Original Article

N-hexane Alters the Maturation of Oocytes and Induces Apoptosis in Mice*

LIU Jin[1], HUANG Lei[1], SUN Yan[2], LI Yu Chen[1], ZHU Jian Lin[1],
WANG Wen Xiang[3], and ZHANG Wen Chang[1,#]

1.Key Laboratory of Environment and Health of Fujian key universities, Fujian Medical University, Fuzhou 350004, Fujian, China; 2.Fujian Provincial Hospital of Maternal and Child Health Care, Fuzhou 350004, Fujian, China; 3.Department of Food, Health and Nutrition Science, University of Public Health and Key Laboratory of Environment and Health, Fujian Medical University, Fuzhou 350004, Fujian, China

Abstract

Objective This study was aimed to determine the effects of n-hexane on the maturation of mouse oocytes.

Methods Cell culture was used to observe the maturation of mouse oocytes and CLSM was employed to determine their apoptosis.

Results Germinal vesicle breakdown (GVBD) and extrusion of the first polar body in mouse oocytes were significantly inhibited by n-hexane. After fertilization, the number of eggs in the mouse was significantly reduced by n-hexane. Mitochondrial membrane potentials (ΔΨm) were altered in mouse oocytes that were leading to apoptosis of the oocytes.

Conclusion N-hexane might have affected the maturation of oocytes, causing alteration of ΔΨm and leading to apoptosis which maybe one of the most important mechanisms.

Key words: N-hexane; Maturation; Fertilization; Mitochondrial membrane potential

Biomed Environ Sci, 2013; 26(9):735-741 doi: 10.3967/0895-3988.2013.09.004 ISSN:0895-3988

www.besjournal.com(full text) CN: 11-2816/Q

INTRODUCTION

Gradual decline in human fertility coincides with intensive industrial and agricultural development and the concomitant release of chemical wastes into the environment. Adults are exposed mainly through the ingestion of contaminated drinking water, meat, fat-dairy products and by breathing polluted air. Contaminants also seem to accumulate in organisms and can cause endocrine disruption at environmentally realistic exposure levels[1].

A large amount of n-hexane is widely used in industry as a solvent or a component of mixed solvents used in the production of adhesives, paints, and cleaning products, etc. Studies have found that n-hexane is toxic to the female reproductive system. They found that the exposure to n-hexane and other organic solvents is hazardous for reproduction in females[2]. In Fujian Province of China, thirty workers in a shoemaking factory were poisoned with n-hexane, which damaged their reproductive systems and caused them to develop various symptoms such as a decline in sexual function and menstrual abnormalities[3]. Also in the mainland of China, eleven workers in Hebei province experienced menstrual abnormalities after exposure to n-hexane[4]. In China, workers in thousands of shoemaking factories are often exposed to organic solvents such as n-hexane. The majority of these

*This work was supported by the National Nature Science Foundation of China, 30972514.

#Correspondence should be addressed to ZHANG Wen Chang. Tel: 86-591-83569235. Fax: 86-591-83569200. E-mail: wenchang2008@126.com

Biographical note of the first author: LIU Jin, female, born in 1983, Ph. D, majoring in Toxicology.

Received: April 28, 2012; Accepted: July 6, 2012

workers are female. In the context of potential risks to the female reproductive system, this study is focused on the effect of n-hexane on maturation and fertilization of mouse oocytes.

N-hexane has been reported to induce impairment of mouse ovaria via alterations in hormone secretion and promote apoptosis of granulosa cells[5]. In our previous study, we found that n-hexane could affect follicle maturation in the mouse. After treatment, the tertiary follicles reduced and the atretic follicles increased. Furthermore, there were fewer eggs in the mouse after super-ovulation[6]. 2, 5-hexanedione is a metabolite of n-hexane in the human body, and the toxicities of 2, 5-HD are related with n-hexane. We also found that 2, 5-HD might cause increased apoptosis in human ovarian granulosa cells through BCL-2, BAX, and CASPASE-3 signaling pathways[7]. Furthermore, we found that 2, 5-HD induced the apoptosis of ovarian granulosa cells by inhibiting protein expression of NF-κB, activating NF-κB, up-regulating mRNA levels of fasl and bax, and down-regulating mRNA levels of xiap and bcl-2[8].

