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GAMETE BIOLOGY

Effects of L- and T-type Ca^{2+} channel blockers on spermatogenesis and steroidogenesis in the prepubertal mouse testis

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Abstract
Purpose To assess the involvement of L-type and T-type Ca^{2+} channel blockers in inducing male infertility.
Methods Prepubertal male mice were fed Ca^{2+} channel blockers nifedipine and ethosuximide for 20 days at dosages below maximum tolerated dose (MTD) and assayed for gross morphological changes in the testis such as body weight, testis size and weight. Sperm and Leydig cell counting were conducted concomitantly with serum testosterone level measurement by radioimmunoassay (RIA) and StAR protein mRNA measurement by reverse transcription and polymerase chain reaction (RT-PCR).
Results A chronic exposure to nifedipine or ethosuximide caused a significant reduction in body weight, testis size/weight and sperm production in a dose-dependent fashion associated with a spermatogenic arrest largely at the elongating spermatid stage. The number of Leydig cells, the serum testosterone level but not the luteinizing hormone level, and the content of StAR protein mRNA were also drastically reduced relative to the controls.
Conclusions Both T- and L-type Ca^{2+} channel blockers play an adverse role in normal spermatogenesis and steroidogenesis partly by blocking postmeiotic germ cell maturation and/or by abrogating StAR protein expression, contributing to male sterility. Therefore, any therapeutic application of Ca^{2+} channel blockers must be used with caution due to its potential adverse side effects on male infertility.

Capsule A chronic exposure to T- or L-type Ca^{2+} channel blockers causes an adverse side effect on spermatogenesis and steroidogenesis partly by blocking the postmeiotic germ cell maturation and by diminishing the testosterone production, both of which may contribute to male sterility.

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Introduction

Ca^{2+} is implicated in diverse cellular functions in both germ cells and somatic cells in the testis, particularly, mediating the responses to endocrine hormones and local regulators in genital tracts [1, 2]. A common belief is that the Ca^{2+} influx and outflux should be tightly regulated to maintain the intracellular Ca^{2+} homeostasis, and an alteration in the Ca^{2+}

transport across the cell membrane could result in a drastic impact on spermatogenesis and steroidogenesis [3, 4].

Based on the gating stimulus, Ca^{2+} channels are divided into ligand-dependent Ca^{2+} channels (LDCC) and voltage-dependent Ca^{2+} channels (VDCC). VDCCs that are open in response to transmembrane voltage changes have been identified in various cell types, including testicular germ cells and somatic cells, and further subdivided on the basis of their voltage activation threshold and pharmacological properties. For instance, a testicular specific Ca^{2+} channel has been identified and subsequently classified as L-type Ca^{2+} channels [5]. Recent studies have documented the presence of another type of VDCC, namely, the T-type Ca^{2+} channel in meiotic and post-meiotic germ cells [6, 7]. Physiological importance of VDCCs in spermatogenesis and steroidogenesis is demonstrated by the finding that Ca^{2+} carrying VDCCs are required for germ cell development [8, 9], and its channel activity can be developmentally modulated by protein tyrosine phosphorylation or intracellular pH [10]. A hormonal stimulation of testicular development by follicle stimulating hormone (FSH) or luteinizing hormone (LH) involves a Ca^{2+} influx possibly through VDCCs in Sertoli cells [11, 12] and Leydig cells [13]. Not surprisingly, abnormal Ca^{2+} currents through conformational defective dihydropyridine-sensitive Ca^{2+} channels have been frequently observed in infertile but not in fertile men [14], attributing male sterility to a defect in the Ca^{2+} channel. Nonetheless, a genomic approach using VDCC knockout animals has been less informative on pathophysiological roles of VDCCs in male sterility mainly due to lethality of Ca^{2+} channel knockouts [15].

