelia have been damaged [18]. In this study, we observed significantly increased serum FSH levels in the rats of the model control group, indicating that adenine caused severe testicular damages and impaired spermatogenesis. The high FSH levels were maintained even four weeks after the adenine treatments, indicating that the model was stable. At the same time, the testosterone levels were decreased, indicating that the reduced testicular function is related to interstitial cell atrophy.

Adenine is usually administered intragastrically or through diet. Wang et al. applied the feeding method to administer 0.5% adenine at 300 mg/kg for 30 days to generate animal models [19]. However, variant modeling effects may be obtained with different food intakes from individual to individual. Therefore, intra-gastric administration is more widely applied, as Yang et al. has reported previously [20]. The usual

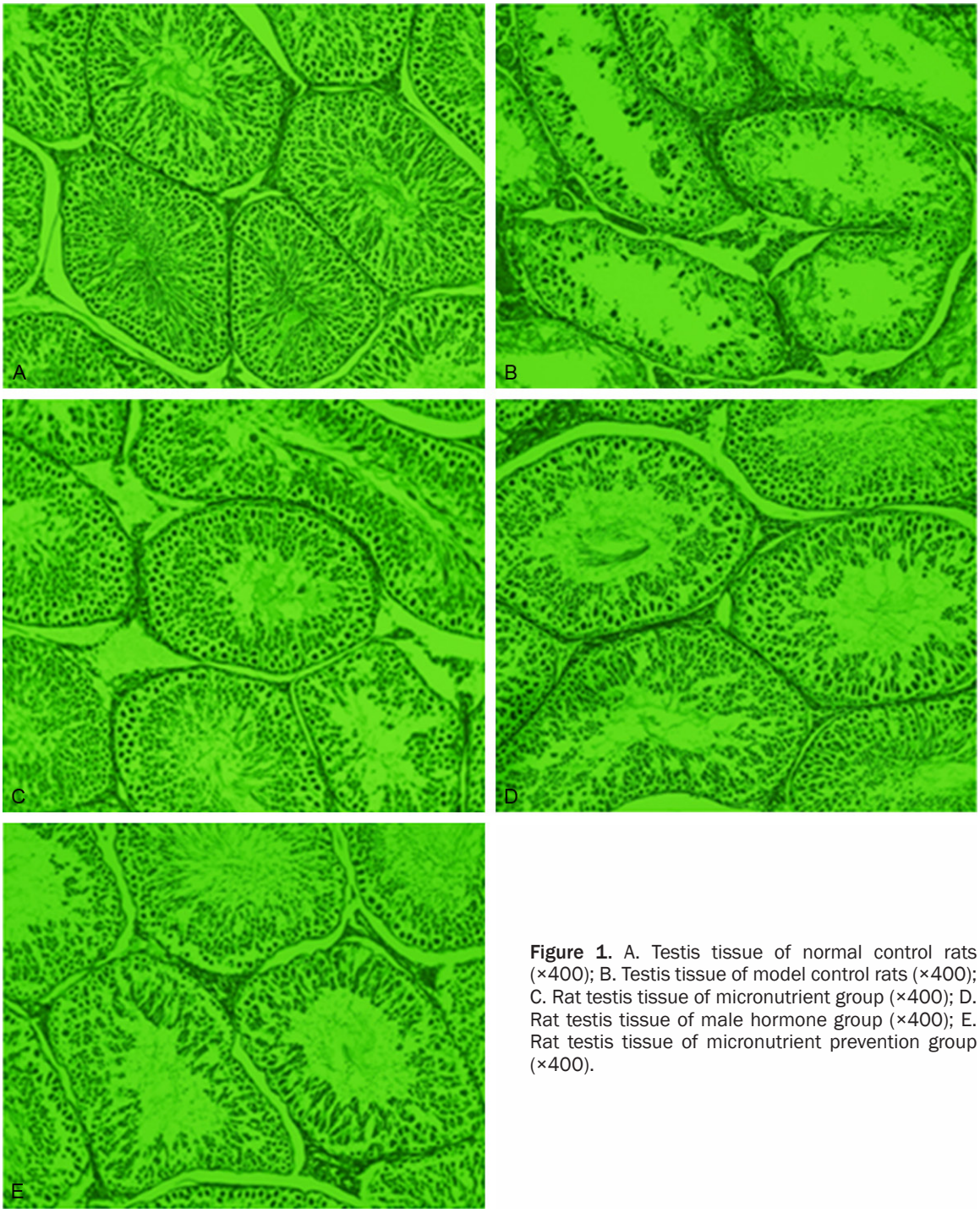

Figure 1. A. Testis tissue of normal control rats (×400); B. Testis tissue of model control rats (×400); C. Rat testis tissue of micronutrient group (×400); D. Rat testis tissue of male hormone group (×400); E. Rat testis tissue of micronutrient prevention group (×400).

dosage is 250-300 mg/kg for 30 days, although large dose of 500 mg/kg has also been used [21]. In this study, we treated the rats with daily intra-gastric administration of adenine at 300 mg/kg body weight for 30 days, which generated successful and sustainable infertility rat models. This demonstrated that intra-gastric administration of adenine is a stable and reliable method to develop infertility animal models.

Mechanism of micronutrients' effects on reproduction

Many micronutrients are related to reproduction, spermatogenesis, androgen synthesis and

secretion, free radical scavenging, and anti-oxidation, especially vitamin A, vitamin E, vitamin C, zinc and selenium.

Zinc is an essential trace element of human body that is a co-factor of varied human enzymes and closely related to many dehydrogenases in reproductive organs. The level of seminal plasma zinc is more than 100 times higher than that of the plasma zinc. Zinc deficiency can delay the development of testis, epididymis, and prostate, which may result in reduced sperm quality, density, and motility. Selenium is an important trace element that is required for male fertility. It is essential for the biosynthesis of testosterone and sperm formation and development. Sperm selenium concentrations below 0.46 mol/L can cause male infertility [22].

Vitamin A is an unsaturated monohydric alcohol containing β-ketone ring. Its role in reproduction is related to its effects on the epithelial tissues in the reproduction system. Deficiency in vitamin A has been reported to hinder the growth and development of different levels of spermatocytes, leading to testicular atrophy (reduction in organ coefficient), reduced sperm counts, and increased malformation rates. In addition, vitamin A may also change the functions of supporting cells in the reproduction system, which play important role in spermatogenesis because they secret many important proteins and provide nutrients to spermatocytes [23, 24].

The level of vitamin C (ascorbic acid) in semen is 8-10 times of that in blood. Oral administration of vitamin C leads to its quick distribution in the whole body, with the seminal vesicle containing the highest level.

Vitamin E contains the benzodihydropyran structure and tocopherol activity. It can protect the structural integrity of different tissues and promote the body's normal immune and reproductive functions. Vitamin E deficiency in rats led to damaged reproductive organs and testicular atrophy [25]. The ability of vitamin E's to improve semen quality has also been reported in other animal models, probably through its regulation of pituitary and adrenal glands [26].

Conclusion

Micronutrient mixture containing vitamin A, vitamin C, vitamin E, zinc, and selenium was able to improve the semen quality, increase the androgen levels, and repair the testicular seminiferous epithelia of adenine-induced infertility rat models.

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