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Expression of calmodulin in germ cells is associated with fenvalerate-induced male reproductive toxicity

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Abstract

Exposure to fenvalerate was demonstrated to be toxic to the male reproductive system. Our previous data revealed that intracellular calcium plays an important role in regulating the above toxicity, through actions on both T-type calcium channels and endoplasmic reticulum calcium signals. The present study explored the effects of fenvalerate on the expression of calmodulin in mouse testis and GC-2spd(ts) cells, and its association with fenvalerate-induced male reproductive toxicity. Male mice were subjected to different doses (3.71, 18.56, 37.12, 92.81 mg/kg bw) of fenvalerate or vehicle control for 4 weeks. Expression of calmodulin was determined by real-time polymerase chain reaction (PCR) and Western blot analysis in mouse testis. Similar approaches were utilized in GC-2spd(ts) cells cultured with 5 μ M fenvalerate at different time points. In the in vivo study, all mice survived through the entire 4 weeks. Administration of fenvalerate resulted in a dose-dependent reduction in testis weight/body weight, sperm motility, and increased head abnormality rate. By histological staining, mice treated with fenvalerate at higher doses showed dilated seminiferous tubules and disturbed arrangement of spermatogenic cells. Meanwhile, both mRNA and protein expression of calmodulin were significantly increased in the testes of mice exposed to fenvalerate compared to control mice. Moreover, in the in vitro study, 5 μ M fenvalerate significantly increased the expression of calmodulin at the mRNA and protein levels in GC-2spd(ts) cells after 8 h of incubation and sustained these levels for at least 24 h. Collectively, these data suggested that enhanced expression of calmodulin correlates with male reproductive damage induced by fenvalerate.

Keywords

Calmodulin; Fenvalerate; Testis; Germ cells; Male reproductive system

Introduction

The widespread environmental exposure to chemical substances has continuously been a major threat to the health status of both humans and wildlife (Diamanti-Kandarakis et al. 2009). Pesticides, especially synthetic pyrethroids have been extensively used in daily life and industry, and documented to be associated with increased incidences of reproductive dysfunction. Fenvalerate, a main-stream pyrethroid pesticide, was initially recommended as a low-toxicity agent, while recent scientific evidence suggests that accumulation of fenvalerate in humans and rodents is detrimental, specifically to the reproductive system (Arena et al. 2008; Gao et al. 2010). For instance, occupational exposure to fenvalerate was associated with poor sperm quality and sexual function (Giri et al. 2002; Shi et al. 2011).

In contrast to its well-accepted toxic effects, the detailed mechanisms involved in fenvalerate-induced dysfunctions remain unclear. Some earlier studies indicated that fenvalerate had weak estrogenic activity; thus, an “endocrine-disrupting chemical” working model was proposed (Chen et al. 2002). However, this working hypothesis was challenged by inconsistent estrogenic activity measured by different assays and exclusion of binding affinity between fenvalerate and estrogen receptors, alpha and beta (Gao et al. 2010; Kunimatsu et al. 2002; Saito et al. 2000). Meanwhile, several different groups revealed that calcium signals might mediate fenvalerate-induced effects in different subjects. He et al. (2004) found that fenvalerate modulates Ca^{2+} homeostasis and subsequently interferes with

FSH-stimulated ovarian steroidogenesis. Our previous data also demonstrated that intracellular calcium plays an important role in regulating the response of mouse spermatogenic cells to fenvalerate stimulation, through actions on both T-type calcium channels and endoplasmic reticulum calcium signals (Xiao et al. 2006; Wang et al. 2009). Because that transient increment in intracellular calcium is a generalized event that exists in many physiological and pathological actions, we sought in these studies to more specifically define the molecular mechanisms involved in fenvalerate-induced male reproductive disorders.

Calmodulin (CaM), a 17 kD protein, is highly conserved throughout evolution. CaM binds to intracellular-free Ca^{2+} , serving as an intermediary protein to transduce signals into downstream effects (Williams et al. 2010; Kaetzel and Dedman 2003). CaM is capable of modifying biological activities of calcium signal-related proteins. Alterations in CaM have been detected in many disease states such as tumor metastasis, neurological disorders, and male infertility (Reyes et al. 1987; van Erp and van de Kerkhof 1987; Martinez et al. 2003). However, there is little known regarding the role of this calcium sensor in fenvalerate-induced reproductive toxic effects. In order to obtain proof of concept, we first hypothesized that CaM modulation may correlate with fenvalerate-induced male reproductive damages. We integrated both in vivo and in vitro approaches to test our hypothesis.