Calcium channel antagonists have been widely used in the treatment of various heart diseases and neurological disorders [16, 17]. In humans, the maximum daily dose of nifedipine, a putative L-type Ca^{2+} channel blocker, to relieve hypertension is about 120 mg [18] while that of ethosuximide, a putative T-type Ca^{2+} channel blocker, for the treatment of epilepsy is about 2,000 mg [19, 20]. However, treatments with L-type Ca^{2+} channel blockers are likely to cause reversible male infertility, raising a speculation on a pathophysiological implication of L-type Ca^{2+} channel blockers in male infertility [21]. Particularly, pharmacologic treatment of pediatric hypertension with calcium channel antagonists has recently increased owing to their low adverse effect profiles [22]. Growing concern for infertility-causing effects of Ca^{2+} channel antagonists, in particular, in children has prompted us to systematically evaluate their effects on male sterility.

As a step toward this goal, we have set up a simple in vivo experiment to assess sterility-causing effects of Ca^{2+} channel blockers by utilizing a chronic administration of L-type and T-type Ca^{2+} channel blockers to prepubertal male mice at dosages comparable to human therapeutic use.

Materials and methods

Products and reagents

Nifedipine (N6792, purity 99%) and ethosuximide (E7138, purity 99%) were obtained from Sigma-Aldrich (St Louis, MO). Radioimmunoassay (RIA) kits for testosterone and for LH and prolactin were from Orion Diagnostica (Espoo, Finland) and Byk-Sangtec Diagnostica (Dietzenbach, Germany), respectively. Alkaline phosphatase-conjugated anti-rabbit antibodies and chromogenic reaction kits were from Dako (Carpinteria, CA). A rabbit polyclonal antiserum against nuclear transition protein 2 (TP2) was provided by Dr. Kistler at the University of South Carolina. The TRIZOL reagent was from Invitrogen (Carlsbad, CA). Reverse transciptase Superscript II™ was from Gibco-BRL (Gaithersburg, MD). Ex Taq™ DNA polymerase was from Takara (Otsu, Japan). Other chemicals, unless otherwise stated, were from Sigma-Aldrich (St Louis, MO).

Animals and drug administration regimens

Imprinting control region (ICR) male mice of postnatal day (PND) 18 weighing 11–13 g, which are known to produce only immature, pachytene stage spermatocytes but can produce mature sperms after about 3 weeks at PND 38, were purchased from Samtaco (Osan, S. Korea). Animals were housed in cages placed in an animal room with a temperature of 23±2°C, a relative humidity of 55±10%, and lighting for 12 consecutive hours per day. Animals were fed a rodent chow and tap water ad libitum. All procedures involving animals and their care were conducted in conformity with the Institutional Animal Research Ethics Committee at Ajou University, S. Korea. Ninety mice were randomly divided into three groups with 30 mice per group. In group 1, nifedipine was dissolved in sesame oil and administered orally for 20 days at dosages of 20, 40 or 80 mg/kg body weight per day (10 mice at each dosage). In group 2, ethosuximide was dissolved in distilled water and administered at 100, 500 or 1,000 mg/kg body weight per day (10 mice at each dosage). In group 3 (control), distilled water or sesame oil was administered orally for 20 days.

Histological and immunohistochemical evaluations

At the end of the experiment, animals were sacrificed by cervical dislocation, and their testes were carefully removed, washed in a normal saline solution, and blotted. The size and weight of each testis were measured before fixed in Bouin's fixative (right testis) or snap frozen in liquid nitrogen (left testis). Fixed testes were embedded in paraffin, sectioned at 5 μm thickness, and stained with haematoxylin and eosin (H&E staining) for histological evaluation. Immunohisto-

chemical staining was performed with antibodies against TP2, a stage specific marker for the early-elongated spermatid of rodent [23], and alkaline phosphatase-conjugated secondary antibodies. Chromogenic reaction was according to the manufacture's protocols.

Serum hormone measurement

Serum concentrations of testosterone, LH, and prolactin were measured by RIA using commercial assay kits according to the manufacturer's protocols.

Reverse transcription and polymerase chain reaction (RT-PCR) analysis of StAR proteins

Total RNA was isolated from decapsulated testes with the TRIZOL reagents, and approximately 5 μg total RNA was reverse-transcribed by Superscript II™ reverse transcriptase. The primers for StAR protein were 5′-GACCTTGAAAGG CTCAGGAAGAAC-3′ (forward) and 5′-TAGCTGAAGA TGGACAGA CTTGC-3′ (reverse); Primers for β-actin were 5′-GACCTTGAAAGGCTCAGGAAGAAC-3′ (forward) and 5′-TAGCTGAAG ATGGACAGACTTGC-3′ (reverse). PCR was subsequently performed using Ex Taq™ polymerase with 25~28 cycles of amplifications at 95°C for 1 min, 55°C for 1 min and 72°C for 3 min.