The effects of n-hexane on neuropathy have been known for a long time, but only some data have been reported concerning its effects on reproduction, specifically on oocyte maturation, including maturation and fertilization. One of the crucial steps during maturation is the formation of mitochondria, which provides energies required for the maturation, such as asymmetric cellular division and formation of a polar body. At the same time, our studies found that n-hexane had toxic effects on the reproductive system of female mice, and induced the apoptosis of ovarian granulosa cells. Oocyte maturation connects with granulosa cells, and the apoptosis of ovarian granulosa cells may induce immature oocytes.

Apoptosis is a cellular process involving a genetically programmed series of events leading to the death of a cell. During this process, several key events occur in the mitochondria, including the release of caspase activators such as cytochrome C, changes in electron transport, and loss of ΔΨm. For this reason, ΔΨm is an important parameter of mitochondrial function and has been used as an indicator of cell health. The present study is focused on the effect of n-hexane on maturation and fertilization of mouse oocytes. At the same time, it attempts to explore the mechanisms of such effect on oocytes.

MATERIALS AND METHODS

Animals

Forty 21 days old immature female ICR mice, forty 56 days old mature female ICR mice and twenty 56 days old mature male ICR mice were used (provided by Fuzhou CDC Laboratory Animal Co. Ltd, China, 2005-0001). Ten mice per cage were housed in a room with controlled lighting (lights on 06:00-20:00 h), temperature (25±1 °C) and humidity (50%-60%). The animals were fed with a mouse diet and water ad libitum. The selected mice had at least three consecutive regular estrous cycles of four to five days in length before initiation of the study.

Animal Models

To study the effect of static inhalation of n-hexane (purchased from Sigma Chemical Corp. St. Louis, MO, USA, 100%) on the reproductive function of female mice, the mice were housed in a L-RDJ/1000 automatic static type exposure cabinet (Jiufang Electronics Corp. Guangzhou, China). N-hexane was given to four groups of female mice for a one-week period (8 h of exposure per day, 7 days/week) at concentrations of 0, 5.7, 22.5, and 90.9 mL/m^3.

Collection, Culture, and Maturation of Oocytes Mouse Oocytes

After treatment with n-hexane, all protocols followed those described by Polanski et al. (2005). To obtain oocytes arrested at prophase I of meiosis (MI), the ovaries were isolated from 21 days old ICR female mice and transferred to pre-warmed (37 °C) M2 medium[9] supplemented with 4 mg/mL BSA and 50 g/mL dibutyryl cyclic AMP (dbcAMP) to prevent immature oocytes from undergoing germinal vesicle breakdown (GVBD). The ovarian follicles were punctured to release the enclosed oocytes, and the immature oocytes displaying a germinal vesicle (GV) were collected. The groups of oocytes used to examine the effects of n-hexane were washed to remove dbcAMP to induce maturation, and were cultured in a M2 medium containing n-hexane under paraffin oil at 37 °C. GVBD and formation of the first polar body were observed under confocal laser scanning microscope (CLSM, Leica).

ΔψM Identification

The mitochondrial membrane potentials (ΔΨm) were visualized using Rhodamine123 (Sigma; 10 μg/mL). Fixation and labeling of mouse oocytes

were performed as follows: mouse oocytes in each group were collected and then washed three times with M2. Then they were stained using the mito-chondrion-specific fluorophore rhodamine 123 prepared from a solution of 10 μg/mL R123 (Sigma Aldrich) for 15 min at 37 °C. Afterwards they were washed three times with M2 again, and then were quickly put under CLSM (Leica) to identify their condition. Their excitation and emission wavelength were at 488 nm and 530 nm, respectively. Bright red fluorescence could be observed.

Apoptosis Identification

Early apoptosis was visualized using JC-1 (sigma; 10 μg/mL), a new cytofluorimetric, lipophilic cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'- tetraethylb-enzimi-dazolylcar-bocya-nine iodide (JC-1). JC-1 selectively enters mitochondria and reversibly changes color from green to red as the membrane potential increases. In healthy cells with high mitochondrial ΔΨm, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic or unhealthy cells with low ΔΨm, JC-1 remains in the monomeric form, which shows only green fluorescence. The samples were observed with a Leica CLSM.

