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TITLE:

Experimental protocol for detecting mitochondrial function in hepatocytes exposed to organochlorine pesticides

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KEYWORDS:

Mitochondrial function, mitochondrial ultrastructure, mitochondrial fluorescence intensity, adenosine triphosphate, mitochondrial membrane potential, oxygen consumption rate

SHORT ABSTRACT:

Understanding the influence of environmental organochlorine pesticides (OCPs) on mitochondrial function in hepatocytes is important in exploring the mechanism of OCPs causing metabolic disorders. This paper presents detailed methods on detecting hepatic mitochondrial function.

LONG ABSTRACT:

This paper presents detailed methods on detecting hepatic mitochondrial function for a better understanding the cause of metabolic disorders caused by environmental organochlorine pesticides (OCPs) in hepatocytes. HepG2 cells were exposed to β-hexachlorocyclohexane (β-HCH) for 24 h at an equivalent dose of internal exposure in general population. Ultrastructure in hepatocytes was examined by transmission electron microscopy (TEM) to show the damage of mitochondria. Mitochondrial function was further evaluated by mitochondrial fluorescence intensity, adenosine 5'-triphosphate (ATP) levels, oxygen consumption rate (OCR) and mitochondrial membrane potential (MMP) in HepG2 cells incubated with β-HCH. The mitochondria fluorescence intensity after stained by mitochondrial green fluorescent probe was observed with a fluorescence microscopy. The luciferin-luciferase reaction was used to determine ATP levels. The MMP was detected by the cationic dye JC-1 and analyzed under flow cytometry. OCR was measured with an extracellular flux analyzer. In summary, these protocols

were used in detecting mitochondrial function in hepatocytes with to investigate mitochondria damages.

INTRODUCTION:

The effects of organochlorine pesticides (OCPs) on health, *e.g.* reproductive interference, immunological toxicity, metabolic changes have been previously studied[1-3]. The methods to detect cellular metabolism and find out mitochondrial dysfunction have enabled scientists to understand the role of mitochondrial function (*i.e.*, mitochondrial STAT3 levels, lactate, pyruvate, lactate-to-pyruvate ratio, coenzyme Q10, mitochondrial proton leak, bioenergetics, biogenesis, and dynamics) in areas such as aging, obesity, diabetes, cardiovascular function, cancer, and safety toxicity[4-7]. In this paper, we describe the methods on assessing mitochondrial dysfunction caused by OCPs.

We exposed HepG2 cells to β-hexachlorocyclohexane (β-HCH), one representative OCPs, for 24 h at a dose equivalent to internal exposure of human. Firstly, TEM was applied to observe the ultrastructure of hepatocytes, such as nuclei, mitochondria and endoplasmic reticulum[8]. Compared with ordinary microscopes, TEM enables to explore the 2D and 3D ultra-structure of cells and cell components (cell lines or tissue), the morphology, chemical composition, as well as function of natural or artificial materials that play a pivotal role in modern science and technology. Mitochondrial function was further evaluated by mitochondrial fluorescence intensity, adenosine 5'-triphosphate (ATP) levels, oxygen consumption rate (OCR) and mitochondrial membrane potential (MMP)in HepG2 cells incubated with β-HCH. Mito-tracker green is a mitochondria green fluorescent probe that can be used for live cell mitochondrial-specific fluorescent staining. The mitochondria in hepatocytes were stained by mito-tracker green solution and mitochondrial fluorescence intensity, number and pattern were observed with a confocal microscopy[9]. Mitochondrial green fluorescent probe can be used to stain live cells. Compared with rhodamine 123 or JC-1, mitochondrial green fluorescent probe does not depend on mitochondrial membrane potential for mitochondrial staining. ATP levels were determined by a luciferase-luciferin kit and normalized by protein concentration. ATP assay kit can be used to detect ATP levels in common solutions, cells or tissues. This kit is based on firefly luciferase catalyzed by fluorescein to generate fluorescence, when ATP is required to provide energy. When the firefly luciferase and fluorescein are excessive, in a certain concentration range, the generation of fluorescence is proportional to the concentration of ATP. In addition, this kit has been specially designed to optimize the chemiluminescence of ATP. ATP, as the most important energy molecules, plays an important role in the various physiological or pathological processes of cells. Changes in ATP levels can reflect defects in cell function, especially mitochondrial energy production. Usually under apoptosis, necrosis or in some toxic state, cellular ATP levels reduced [10]. MMPs assay kit with JC-1 is a kit that uses JC-1 as a fluorescent probe to detect cells, tissues or purified MMPs quickly and sensitively. It can be used for early detection of apoptosis. The cationic dye JC-1 is a fluorescent probe used to detect the MMP which can be analyzed under flow cytometry indicated by changes of green and red fluorescence ratio. When the MMP is high, JC-1 is aggregated in the matrix of the mitochondria to form a polymer (J-aggregates), which produces red fluorescence. When the MMP is low, JC-1 cannot accumulate, and forms monomer which produces green