Statistical analysis

The statistical significance between the treated groups and controls was determined by Student's *t*-test. Differences were considered significant at $p<0.05$.

Results

Gross morphological changes in the testis caused by Ca^{2+} channel blockers

The ability of nifedipine and ethosuximide to exert their deleterious actions on testicular development and maturation was evaluated using a daily oral dosing of prepubertal mice for 20 days beginning at PND 18. Figure 1 visualizes the efficacies of the treatment against testicular development. All treatments caused a dose-dependent decrease in body weight, testis size, and testis weight albeit a significant reduction ($p<0.05$) observed at the highest doses of nifedipine and ethosuximide (Fig. 1a–c): The testis size was reduced by 23.3% and 33.9% at 80 mg/kg/day of nifedipine and 1,000 mg/kg/day of ethosuximide administration, respectively, compared with control values; The corresponding reduction in testis weight were by 52.1% and 51.3%. The gonadosomatic indices (GSI), which

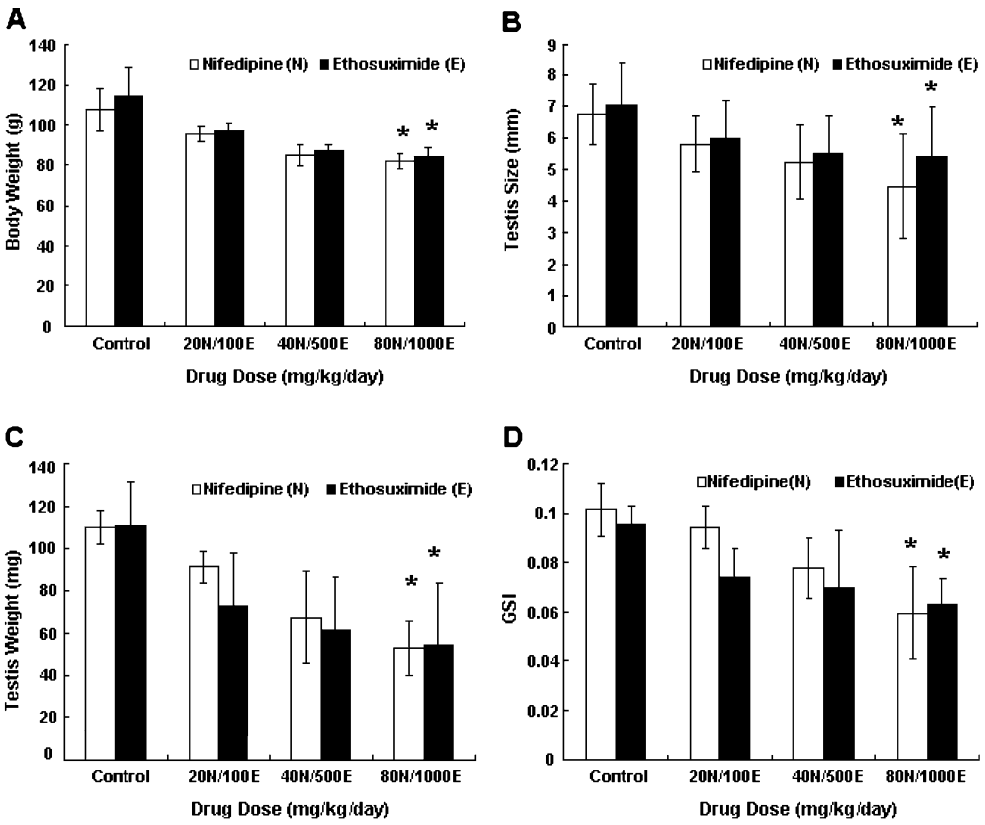

Fig. 1 The effects of Ca^{2+} channel blockers on gross morphological changes in the testis. Effects of nifedipine and ethosuximide on body weight (**a**), testis size (**b**), testis weight (**c**) and gonadosomatic index (**d**) of mice to which Ca^{2+} channels blockers were orally administered for 20 days at varying dosages as indicated. Data were represented as means ± SD ($n=10$/each treatment). *Significantly different from corresponding controls by Student's *t*-test ($p<0.05$)

were calculated from ratios of testis weight to body weight, were also substantially lowered at 80 mg/kg/day of nifedipine and 1,000 mg/kg/day of ethosuximide administration ($p<0.05$) (Fig. 1d).