Fixation and labeling of mouse oocytes were performed as follows: Mouse oocytes in each group were collected and then washed three times with M2. All oocytes were stained for 30 min at 37 °C with JC-1 at a concentration of 1 mmol/L[11]. After staining, the oocytes were washed through sequential changes of the medium. An inverted CLSM (DFC-480, Leica) was used to observe the results. Their excitation and emission wavelength were at 488 nm and 543 nm, respectively. Then quantitative analyses were made upon the stored image to obtain the mean intensity of the fluorescence. After the ratios of red and green fluorescence were determined, the early apoptosis of oocytes could be observed.

Fertility Assessment

After exposure to n-hexane, the adult control and the tested female mice were super ovulated by intraperitoneal injections of a pregnant mare's gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) at 48 h apart. Then, in the morning, the females were mated with normal male mice (ICR) overnight (two females per one male). The presence of spermatozoa in the vaginal smear in the next morning was indicative of copulation and was considered as day-zero of pregnancy. Fertilized eggs were collected at 24 and 48 h post-pregnancy for the determination of the number of embryos under a light microscope.

Statistical Analysis

Data were expressed as mean±S.E.M. and were subjected to one-way ANOVA followed by a Dunnett test or a Student-Newman-Keuls multiple comparison tests based on homogeneity of variance. Data were expressed as percentage and were subjected to the Chi-square test. Sigma Stat statistical software (SPSS 13.0) was employed. Significant differences were established at $P \leq 0.05$.

RESULTS

N-hexane Affects Oocyte Germinal Vesicle Breakdown

First, the maturation of the immature oocytes, arrested at phase I of mitosis in the M2 medium containing increasing concentrations of n-hexane was examined (shown in Figure 5A).

Figure 1 indicates that under all conditions and even at high concentrations of n-hexane, GVBD occurred, normally with the same efficiency and significant delay as compared to the control at the start of the experiment.

In 0 h, The GVBD rate of the control group was 57.23%, while that of the treated groups was lower (the GVBD rate in the 90.9 mL/m^3 group was 52.51%).

After 24 h culture, under the same conditions, GVBD occurred, but the experiment groups (5.7 and 90.9 mL/m^3 n-hexane) behaved abnormally with the different efficiency and significant delay (or not shown), as compared to the control ($P<0.01$). The GVBD rate of the control group was 57.38%. The GVBD rate of the 5.7 and 90.9 mL/m^3 groups was 40.79% and 34.43%, lower than that of the control group. In the 22.5 mL/m^3 group the GCBD rate was 52.42%, also lower than in the control, but the difference was not significant.

N-hexane Inhibits the Release of the Oocyte First Polar Body

After the GVBD occurred, the oocyte went to the formation of the first polar body (shown in Figure 5D). We found that the presence of n-hexane prevented the formation of the first polar body (Figure 2).

This event occurred both in 0 h, and after 24 h culture 90.9 mL/m^3 n-hexane-treated eggs expelled polar bodies that were significantly lower in number than those observed in the control eggs (P<0.01).

In 0 h, the release rate of the first polar body in the control group was 3.38%, and then the release rate decreased to 1.08% in the 90.9 mL/m^3 n-hexane-treated group (Figure 2).

After 24 h culture under the same conditions, the first polar body released, but the percentage of the released first polar body in the 90.9 mL/m^3 group was 5.21%, as compared to 12.03% in the control group.

N-hexane Affect Oocyte Germinal Vesicle Breakdown

Cell deaths happened when oocytes were exposed to n-hexane (Figure 5B), some cells in the high group appeared to display abnormal cell morphology, and the cytoplasmic distribution and cell membrane had ruptured (Figure 5C).

The death rates in the groups treated with n-hexane at different concentrations, were higher than those in the control group (P<0.01 or P<0.05).

In 0 h, the death rates in the control group were 14.81%, and then the death rates increased to 20.86% in the 90.9 mL/m^3 n-hexane-treated group (Figure 3).

After 24 h of culture, the death rate induced by 22.5 and 90.9 mL/m^3 n-hexane was higher than that in the control group (P<0.01 or P<0.05). The death rate in the control group was 27.49%, while it increased to 34.8% and 58.85% in the 22.5 and 90.9 mL/m^3 n-hexane-treated group, respectively.