fluorescence[11]. So the ratio of red and green fluorescence can reflect the level of MMP. The OCR of cells is a crucial indicator of normal cellular function[12]. It is regarded as a parameter to research mitochondrial function. Cell mito stress test kit provides a stable method for analyzing key parameters of mitochondrial function. The kit provides quality control and predictive reagents as well as a standard method for performing cell mitochondrial stress test. It can be used to detect all cell types, including primary cells, cell lines, suspended cells, and also for islets, nematodes, yeasts and isolated mitochondria.

OCR measurement can provide valuable insight into the physiological status or alterations of cells. It was determined with an extracellular flux analyzer to detect breathing baseline, proton leak, maximal respiratory, ATP turnover and reserve capacity. In brief, after baseline measurements of OCR, OCR was detected after sequentially adding to oligomycin (ATP Coupler), FCCP (mitochondrial oxidative phosphorylation uncoupler) and antimycin A/rotenone (an inhibitor of oxygen consumption) per well.

In an effort to facilitate the development of more specific protocol for detecting mitochondrial function in hepatocytes in vitro, we present here experiments by TEM, confocal microscopy, luminometer, flow cytometry and extracellular flux analyzer with future application in studying mitochondria damage related adverse outcomes.

PROTOCOL:

All experiments and the experiment protocols were performed in accordance with relevant guidelines and regulations and approved by the local Ethical Committee of Nanjing Medical University.

1. Mitochondrial ultrastructure by TEM

1.1. Collecting HepG2 cells

1.1.1. Seed HepG2 cells in 100 mm dishes. Store at 37 °C and 5% CO_2.

1.1.2. Digest cells with 0.25% EDTA in 1.5 mL EP tube.

1.1.3. Centrifuge at 1000 x g for 3 min at room temperature (RT). Discard the supernatant.

1.1.4. Collect 4-6 x 10^5 HepG2 cells.

1.2. Add 1 mL of 5% glutaraldehyde (Solvent: double distilled water) with pipettes and incubate at 4°C for 2 h.

1.3. Add and wash in 4 changes of 1 mL of phosphate buffer (containing Na_2HPO_4, KH_2PO_4, NaCl and KCl, pH 7.4), 15 min each. Suck out phosphate buffer with pipettes.

1.4. Add 200 μm of 1% osmium (Solvent: double distilled water) and incubate at 4 °C for 2 h

to black sample.

Caution: Osmium is highly toxic and volatile substance. It should be operated in the drug cabinet carefully.

1.5. Add and wash in 2 changes of 1 mL of phosphate buffer, 5 min each. Suck out phosphate buffer.

1.6. Stain in 2% uranyl acetate solution (Solvent: double distilled water and acetic acid) in 1.5 mL EP tube for 2 h.

1.7. Dehydrate and submerge through 50% acetone, 70% acetone, 90% acetone (Solvent: double distilled water), 2 changes of absolute acetone, 15 min each.

1.8. Penetrate in 2 drops acetone (100%)/embedding agent (1:1) for 1.5 h at RT. Epon812 embedding agent, the recipe is as follows: A: Epon812, 62 mL and DDSA, 100 mL; B: Epon812, 100 mL and MNA 89 mL. A:B=2:8 (v:v, in winter), A:B=1:9 (v:v, in summer). Embedding in embedding molds (Soft plastic plate with checkered grid).

1.9. Incubate sequentially at 37 °C for 12 h, 45 °C for 12 h, and then 60 °C for 48 h.

1.10. Prepare and observe ultrathin sections (the largest area cannot exceed 0.5 mm × 0.3 mm) by ultrathin sections machine and TEM (2 μm and 500 nm).