Histological influence by Ca^{2+} channel blockers

The inhibitory effects of nifedipine and ethosuximide on prepubertal testis development were further evaluated

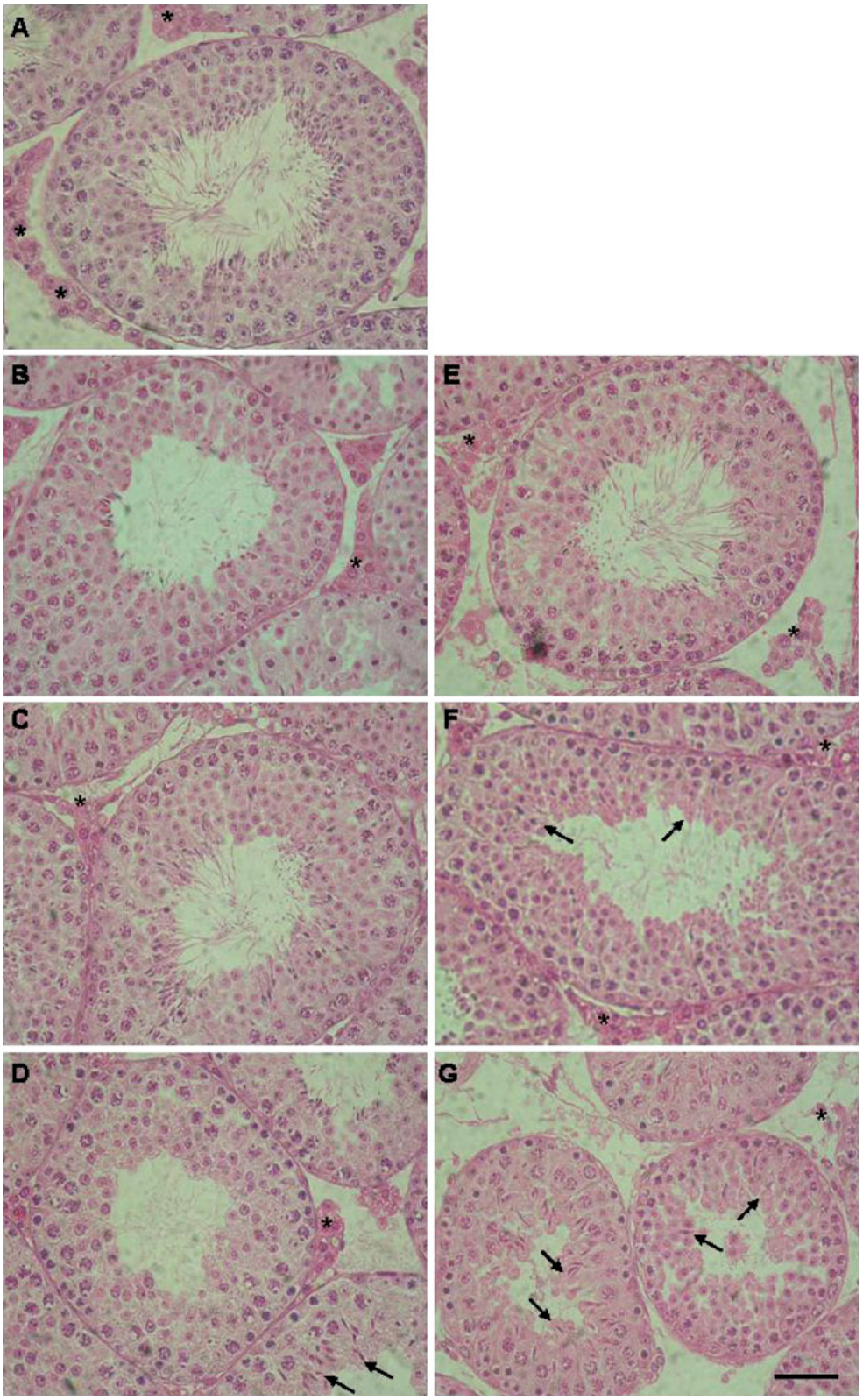

Fig. 2 Histological examinations of mice testicular tissues following Ca^{2+} channel blocker treatments. Nifedipine or ethosuximide was orally administered at varying dosages to prepubertal mice for 20 days before the testes were taken for a histological examination following H&E staining: (**a**) Control mice given vehicles only (water or sesame oil); (**b**) Nifedipine given daily at 20 mg/kg/day; (**c**) Nifedipine given daily at 40 mg/kg/day; (**d**) Nifedipine given daily at 80 mg/kg/day; (**e**) Ethosuximide given daily at 100 mg/kg/day; (**f**) Ethosuximide given daily at 500 mg/kg/day; (**g**) Ethosuximide given daily at 1,000 mg/kg/day. *Asterisks* point to Leydig cells; *Arrows* point to round, elongating spermatids; *Scale bar*, 100 mm

histochemically following H&E staining. Whereas control mice had the seminiferous tubules with fully developed tubular lumens and seminiferous epithelia harboring mature sperms, the treated mice showed a lesion in the seminiferous tubules with incomplete luminal development and few sperms (Fig. 2a–g). Dosing at 1,000 mg/kg/day of ethosuximide, for instance, featured a lack of mature spermatozoa and a spermatogenic arrest primarily at the round, elongating spermatid stage, and a loss of Leydig cells.

Because of the potential to underestimate their effects based on external measurement, we investigated alternative methods of evaluating a premature arrest of spermatids by using a stage-specific TP2 expression. The results of such an experiment are shown in Fig. 3. Whereas control testes revealed abundant TP-2 positive immuoreactivities that mainly localized to epithelia surrounding the tubular lumen, the drug-treated