Effect of N-hexane on Fertilization of Mouse Oocytes

Normally, the development of matured oocytes stops at metaphase II of meiosis, where they wait to be fertilized. The present study was designed to clarify whether n-hexane was capable of preventing the oocytes from being fertilized. While establishing cohorts of female mice for short-term observation, we noticed that the females yielded smaller offspring, which prompted us to measure the impact of n-hexane on female fertility. In the experiment, we noticed that female fertility abnormalities were higher with the increased concentrations of n-hexane. These results are not shown in this article. We also found that after the female mice became pregnant, the embryos that we collected were different in each group. Figure 4 indicate that after exposure to n-hexane, the number of embryos were

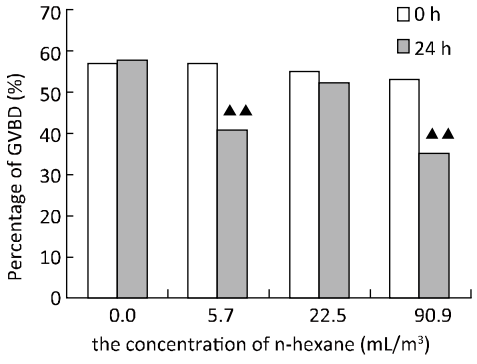

Figure 1. Percentage of GVBD observed in mouse oocyte populations matured in the presence or absence (control) of n-hexane after 0 and 24 h culture in the experiment. 150 to 300 eggs were counted in each group in the experiment reported here. ▲▲P<0.01 compared with control.

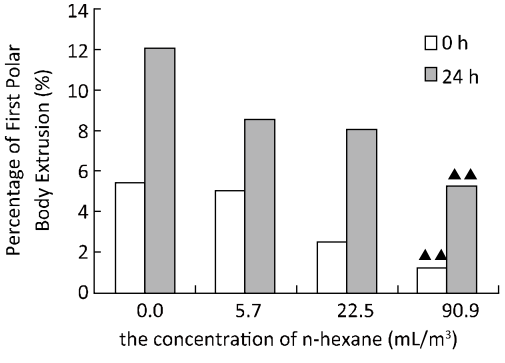

Figure 2. Extrusion of the first polar body by mouse oocytes matured in the presence or absence (control) of n-hexane after 0 and 24 h culture in the experiment. 150 to 300 eggs were counted in each group in the experiment reported here. ▲▲P<0.01 compared with control.

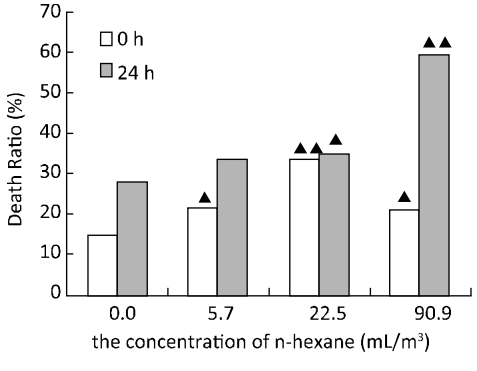

Figure 3. Death rate in the presence or absence (control) of n-hexane after 0 and 24 h culture in the experiment. 150 to 300 eggs were counted in each group in the experiment. ▲▲P<0.01, ▲P<0.05 compared with control.

significantly decreased as compared with those observed in the control eggs (P<0.01).

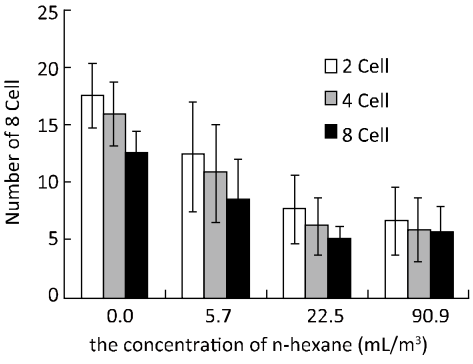

Figure 4. *In vivo* development of embryos from ICR female mice crossed with ICR male mice. 8 cell embryos were collected at 24, 48 and 56 h post-pregnancy and 8 to 10 female mice were counted in this experiment. Results are mean±SE. ▲▲P<0.01 compared with control.