Materials and methods

Chemicals and reagents

Fenvalerate (α -Cyano-3-phenoxybenzyl α -(4-chlorophenyl)-isovalerate, $\text{C}_{25}\text{H}_{22}\text{ClNO}_3$, analytical standard, product number: 45495, purity: 99.9 %) was obtained from Sigma (St. Louis, MO, USA). All other reagents were also from Sigma if not otherwise specifically stated.

In vivo experiments

Animal maintenance—Male CD1 mice weighing 18–20 g were purchased from the Animal Center of Jiangsu Province, Nanjing, China (SCXK (Su)2002-0031). All animals were housed in independent ventilation cages (IVC) at an ambient temperature of $23 \pm 1^\circ\text{C}$ and humidity of $55 \pm 5\%$ with 12 h light/dark cycle. They were allowed access to diet and tap water ad libitum. All animal procedures were in strict conformation with the Institute Ethical Committee guidelines for the care and use of laboratory animals. A pilot experiment was executed to determine the median lethal dose (LD_{50}) of fenvalerate by the Bliss method. After that, 50 mice were randomly divided into 5 groups ($n = 10$ in each group) and continuously administered fenvalerate in corn oil at a dosage of 3.71, 18.56, 37.12, 92.81 mg/kg bw, or 0.1 mL/10 g bw of corn oil by gavage for 30 days.

Tissue harvest—Mice were killed by cervical dislocation 28 days later. Body weight and testis weight were recorded at that time. Sperm samples were isolated from epididymides immediately and analyzed for sperm motility and abnormalities by a previously reported method (Cooper et al. 2010). Testes were saved for histological changes and other analyses.

Testicular histology—The testes were fixed in Bouin's solution, processed, and embedded in paraffin. Sections ($4\ \mu\text{m}$) were stained with hematoxylin and eosin (H&E) and were observed under phase-control microscopy (Olympus, Japan) for morphological analyses. Two independent, trained pathologists reviewed the slides from all groups.

In vitro experiments

Cell culture and treatment—The GC-2spd(ts) (GC-2) cell line was purchased from American Type Culture Collection (ATCC® Number: CRL-2196™). The cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). For experiments, cells were seeded at a density of 5×10^5 cells per 10 cm Petri dish with 10 mL of complete medium. After cells attained 80 % confluence, the media were substituted to phenol red-free DMEM containing 5 % charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT, USA) for 24 h before co-culture with fenvalerate or 0.01 % DMSO as vehicle control, respectively. Cell culture photos were taken under phase-control microscopy (Olympus, Japan) to check cell morphology.

MTS assay—Cell viability was measured by the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Briefly, GC-2 cells were seeded into 96-well plates at a density of 4×10^3 per well. Various concentrations of fenvalerate (0, 0.1, 0.5, 1.0, 5.0, 10, 50, 100 µM) were added to the medium after cells were pretreated with phenol red-free DMEM containing 5 % charcoal/dextran-treated fetal bovine serum for 24 h. 20 µL of MTS reagent was then administered into each well and incubation continued for an additional 2 h. Subsequently, absorbance values were then read at 490 nm by a microplate spectrophotometer (SpectraMax M5, Molecular Devices Corporation, Sunnyvale, CA, USA).

LDH release assay—The GC-2 cells (4×10^3 cells per well) were seeded in 96-well opaque-walled tissue culture plates with clear bottoms (BD, Franklin Lakes, NJ, USA). Prior to treatment, the media were changed to phenol red-free DMEM with 5 % charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT, USA). After co-stimulation with various concentrations of fenvalerate (0, 0.1, 0.5, 1.0, 5.0, 10, 50, 100 µM) for 24 h, the release of lactate dehydrogenase (LDH) from cells with damaged membranes into the culture medium was measured by using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, WI, USA). Eight wells were used for each concentration of fenvalerate. Briefly, 100 µL resazurin-labeled substrate was added into each well to react with released LDH. The resulting resorufin product was determined by adding 50 µL stop solution to each well and fluorescence. Fluorescence data were collected using a 96-well fluorometer with an excitation wavelength of 560 nm and an emission wavelength of 590 nm (SpectraMax M5, Molecular Devices Corporation, Sunnyvale, CA, USA). Similarly, at least 6 wells were used for each concentration of fenvalerate.