testes exhibited a complete, if any, lack of such immunoreactivities in surrounding epithelia. A more quantitative analysis conducted by counting TP2-positive spermatids revealed that both nifedipine and ethosuximide at all doses resulted in a decrease in the number of mature spermatids in a dose-dependent manner reaching a significant reduction ($p<0.05$) even at the lowest doses (e.g. 20 mg/kg/day nifedipine or 100 mg/kg/day ethosuximide) compared to the control mice (Fig. 4a). Similarly, Leydig cells was also significantly decreased in number in all treatments ($p<0.05$) (Fig. 4b).

Serum level of testosterone was lowered by Ca^{2+} channel blockers

A failure of steroidogenesis in Leydig cells must lead to a reduced plasma testosterone level. Thus, we investigated whether a chronic exposure to Ca^{2+} channels blockers caused reduced serum levels of testosterone, prolactin, and LH by RIA. Nifedipine or ethosuximide at all doses clearly lowered testosterone level in a dose-dependent manner while leaving prolactin and LH levels unaffected (Fig. 5a, b). The testosterone level at 20 mg/kg/day nifedipine, for instance, was approximately 4-fold lower than basal levels (0.61± 0.27 ng/ml vs. 2.42±0.30 ng/ml, $n=5$).

StAR protein expression was abolished by Ca^{2+} channel blockers

It is assumed that StAR proteins are at the late-limiting step of steroidogenesis in Leydig cells by transporting cholesterols into mitochondria [24]. Therefore, we sought to see whether the same chronic exposure also inhibited StAR protein expression. A representative RT-PCR analysis showed the time course of StAR mRNA expression profiles starting at PND 7 until PND 42 (Fig. 5c). The content of StAR mRNA increased gradually reaching the peak at PND 28 and then remained constant until PND 42. However, a 20-day-administration of nifedipine and ethosuximide at 80 mg/kg/day and 1,000 mg/kg/day,