N-hexane Triggers a Decrease in the Mitochondrial Membrane Potential (ΔΨm)

After 24 h of culture, a laser scanning confocal microscope with an excitation wavelength of 505 nm and emission wavelength of 534 nm was used for analytical purpose. The experiment was repeated three times and the average values were used. The mitochondrial membrane potential (Rhodamine 123) began to decline after the n-hexane treatment, which was positively correlated with the dose. The mitochondrial potential in the oocyte cells after 5.7, 22.5, and 90.9 mL/m^3 treatments was significantly lower than that of the control group (P<0.01) (Table 1). The mitochondrial distributions of all of the dosage groups were variable; however, the mitochondria of the control group were more concentrated in the perinuclear regions, displaying higher red fluorescence (Figure 5E), and the mitochondria of the treated groups were scattered in the cytoplasm, while the presence of the highest concentration of n-hexane displayed lower red fluorescence (Figure 5F).

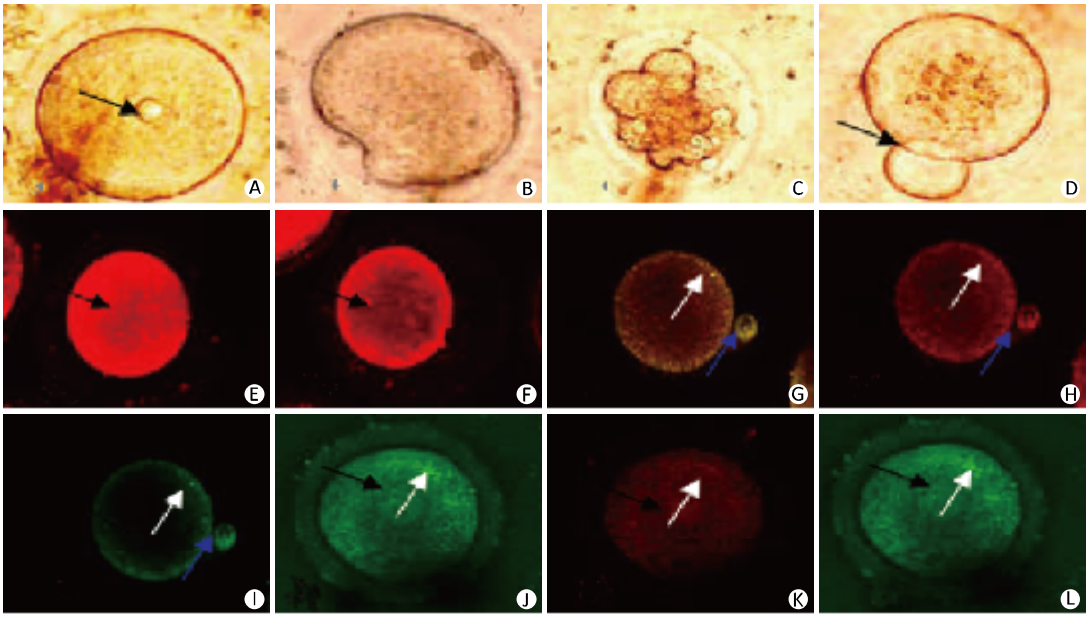

Figure 5. The typical pattern, mitochondrial polarization and distribution of pericortical J-aggregate fluorescence in mouse oocyte. The typical patterns of oocyte in different stages are shown from A-D. A is the normal oocyte with germinal vesicle (black arrow). B and C are abnormal oocytes when they are exposed to n-hexane. D is the normal oocyte with first polar body (black arrow). E is the normal mature oocyte stained with Rhodamine 123 and has higher red fluorescence than the abnormal cell (black arrow F). In the control group, the MII oocyte with normal first polar body (blue arrows) stained with JC-1 (G-I). G was mixed with red fluorescence (H) and green fluorescence (I). The oocyte in the treated group, with nuclear (white arrow) stained with JC-1 (J-L). G was mixed with red fluorescence (K) and green fluorescence (L). A-D were indicated by CLSM (DFC-480, Leica). E-L was CLSM (DFC-480, Leica).