ATP assay—Cells were plated into 96-well opaque-walled tissue culture plates with clear bottoms (BD, Franklin Lakes, NJ) and treated with the various concentrations of chemicals as describe in the previous two sections. After 24 h of incubation, plates were taken out and equilibrated at room temperature for 30 min. CellTiter-Glo® Luminescent Cell Viability Assays (Promega, Madison, WI, USA) were used. A volume of 100 µL of reagent was added to each well and mixed for 2 min on an orbital shaker to lyse the cells. After incubation for 10 min at room temperature, luminescence was recorded by a 96-well luminometer (SpectraMax M5, Molecular Devices Corporation, Sunnyvale, CA, USA). Similarly, at least 6 wells were used for each concentration of fenvalerate.

Expression of CaM in GC-2 cells—To determine the effect of fenvalerate on the expression of CaM, GC-2 cells were placed in 10-cm Petri dishes and subjected to 5 µM fenvalerate for various time points. Cells were harvested for mRNA analysis or Western blot

by the protocols described later. Of note, each sample consisted of a pool of duplicated wells, and whole experiments were executed at least 3 times.

Real-time RT-PCR in cells and tissue—Total RNA was isolated using the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA pellets were dissolved in RNase-free and quantified by Nanodrop 1000 (Thermo scientific, Rockford, IL, USA). Two micrograms of total RNA were reverse transcribed into first-strand complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and used as a template for real-time PCR analysis. The primers used were as follows: CaM (NM_009790), sense, 5'-TTGCCGTCTATGACCACGTAAG-3', and antisense, 5'-CCTGCTTTTGGCATAACACAGTG-3'; GAPDH (NM_001001303), sense, 5'-ACAAAATGGTGAAGGTCGGTG-3' and antisense, 5'-ACAAAATGGTGAAGGTCGGTG-3'. Real-time PCR was carried out on the ABI Prism 7900HT Sequence Detection System with SYBR® Green PCR Master Mix reagents (Applied Biosystems, Foster City, CA, USA) in 10 µL reaction mixture. Fold changes in mRNA levels were calculated by using the $\Delta\Delta CT$ method and GAPDH as reference gene. Specificity of PCR reaction was double-confirmed by electrophoresis and melting curve analysis.

Western blot analysis—The levels of CaM protein were determined by Western blot analysis. Cell lysates were prepared by using the RIPA (Radio-Immunoprecipitation Assay) buffer essentially as described by the manufacturer (R0278, Sigma, St. Louis, MO, USA). Briefly, washed cell pellets were resuspended in extraction lysis buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0 % Igepal CA-630 (NP-40), 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate) with Protease Inhibitor Cocktail (P8340, Sigma, St. Louis, MO, USA) and then incubated for 30 min at 4 °C. The protein concentration was determined by Pierce BCA Protein Assay (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instruction. Samples containing equal amounts of protein were loaded onto 4–12 % sodium dodecyl sulfate–polyacrylamide gels, subjected to electrophoresis, and subsequently blotted onto 0.22-µM PVDF membrane (Millipore, Bedford, MA, USA). After blocking with nonfat dry milk (5 % w/v), the membrane was incubated with mouse monoclonal CaM antibody (1:1000, Upstate, Lake Placid, NY, USA) to detect the total forms of CaM. Mouse anti- β -actin (1:2000, Sigma, St. Louis, MO, USA) was used to detect reference protein expression. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution, Zhongshan BioTech, Beijing, China). The blots were detected with an enhanced chemiluminescence system (ECL, GE Healthcare, UK), and the membranes were exposed to X-ray film (Kodak, Rochester, NY, USA). The density of the respective bands was quantified by a densitometer using AlphaView software (FluorChem SP, Alpha Innotech Corp., San Leandro, CA, USA).

Statistical analysis

Data were expressed as mean $\pm$ SEM. One-way ANOVA was performed for the statistical analysis of data; when group comparisons showed a significant difference, the Dunnett's test was used. $p < 0.05$ was accepted as statistically significant.