2. Mitochondrial fluorescence intensity detection

2.1. Seed 2 x 10^4 HepG2 cells in 6-well plates. Add β-HCH for 24 h.

2.2. Add anhydrous DMSO to formulate a final concentration of 1 mM mitochondrial green fluorescent probe.

2.3. Add 1 mL mitochondrial green fluorescent probe solution (final concentration: 200 nM) to each well of 6-well plates, incubate at 37 °C for 45 min.

2.4. Remove the mitochondrial green fluorescent probe solution and add freshly prepared cell culture medium (DMEM) at 37 °C prior to imaging.

2.5. Observe mitochondrial green fluorescence by a fluorescence microscope (10x). The maximum excitation wavelength at detection is 490 nm and the maximum emission wavelength is 516 nm.

3. Assay of the cellular ATP levels

3.1. Seed HepG2 cells in 6-well plates. Add β-HCH for 24 h.

3.2. Add 200 µL of lysis buffers from the luciferase-luciferin ATP assay kit to each well of 6-well plates. Centrifuge at 12,000 x g for 5 min at 4 °C, collect the supernatant with pipettes into new tubes.

3.3. Dilute the ATP detection reagent with the ATP dilution at a ratio of 1: 5, as ATP detection buffer.

3.4. Prepare standard curve determination: dilute the 0.5 mM of ATP standard solution to 10, 5, 1, 0.5, 0.1, 0.05, 0.01 µM by ATP lysis buffers.

3.5. Add 100 µL of supernatant of cell in a 96-well plate for 5 min at RT, and add 100 µL ATP detection buffer, detect by a luminometer (chemiluminescence detector). Calculate ATP concentration (nmol/L) though the standard curve.

3.6. Detect protein concentration by a BCA Protein Assay Kit with multimode reader.

3.6.1. Prepare of standard curve determination: dilute the 5 mg/mL of protein standard solution to 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL by double distilled water.

3.6.2. Add 1 µL of supernatant of cell in a 96-well plate and add 19 µL of double distilled water.

3.6.3. Add 200 µL of BCA detection solutions for each well. Incubate at 37 °C for 30 min. Detect OD (optical density) value by a multimode reader with 562 nm. Calculate the protein concentration (mg/mL) though the standard curve.

3.7. Correct the ATP content per mg protein concentration (unit: ATP concentration/protein concentration, nmol/mg).

4. Mitochondrial membrane potential (MMP) assessment by JC-1

4.1. Collect HepG2 cells seeded in 6-well plates. Digest cells with 0.25% EDTA in 1.5 mL EP tube, centrifuge at 1000 x g for 3 min, discard the supernatant and collect cells.

4.2. Add 50 µL of JC-1 (200X) to 8 mL of distilled water to vortex and mix. Add 2 mL of JC-1 (5X) staining dyeing buffer as JC-1 detection solution.

4.3. Incubate cells with a mixture of 0.5 mL of cell culture medium and 0.5 mL of JC-1 detection solution for 20 min at 37 °C.

4.4. Centrifuge at 600 x g for 3 min at 4 °C. Discard the supernatant.

4.5. Rinse in 2 changes of 1 mL of JC-1 (1X) dyeing buffer, centrifuge at 600 x g for 3 min at 4 °C. And discard the supernatant.

4.6. Suspend cells in 0.5 mL of JC-1 (1X) dyeing buffer, analyze via flow cytometry to detect green and red fluorescence. When the JC-1 polymer is detected, set the excitation light to 490 nm, and the emission light to 530 nm. When the JC-1 polymer is detected, set the excitation light to 525 nm, and the emission light to 590 nm.

5. Oxygen consumption rates (OCR) measurements

5.1. Seed HepG2 cells in cell culture microplates of 96-well at a density of 4500 cells/100 µL per well. Ensure cells covered with each well after 24 h.

5.2. Add the appropriate volume of the prepared reagents into the appropriate injection port, according to previous literature[13]. Port A: 25 µL 1 µM of oligomycin (ATP Coupler); Port B: 25 µL 0.75 µM of FCCP (Electronic Throttle Control, ETC Accelerator); Port C: 25 µL 0.5 of µM antimycin A/rotenone (mitochondrial Inhibitor A and mitochondrial Inhibitor B).