N-hexane Induced Oocyte Apoptosis

JC-1 was used to identify the early apoptotic oocyte. With the intensity of n-hexane, the red fluorescence in the oocyte cells increased and then decreased. On the other hand, the green fluorescence in oocyte cells increased with the dose intensity. The apoptotic or unhealthy oocyte cells increased after 22.5 and 90.9 mL/m^3 treatments, which were significantly higher than those of the control group (P<0.01, P<0.05) (Table 1). Figure 5 shows that the oocytes treated in the highest presence of n-hexane displayed a lower red fluorescence and higher green fluorescence. On the contrary (Figure 5), the oocytes matured in the control group showed higher red fluorescence and lower green fluorescence.

Table 1. Mitochondrial Polarization and Apoptosis in Oocytes after Exposure to N-hexane in Mice

Group (mL/m^3)	Oocytes Treated	
	Rhodamine 123	JC-1 (Red/Green)
Control	27.26±20.37	2.80±1.45
5.7	3.23±0.42▲▲	1.49±2.91
22.5	7.47±0.38▲▲	0.69±0.36▲▲
90.9	2.89±0.37▲▲	0.72±0.27▲

Note. One-way ANOVA, ▲▲P<0.01, ▲P<0.05 compared with control.

DISCUSSION

The results presented in this paper suggest that the solvent can alter oocyte maturation in mammals. The effects were observed at relatively low doses of the solvent. Such concentrations in contaminated areas, such as shoe manufacturing establishments are likely to occur. Acute solvent poisoning may also occur, particularly in the developing countries of Asia, South America, or Africa where the control is poor, and where various solvents are used. Workers, who may not always wear protective gloves and respirators, may be exposed during the manufacturing, handling, or spraying these solvents.

N-hexane solvent can also accumulate in mammals including humans, especially females. Female workers were found to experience menstrual abnormalities after exposure to n-hexane and a concentration of 2, 5-hexane was detected in their blood[4]. In our study, it was found that the development of reproductive organs such as ovaries and follicle altered after exposure to n-hexane[5]. Therefore, at the same dose of n-hexane and in cases of poisoning, the oocytes could also have possible exposure to concentrations of n-hexane.

Oocyte maturation includes nuclear maturation and cytoplasmic maturation, and both of them are essential for fertilization and embryo development[12]. Nuclear maturation is characterized by the release of the first polar body[13], however, it is difficult to evaluate cytoplasmic maturation. Some reported that the capability of fertility was the most important characteristic, while the others assumed that mitochondria might also be used to estimate cytoplasmic maturation[14]. Mitochondria in the oocyte can provide adenosine triphosphate (ATP) for fertilization and pre-implantation in embryo development[14], and they can act as stores of intracellular calcium (Ca) and pro-apoptotic factors as well[15].

In this experiment, we observed that n-hexane inhibited GVBD in mouse oocytes, even when used at 5.7 mL/m^3. However, n-hexane could prevent the formation of the first polar body only at a concentration of 90.9 mL/m^3. The death of oocytes in the experiment increased after treatment with different concentrations of n-hexane. We found that there were no clear dose-response relationships in the results. The reason maybe that 5.7 mL/m^3-n-hexane is toxic and inhibits GVBD in mouse oocytes, after exposure to 22.5 mL/m^3-n-hexane. Oocytes can regulate themselves for a moment, but when the dose increases to 90.9 mL/m^3, the toxicity becomes stronger and oocytes lose their ability. We also observed elevated abnormal fertilization in the fertilization of mouse oocytes that matured in the presence of n-hexane, during which 2-cell, 4-cell, and 8-cell embryos decreased after exposure to n-hexane. All these findings strongly suggest that n-hexane can alter the maturation of oocytes. This can be at the origin, altering further development, as seen in mouse. It is then clear that the exposure of animals reaching sexual maturity to this organochlorine pesticide can severely affect their fertility and lineage.

The role of mitochondria during oocyte and early embryonic development in a mammal is usually viewed in terms of a singular purpose, the generation of ATP[16]. Inappropriate culture conditions may inhibit mitochondrial movement to the inner cytoplasm, thus affecting its cytoplasmic maturation[14]. At the same time, the magnitude of

the potential difference (polarity) across the inner mitochondrial membrane (ΔΨm) determines the levels of several mitochondrial activities, including ATP generation, focal regulation of calcium homeostasis and organelle volume homeostasis[17]. In this experiment, we also found that after n-hexane treatment, mitochondrial membrane potential began to decline and apoptotic oocyte cells appeared. The change of mitochondrial permeability is considered the earliest event in the cell apoptosis cascade. The mitochondrial membrane potential change results in the release of cytochrome C to initiate caspase-9 and -3 activation and subsequent apoptosis[18-19]. The change of mitochondrial membrane potential was investigated by Rhodamine 123 staining. In normal cells, mitochondria can absorb Rhodamine 123, and its absorptivity will decline while the membrane potential decreases. The findings of the present study have revealed that the mitochondrial membrane potential begins to decline after the n-hexane treatment. The same outcomes were observed using JC-1 staining. JC-1 can also reflect mitochondrial membrane potential citation and the apoptosis of cells.