Results

Influence of fenvalerate exposure on male reproductive system

All mice survived through the 4-week treatment period. There were no significant differences in mean body weights among the five experimental groups. As shown in Table 1, testis weight normalized by body weight was significantly ($p < 0.05$) reduced by 16.2 and

20.1 % in groups treated with 37.12 and 92.81 mg/kg bw of fenvalerate, compared with vehicle control-treated mice, respectively (6.21 ± 0.54 mg/g, 5.92 ± 0.29 mg/g vs. 7.41 ± 0.57 mg/g). Furthermore, sperm motility and sperm head abnormality rates, which are important parameters in the assessment of male reproductive function, were also altered and are presented in Table 1. Sperm motility was significantly affected in groups D and E ($p < 0.05$), accompanied by increased head abnormality rates ($p < 0.01$). Collectively, these data suggest that fenvalerate is detrimental to the mouse male reproductive system at a certain dose range.

Influence of fenvalerate exposure on mouse testicular morphology

In order to evaluate the potential impact of fenvalerate exposure on testicular morphology, H&E staining was performed on sections from mice treated with vehicle control or fenvalerate. Representative images are present in Fig. 1. As shown at a lower magnification, seminiferous tubules apparently resemble normal “back-to-back” morphology in mice treated with vehicle control. In contrast, some of the seminiferous tubules in mice treated with fenvalerate at the doses of 37.12 and 92.81 mg/kg bw were dilated. Moreover, at a higher magnification, the arrangement of spermatogenic cells was disturbed in sections from groups D and E (37.12 and 92.81 mg/kg bw, respectively). Specifically, vacuolization was detected in spermatogenic cells in slides of testes from mice exposed to fenvalerate.

Expression of CaM in mouse testes

Calcium signals have been shown to be important mechanism responses for multiple physiological and pathological events (Erickson et al. 2011). The binding of intracellular calcium and CaM is a key step for calcium signaling and subsequent actions. We investigated the expression of CaM in mice from the different treatment groups. As shown in Fig. 2a, mRNA expression of CaM was significantly higher in mice exposed to 18.56 mg/kg bw of fenvalerate compared to control mice (1.53 ± 0.31 vs. control group, $p < 0.05$) and was even greater in mice treated with 37.12 mg/kg bw (2.72 ± 0.44 vs. control group, $p < 0.05$). Moreover, by Western blot, expression of CaM protein was increased in the testes of mice exposed to fenvalerate. As quantitated by optical density, CaM expression was shown to be 5.06-fold greater in group D than in group A (5.06 ± 0.66 vs. control group, $p < 0.01$).

Influence of fenvalerate on cell growth of GC-2 cells

The next question was whether the increment of CaM in the testis induced by fenvalerate was contributed totally or at least partially by germ cells. It has been demonstrated by several investigators that primary culture of germ cells still has unresolved technical challenges especially for subacute and chronic experimental interventions. In order to bypass this barrier, we employed well-established GC-2 cells as alternative in vitro subjects. First of all, the influence of fenvalerate on cell growth of GC-2 cells was determined by observing cell morphology and measuring cell viability. As shown in Fig. 3a, addition of 10 μ M of fenvalerate in culture medium for 24 h resulted in a significant reduction in cell density compared with vehicle control-treated cells. Accordingly, quantitation by an MTS assay showed that treatment with 10 μ M fenvalerate resulted in a 20.99 % decrease in cell viability compared to the vehicle control and that decrease occurred in cells treated with larger doses. Moreover, we also measured cellular release of LDH as a marker for membrane integrity. As illustrated in Fig. 4a, LDH release was roughly unchanged in cells treated with various concentrations of fenvalerate compared to medium control, suggesting that cellular membranes remain intact following exposure to fenvalerate. Furthermore, ATP content was determined as a marker for intracellular energy. Differently, stimulation of fenvalerate substantially depleted cellular ATP levels in a dose-dependent manner (Fig. 4b; 0.1 μ M vs. 0 μ M, $p < 0.05$, peak at 50 μ M). Based on these data and our previous

experience (Wang et al. 2009; Xiao et al. 2006), we chose 5 μ M as an optimized working concentration for the following experiments.

Effects of fenvalerate on the expression of CaM in GC-2 cells

Our previous studies demonstrated that fenvalerate induced increased intracellular calcium concentrations via intracellular calcium release and extracellular calcium influx (Xiao et al. 2006; Wang et al. 2009). CaM has been identified as a crucial molecular partner for actions of intracellular calcium signals. Expressional levels of CaM are associated with the sustainability and magnitudes of calcium-related cellular signal pathways. We next sought to examine whether fenvalerate impacts the expression of CaM in GC-2 cells. As shown in Fig. 5a, 5 μ M of fenvalerate increased mRNA expression of CaM as early as 4 h incubation and reached statistical significance at 1.90-fold change after incubating for 12 h (1.90 ± 0.15 vs. 0 h, $p < 0.05$). Consistently, increased CaM protein levels were detected after incubation with fenvalerate and reached a peak at 12 h at 2.11 ± 0.13 fold change ($p < 0.05$), compared with control.