5.3. Store cartridge in a 37 °C incubator with no CO_2 until ready to use.

5.4. Make a medium change of the cell plate through removing the running medium from each well and adding to the new DMEM. Final volume for each well is 180 µL.

5.5. Store the cell plate in a 37 °C incubator with no CO_2 for 1 h before the assay.

5.6. Record OCR automatically by software.

5.6.1. Open the OCR software.

5.6.2. Choose Cell Mito Stress Test Kit in the Apps drop-down menu.

5.6.3. Click the Start App button.

5.6.4. Click the Run Stress Test button. The Cell Stress Test Setup screen appears.

5.6.5. Do the following steps:

5.6.5.1. Enter the number of cells seeded per well in the Cell seeding # box.

5.6.5.2. Enter the average OCR in the Average basal OCR box.

5.6.5.3. Enter the final working concentration for each reagent and inject the reagents.

NOTE: The average basal OCR value for the cell should have been detected before running the optimization assay when optimizing for cell seeding concentration.

5.6.6. Click the Next button. The group info screen appears.

5.6.7. Assign a group to the unassigned wells through choosing a color and giving a name, and then clicking on the appropriate wells.

NOTE: The different groups are defined as different treatments prior to running of the Cell Mito Stress Test. All wells of microplates will get the same reagent injections when the stress test is run.

5.6.8. Click the Next button. The Stress Test Injection Layout screen appears.

5.6.9. Click Start. The Stress Test is now run on the Analyzer. When the run is over, follow the point s in the software, remove and discard the cartridge and cell plate.

REPRESENTATIVE RESULTS:

The mitochondria cristae of HepG2 cells exposed to β-HCH were markedly damaged. Scattered mitochondria were mildly to markedly expanded, irregularly shaped, and mitochondrial ridge disappeared with relatively abnormal mitochondrial architecture (**Figure 1**).

Average mitochondrial green fluorescence intensity, which represents the mitochondria, decreased in HepG2 cells exposed to β-HCH (**Figure 2**), as well as in ATP levels (**Figure 3**). The fluorescence intensity and ATP level decreased gradually with increasing exposure concentrations. Potentially, the reduction in mitochondria number, or damaged mitochondria, caused the observed mitochondrial fluorescence intensity, and consequently the reduced production of ATP.

The results of flow cytometry demonstrated that the ratios of red/green JC-1 fluorescence in β-HCH group were significantly lower than in the control (**Figure 4**). OCR of HepG2 cells was reduced in a dose-dependent manner after β-HCH exposure. Compared with control group, basal respiration rates, proton leak, maximal respiratory capacity, and ATP turnover were significantly decreased in HepG2 cells exposed to β-HCH (**Figure 5**). These results indicated that mitochondrial function was impaired after β-HCH exposure.

All the instruments used in these experiments were show in **Supplemental Figure 1**.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative TEM micrographs of HepG2 cells exposed to β-HCH (A: 6000×, B: 25000×). Typical damages showed mildly enlarged mitochondria (M), electron-lucent matrices, damaged cristae and loose organelle gaps. M: mitochondria.

Figure 2: Detection of mitochondrial fluorescence in HepG2 cells treated with β-HCH[13]. The amount, location and fluorescence intensity of mitochondria **(A)** were showed in fluorescence microscopic images and quantitative levels of mitochondrial fluorescence intensity were based on mitochondrial green fluorescent per cell **(B)**. *: $P < 0.05$, ***: $P < 0.001$ compared with the

control. Each data point was the mean ± SEM from three separate experiments.

Figure 3: ATP levels in HepG2 cells[13]. ***: *P* < 0.001 compared with the control, ##: *P* < 0.01 compared with 10 ng/mL β-HCH. Data were presented as mean ± SEM of three separate experiments.

Figure 4: Effects on MMP by JC-1 staining and flow cytometry[13]. (A) Flow cytometry plots. The Y-axis showed the ratio of red to green fluorescence. PE: Red fluorescence, FITC: Green fluorescence. **(B)**. **: P < 0.01 compared with the control. Each data point was the mean ± SEM from three separate experiments.