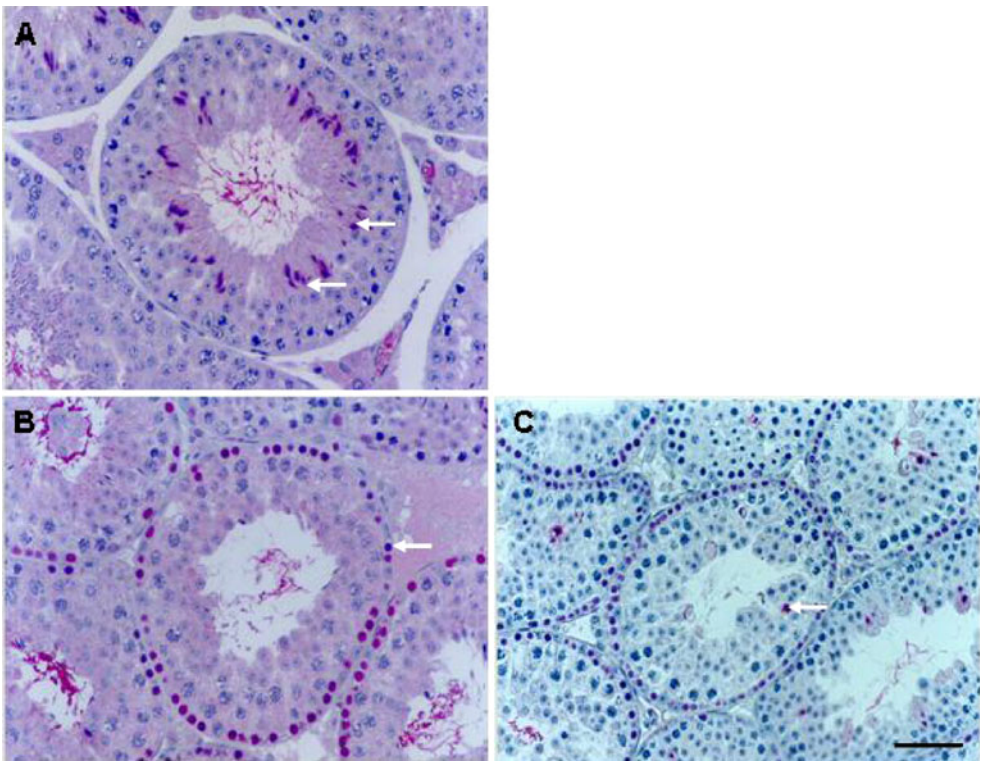

Fig. 3 Immuohistochemical evaluation of sperm maturation following a chronic exposure to Ca^{2+} channel blockers. Nifedipine and ethosuximide were orally administered in a similar regimen to Fig. 2 for 20 days before testicular tissues were taken for immunostaining for TP2, a cellular marker for a mature, elongated spermatid, followed by H&E counter-staining before a microscopic examination. (**a**) Control mice; (**b**) Nifedipine given daily at 80 mg/kg/day; (**c**) Ethosuximide given daily at 1,000 mg/kg/day. *Arrows* indicate TP2-positive cells; *Scale bar*, 100 mm

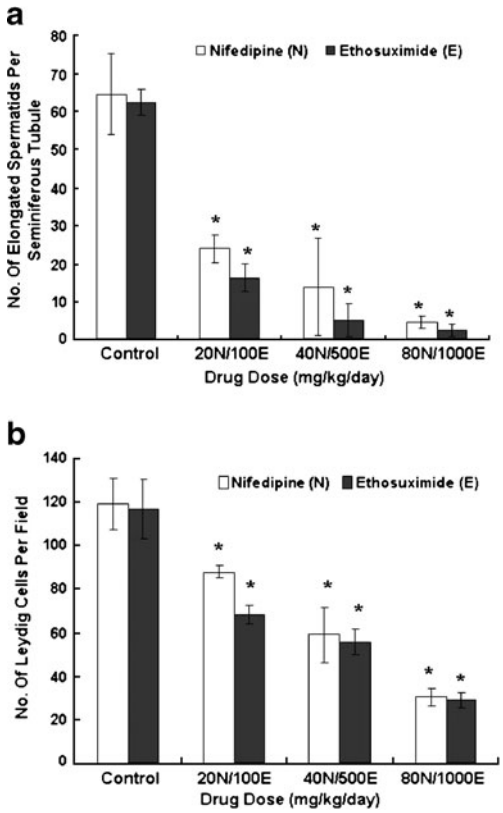

Fig. 4 A quantitative evaluation of spermatid maturation after a chronic administration of Ca^{2+} channel blockers. The histological data obtained in Figs. 2 and 3 were subjected to a microscopic counting. (**a**) The number of mature, elongated spermatids within a seminiferous tubule were microscopically counted and plotted in means ± SD. Spermatids were counted in at least 50 different seminiferous tubule sections in 10 microscopic fields at 400x magnification. (**b**) The number of Leydig cells in the testis was counted and plotted in means ± SD. Leydig cells were counted in at least 50 microscopic fields. *Significantly different from controls by Student's *t*-test ($p<0.05$)

respectively, caused a complete abolition of StAR mRNA expression (Fig. 5d).