In conclusion, the findings of the present study suggest that n-hexane can alter the maturation of oocytes in mice. The change in mitochondrial membrane potential and the occurrence of apoptotic cells appear to be the most important mechanisms.

REFERENCES

1. Vos JG, Dybing E, Greim HA, et al. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Crit Rev Toxicol, 2000; 30, 71-133.
2. Sallmen M, Neto M, Mayan ON. Reduced fertility among shoe manufacturing workers. Occup Environ Med, 2007; 10. (Abstract 1136)
3. Wang WY, Wu AS. Investigated n-hexane poised in QuanZhou and PuTian City. Strait Journal of Preventive Medicine, 2003; 01, 31-2. (In Chinese)
4. Xuechun Xu. Chronic n-hexane poising in clinic. Industrial Disease, 2004; 30, 175-7. (In Chinese)
5. Liu J, Huang HL, Pang F, et al, The Effect of n-Hexane on the Reproductive System of Female Mice. Biomed Environ Sci, 2012; 25(2), 189-96.
6. Huang HL, Jin L, Fen P, et al. The impact of n-hexane on the secretion of mouse estrogen and progesterone. Advances in Bioscience and Biotechnology, 2011; 2, 429-33.
7. Yan S, Yuan L, Hong L, et al. 2011 2,5-Hexanedione induces human ovarian granulosa cell apoptosis through BCL-2, BAX, and CASPASE-3 signaling pathways. Arch Toxicol, 2012 Feb; 86(2), 205-15.
8. Wenchang Zhang, Lei Huang, Cancan Kong, et al. 2012. Apoptosis of rat ovarian granulosa cells by 2,5-hexanedione *in vitro* and its relevant gene expression. J Appl Toxicolo, DOI 10.1002/jat.2714.
9. Whittingham DG. Culture of mouse ova. J Reprod Fertil, 1971; 14, 7-21.
10. George A, Thouas, Alan O, et al. Effect of Female Age on Mouse Oocyte Developmental Competence Following Mitochondrial. Biology of Reproduction, 2005; 73, 366-73.
11. Van Blerkom J, Davis P, Mathwig V, et al. Domains of high-polarized and low-polarized mitochondria may occur in mouse and human oocytes and early embryos. Hum Reprod, 2002; 17, 393-406.
12. Eppig JJ. Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reprod Fertil Dev, 1996; 8, 485-9.
13. Fulka JJ, First NL, Moor RM. Nuclear and cytoplasmic determinants involved in the regulation of mammalian oocyte maturation. Mol Hum Reprod, 1998; 4, 41-9.
14. Torner H, Brüssow KP, Alm H, et al. Mitochondrial aggregation patterns and activity in porcine oocytes and apoptosis in surrounding cumulus cells depend on the stage of pre-ovulatory maturation. Theriogenology, 2004; 61, 1675-89.
15. Wang LY, Wang DH, Zou XY, et al. Mitochondrial functions on oocytes and pre-implantation embryos. Journal of Zhejiang University Science B, 2009; 1581-73. (In Chinese)
16. May-Panloup P, Chretien MF, Malthiery Y, et al. Mitochondrial DNA in the oocyte and the developing embryo. Curr Top Dev Biol, 2007; 77, 5-83.
17. Jonathan Van Blerkoml, Patrick Davis. Mitochondrial signaling and fertilization. Molecular Human Reproduction, 2007; 13, 759-70.
18. Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med, 2000; 29, 222-30.
19. Kowald A, Kirkwood TB. Accumulation of defective mitochondria through delayed degradation of damaged organelles and its possible role in the ageing of post-mitotic and dividing cells. J Theor Biol, 2000; 202, 145-60.