Figure 5: Detection of cellular oxygen consumption rate (OCR). **(A)** The Effect of β-HCH on cellular OCR was measured by the extracellular flux analyzer. The four periods represent cellular basal respiration rate, ATP-synthase-inhibited rate, maximal uncoupled rate, and rotenone-or antimycin-A-inhibited rate. **(B)** Quantitative histograms of OCR results for baseline, proton leak, maximal respiratory capacity, ATP turnover and reserve capacity. *: *P* < 0.05, **: *P* < 0.01, ***: *P* < 0.001 compared with the control. Each data point was the mean ± SEM from three separate experiments.

Supplemental Figure 1: All the instruments used in protocols. (A) Transmission electron microscopy. **(B)** Laser scanning confocal microscope. **(C)** Luminometer. **(D)** Multimode reader. **(E)** Flow cytometry. **(F)** Extracellular flux analyser.

DISCUSSION:

Critical to the success of the detection protocol is the use of a variety of experimental methods that have been covered the study from phenotype to mechanism. In this study, HepG2 cells were cultured in DMEM with penicillin and streptomycin and 10% fetal bovine serum. When cells reached 40-50% confluence, β-HCH (0, 10, 100 ng/mL) were added and incubated for 24 h. We firstly used TEM which showed the ultrastructural changes in hepatocyte caused by the representative OCPs, β-HCH, showing the impairment of mitochondria structure **(Figure 1)**. Furthermore, fluorescent staining assay **(Figure 2)**, luciferase-luciferin ATP assay **(Figure 3)**, JC-1 assay **(Figure 4)** and cell mito stress test assay **(Figure 5)**, which commonly assess mitochondria dysfunction, were performed. These results serve as basis for investigation of the underlying molecular mechanisms.

In the above methods, investigators should pay attention to precautions in some steps. For example, in the preparation of electron microscopy cell, the number of cells should be paid attention, in order to avoid poor fixation caused by too large tissue or cell block. 5% glutaraldehyde acts as a fixative, it is better not to store more than half year so as to avoid failure of detection. Fixed samples are placed at 4 °C for 24 h or up to 1 month before the next step. Mitochondrial green fluorescent dye is easy to quench, and light should be avoided to slow the fluorescence quenching. In cellular ATP levels assay, when using a multifunctional luminometer that can detect chemiluminescence, opaque blackboard or whiteboard 96-well plates should be used to avoid mutual interference between adjacent holes. ATP, specifically

cleavage of ATP in the sample is not stable at room temperature, and the experiment need to be operated at 4 °C or on ice. ATP can be stable on ice for up to 6 h. In the preparation of JC-1 detection solution, JC-1 staining dyeing buffer (5X) can be added after the JC-1 (200X) is thoroughly dissolved and mixed with the ultrapure water, so that JC-1 detection solution will be easy to dissolve completely. JC-1 probe should be loaded and washed within 30 minutes and saved at 4 °C or ice to complete the follow-up test. In OCR measurements, the average basal OCR value for the cell should be detected before the optimization assay, when optimizing for cell seeding concentration.

There are some limitations of these methods. OCR measurement by an extracellular flux analyzer is limited to cell experiments. The detection of mitochondrial function is not deep enough since the metabolic products by mitochondria are not measured, such as measuring fatty acids and metabolites in TCA cycles in hepatocytes. Furthermore, the expression of genes and proteins with regard to hepatic fatty acid synthesis and degradation could be detected by real-time polymerase chain reaction and western blot, which can further confirm the molecular disorders of fatty acids metabolism and mitochondrial dysfunction[13]. Digital images of mitochondria can be captured in living cells under confocal microscopy and analyzed for changes of mitochondrial morphology based on form factor (FF) and aspect ratio (AR) values. The metabolic function of mitochondria was also assessed by measuring extracellular acidification rate (ECAR) using a bioenergetic analyzer[14]. Some mitochondrial marker antibodies (cytochrome c, HSP60, PHB1, SOD1, VDCA and STAT3) can be measured by western blot[4,15-17]. The activities of enzymes respecting mitochondrial function and the tricarboxylic acid (TCA) cycle, such as malic dehydrogenase, succinate dehydrogenase, citrate synthetase, ATPase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase are evidenced[18]. Function of the electron transport chain in cells and in isolated liver mitochondria is detected using high resolution respirometry[19].