Discussion

In the current study, fenvalerate exposure at certain dosages reduced mouse testis weight and sperm motility, increased abnormality rates in sperm, and induced dilation in seminiferous tubules and vacuolization of germ cells. These results indicate that fenvalerate has an adverse effect on the fertility and the reproductive tract of male mice. Meanwhile, we found that fenvalerate treatment resulted in elevated CaM expression in mouse testis and GC-2 cells, suggesting that fenvalerate-induced male reproductive damage is associated with elevated CaM expression at least in spermatogenic cells.

Several studies have found that exposure to fenvalerate adversely impacts on several systems including the immune and endocrine systems (Arena et al. 2008; Dutta and Das 2011). Among these, the male reproductive system has raised major concerns from both the research community and the public. In human epidemiological studies, fenvalerate exposure resulted in reduction in sperm quality and extension of time to pregnancy. In experimental rodents, fenvalerate exposure has been reported to damage spermatozoa, Leydig cells, or to disrupt endocrine function such as hormonal regulation, regardless of whether exposure occurs in the prenatal, pubertal, or adult stage (He et al. 2004; Zhao et al. 2011; Zhang et al. 2009). Our findings, by employing a step-wise strategy, clearly identified a toxic dosage range for fenvalerate in ICR mice, thus providing a baseline experimental condition for future studies. Of note, this dosage is in line with data reported by other independent groups (Zhao et al. 2011). Collectively, current available data including our study point out that there is evident long-term toxicity of fenvalerate on the male reproductive system. Moreover, consumption of food contaminated by fenvalerate may aggravate human exposure risk by the accumulation of remnant chemical and toxicity.

Our previous findings suggest that increment in intracellular calcium emerges as a potential mechanism for fenvalerate-induced effects on spermatogenic cells. Recognizing that CaM is a ubiquitous calcium sensor protein, we sought to examine the expression of this calcium partner in our in vivo model. We did find that 28 days of exposure to fenvalerate dramatically increased expression of CaM in the testis. Furthermore, we questioned whether increased CaM in testis by fenvalerate could be related to effects upon the spermatogenic cells. Due to the technical challenges of isolating and culturing sperm cells, we chose the GC-2 cell line as a surrogate subject. Fenvalerate treatment resulted in a time-dependent increase in both mRNA and protein expression of CaM, suggesting that spermatogenic cells are responsible at least in part for the increment in CaM seen in the testis. This important concept not only provides an additional bond between calcium signaling and fenvalerate-

induced reproductive toxicity, but also sheds light on potential downstream pathways involved in the abovementioned pathway.

Intracellular calcium, by binding CaM, induces conformational changes of CaM, thereby activating downstream effector proteins such as Ca^{2+} /CaM-dependent protein kinase II (CaM Kinase II) and calcineurin, a Calcium/CaM-dependent protein phosphatase (Erickson et al. 2011). CaM Kinase II is a well-known important pathway in neural and cardiac development. Recent studies have also suggested that this kinase is expressed in sperm and is associated with toxicities of several chemicals such as organophosphorus compounds and tobacco (Fan et al. 2003; Schlingmann et al. 2007; Arredondo et al. 2008).

Activation of calcineurin and NFAT (nuclear factor of activated T cell) has also been documented to play pivotal roles in many processes, including cell proliferation, development, and apoptosis (Baine et al. 2009). Specifically, calcineurin is the target of a class of drugs including cyclosporine and tacrolimus. Therefore, it is of particular preventive interest to explore whether calcineurin inhibitors are able to impact fenvalerate-induced male reproductive damage.