Discussion

The primary goal of the present studies was to prospectively address the question 'Do Ca^{2+} channels blockers currently used as therapeutics against hypertension and epilepsy exert deleterious side effects that could eventually contribute to male sterility?' This was accomplished by utilizing a chronic exposure of prepubertal male mice to nifedipine and ethosuximide at dosages comparable to those used for human therapy and of duration corresponding to the testicular maturation period. First, our treatment caused a significant decrease in the body weight, testis size/weight, and the gonadosomatic index. Second, it also caused a lesion of the seminiferous tubule featured with a reduced luminal development and a premature spermatogenic arrest. Finally, the serum testosterone level was drastically lowered by the treatment, concomitant with a reduced expression of StAR proteins. All such observations are most consistent with the interpretation that a blockade of either the T- or L-type Ca^{2+} channel by its respective blockers may lead to an impairment of normal spermatogenesis and steroidogenesis, in particular, during testicular maturation.

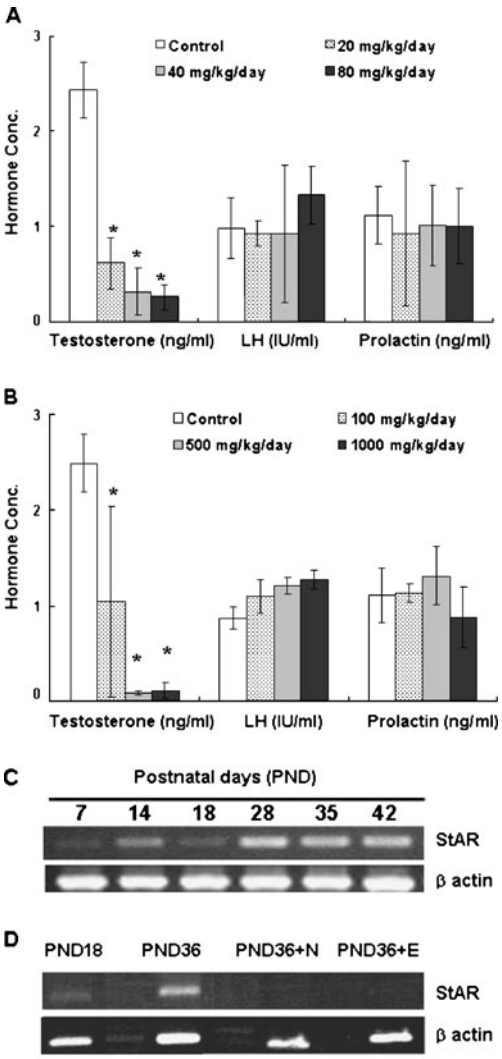

Fig. 5 Steroidogenesis inhibition by Ca^{2+} channel blockers. Nifedipine and ethosuximide were administered to prepubertal mice in a similar regimen to Fig. 2. In (**a**) and (**b**), serum levels of testosterone, prolactin, and LH were measured by RIA: (**a**) Nifedipine-dosed mice; (**b**) Ethosuximide-dosed mice. *Significantly different from controls by Student's *t*-test ($p<0.05$). *Error bar*, SD ($n=5$). In (C) and (D), the mRNA levels of StAR protein were semi-quantitatively measured by RT-PCR expression analysis. (**c**) A time course of StAR protein mRNA expression profile from PND 7 to PND 42. (**d**) Inhibition of StAR mRNA expression by Ca^{2+} channel blockers. *N* indicates nifedipine dosing at 80 mg/kg/day; *E* indicates ethosuximide dosing at 1,000 mg/kg/day. Total RNAs pooled from 5 testes from different animals was analyzed to minimize the testis to testis difference. β-actin mRNA was used as an internal control