It is of interest that Zhao et al. recently reported that a similar dosage (15 and 60 mg/kg bw) exposure of fenvalerate resulted in germ cell apoptosis via the Fas/FasL signaling pathway (Zhao et al. 2011). Coincidentally, CaM has also been implicated to bind directly to Fas and play a critical role in Fas-mediated apoptosis (Wu et al. 2005; Chen et al. 2008). Taken together, these data suggest a link between calcium signals and apoptosis in the setting of fenvalerate-induced male reproductive damage. Our in vitro data confirmed that Fen-induced cell toxicity parallels with the dramatic reduction in intracellular generation of ATP, without apparent destruction of the cell membrane. Furthermore, the significant reduction in ATP at lower doses of Fen suggests that ATP reduction may be secondary to ATP consumption by upstream intracellular cascades in response to Fen-induced cell signaling and damage.

In view of these results, there is also concern as to whether exposure to fenvalerate will have greater risk in certain specific populations. Because fenvalerate can increase the expression of CaM, this subpopulation may include, but not be limited to those who are experiencing a situation of altered CaM expression due to disease. As indicated in the literature, enhanced expression of CaM has been commonly detected in several diseases such as psoriasis, Huntington's disease, and acute pancreatitis (Martinez et al. 2003; Petersen et al. 2011). Therefore, exposure to fenvalerate in patients with the above specific disorders may bear additional risk of exacerbating the primary disease.

In summary, we found that enhanced expression of CaM occurs concomitantly with male reproductive damage induced by fenvalerate. This finding provides new evidence to bind the calcium signaling pathway to fenvalerate-induced toxicity and sheds light on new potential downstream pathways. However, additional work is urged to explore the precise mechanism responsible for fenvalerate-induced toxicity in the male reproductive system. Moreover, caution should be taken in monitoring additional risks of fenvalerate exposure in certain subpopulations.

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Fig. 1. Morphologic change of the testis in mice with treatment for fenvalerate H&E staining. **a, c** Control group; **b, d** Fenvalerate (37.12 mg/kg bw)-treated group; **a, b** $\times 100$; **c, d** $\times 1,000$

Fig. 2.

Effects of fenvalerate on the expression of CaM in testis tissue from mice. **a** mRNA expression of CaM. **b** Representative immune blots of CaM. **c** Semi-quantitation of OD values * $p < 0.05$ versus Group A. Fenvalerate (mg/kg bw): A = 0, B = 3.71, C = 18.56, D = 37.12, and E = 92.81. * $p < 0.05$ versus Group A

Fig. 3. Influence of fenvalerate on cell growth in GC-2 cells. **a** Morphologic changes of GC-2 cells in response to fenvalerate (0, 0.1, 1.0, 5.0, 10, 100 μM) for 24 h. Representative images, Magnification $\times 200$. **b** Influence of fenvalerate on cell survival rates in GC-2 cells, $*p < 0.05$ versus 0 μM fenvalerate

Fig. 4. Impact of Fen on cellular LDH release and ATP content in GC-2 cells. **a** Cellular LDH release in GC-2 cells after fenvalerate (0, 0.1, 0.5, 1.0, 5.0, 10, 50, 100 μM) treatment for 24 h; **b** cellular ATP content in GC-2 cells after fenvalerate (0, 0.1, 0.5, 1.0, 5.0, 10, 50, 100 μM) treatment for 24 h. * $p < 0.05$ versus 0 μM fenvalerate

Fig. 5.

Effects of fenvalerate on the expression of CaM expression in GC-2 cells. **a** Fenvalerate induced mRNA expression of CaM in GC-2 cells in a time-dependent manner. $*p < 0.05$ versus 0 h. **b** Representative immune blots of CaM protein in GC-2 cells. **c** Semi-quantitation of blots density, $*p < 0.05$ versus 0 h

Table 1

Effects of fenvalerate on reproductive system-related markers in mice

Group	Dosage (mg/kg bw)	N	Testis/BW (mg/g bw)	Sperm motility (%)	Sperm head abnormality rate (%)
A	0	10	7.41 ± 0.57	67.43 ± 9.80	5.53 ± 0.57
B	3.71	10	6.97 ± 0.76	60.96 ± 1.36	9.53 ± 0.55
C	18.56	10	6.64 ± 0.69	57.27 ± 0.33	12.98 ± 3.09 [*]
D	37.12	10	6.21 ± 0.54 [*]	49.25 ± 1.77 [*]	15.98 ± 3.38 ^{**}
E	92.81	10	5.92 ± 0.29 [*]	49.50 ± 9.90 [*]	17.35 ± 2.17 ^{**}

^{*} versus Group A, $p < 0.05$ ^{**} versus Group A, $p < 0.001$