Our present findings are overall in line with many previous ones with regard to male sterility caused by calcium channel blockers. Human sperms from patients treated by nifedipine to relieve hypertension fail to undergo acrosome reaction and in vitro fertilization due to a defect in the late stage of spermatogenesis [21, 25]. Most intriguingly, expression level of alpha-1H T-type Ca^{2+} channels in the testis of subjects with Sertoli Cell-Only (SCO) syndrome, a condition of the testes in which only Sertoli cells line the seminiferous tubules thus azoospermic, is substantially lowered compared to the testis retrieved from normal subjects [26].

In considering the results of this study, it is important to recognize that successful spermatogenesis and steroidogenesis are largely dependent on ample support from nearby somatic cells in the testis. For instance, FSH and LH exerts their actions on steroidogenesis by mainly regulating intracellular Ca^{2+} concentration through VDCCs in Sertoli cells [27, 28] and Leydig cells [29]. Thus, it is possible, and even likely, that any malfunction of Ca^{2+} channels in somatic cells may result in a drastic attenuation or a complete failure of spermatogenesis and steroidogenesis. In accordance, the present data show a drastic decline of serum testosterone level on Ca^{2+} channel blocker treatments while LH and prolactin level remained unaffected. Of particular, our present pathophysiological characteristics influenced by Ca^{2+} channel blocker treatments are very similar to those observed in the LH receptor knockout mice. The LH knockout mice also exhibit a reduction in blood testosterone level, a poor development of the testis, and a spermatogenic arrest at round spermatid stage, which are largely attributable to the absence of LH effects [30]. Several other studies have also documented Ca^{2+} channels in disenabling steroidogenesis in the testis. Vigier et al. [31] demonstrate that the blood testosterone level decreases until PND 15 and then reincreases thereafter until reaching to the adulthood level during the rat postnatal testicular development, and the early meiotic stages of spermatogenesis can progress in the lack of testosterone. However, testosterone is crucial for postmeiotic differentiation of male germ cells, and otherwise, germ cell death increases in the absence of testosterone [32]. Based on many previous studies and our own, it is highly likely that one of the major targets of nifedipine and ethosuximide may be on Leydig cells. Then, what is the underlying mechanism for Ca^{2+} channel blockade in association with various aspects of male sterility? At the moment, one possibility might be triggering the cell death of Leydig cells because the cell death of Leydig cells could best account for a reduced blood testosterone levels and StAR protein expressions.

There are some issues, however, that need to be addressed pertaining to proper interpretation of the present study. Are the doses that we used a biological active dose rather than a maximum tolerated dose? In this study, the doses were 20 to 80 mg/kg/day for nifedipine and 100 to 1,000 mg/kg/day for ethosuximide. Within these dose ranges, each drug cause a concentration-dependent disenabling of testicular maturation, implying that the doses tested may not be physiologically saturated or maximally tolerated in mice. The physiological relevance of the present doses can be further validated by a report by Mendel et al. [33], who clearly demonstrated that at 40 mg/kg/day dosing, a tyrosine kinase inhibitor targeting VEGF and PDGF effectively exhibits its antitumor activity causing a substantially reduced growth of xenografted tumors established in nude mice. And they also deduced that this dosage is likely to approximate effective blood concentrations in humans based on various pharmacokinetic and pharmacodynamic evaluations.

In conclusion, we have demonstrated that blocking of either T-type or L-type Ca^{2+} channel by its respective blockers may result in the failure of normal spermatogenesis and steroidogenesis during prepubertal testicular maturation. Particularly, 80 mg/kg/day of nifedipine and 1,000 mg/kg/day of ethosuximide administration were very efficacious doses at which they cause male sterility in mice. We also have provided direct evidence that Ca^{2+} channels blockers are effective in causing a premature arrest of developing spermatids and diminishing Leydig cell abundance by abrogating StAR protein expression and thus testosterone production. Although the studies described here specifically identified nifedipine and ethosuximide as causatuve agents responsible for male sterility, this approach should be easily adapted to other Ca^{2+} channel blockers as long as models and techniques developed in this study are available.

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