Our protocols focus on the detection of mitochondrial morphology, structure, location, quantity, capacity, membrane potential and respiratory chain function. These are basically able to evaluate mitochondrial function from different aspects. What's more, these methods are simple and easy to operate. Mitochondrial function studies have been widely conducted in different areas, such as liver[20], gut microbiota[21], pluripotent stem cells[22], and in some common diseases, such as type 2 diabetes[23], Parkinson's disease[24] and inflammatory bowel disease[25]. There may be more diseases associated with mitochondria, and our protocols can be helpful in the investigation of relevant mechanisms. In addition, further empirical work is also needed on the comprehensive and in-depth scale of these effects with more experimental methods.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

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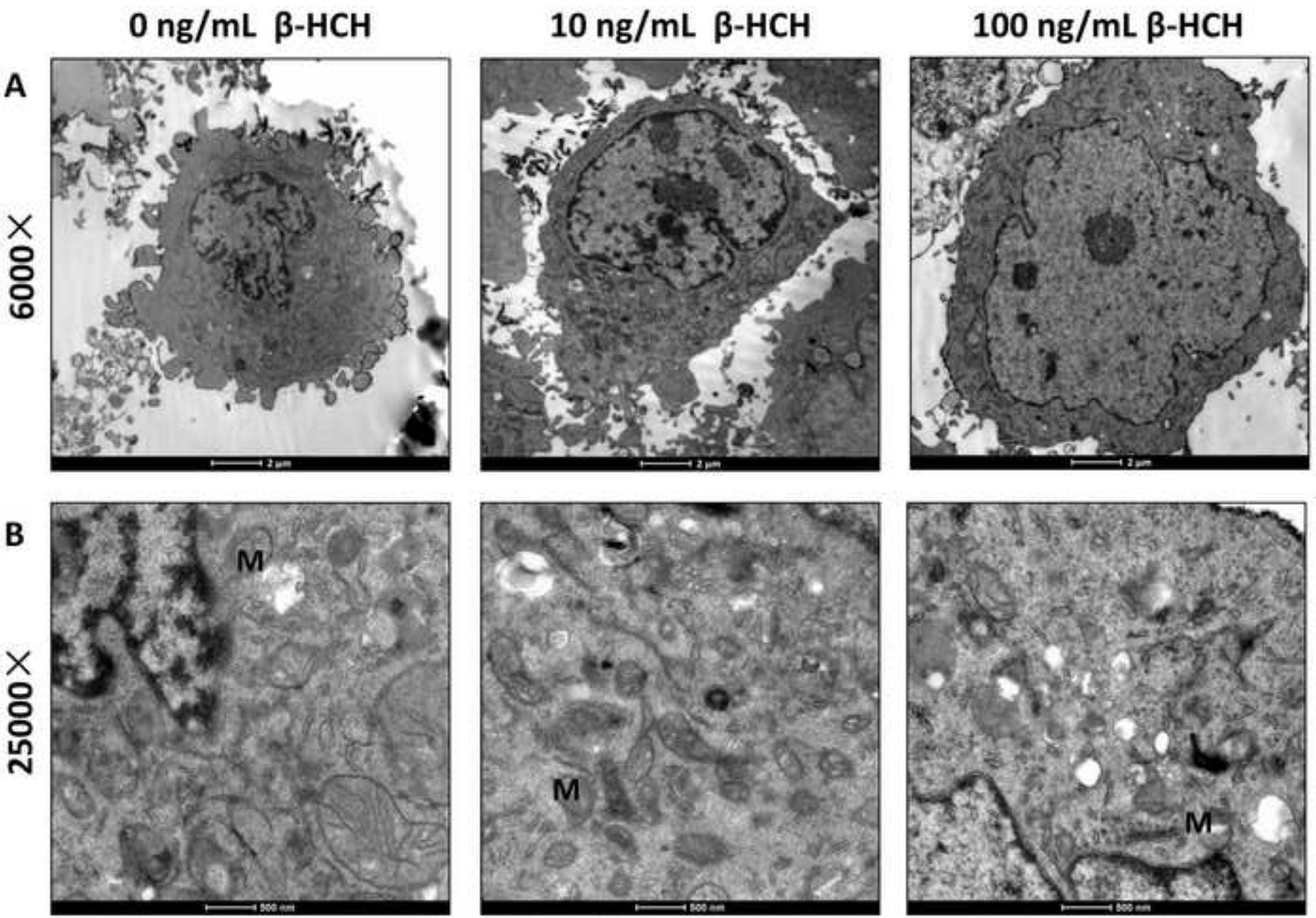

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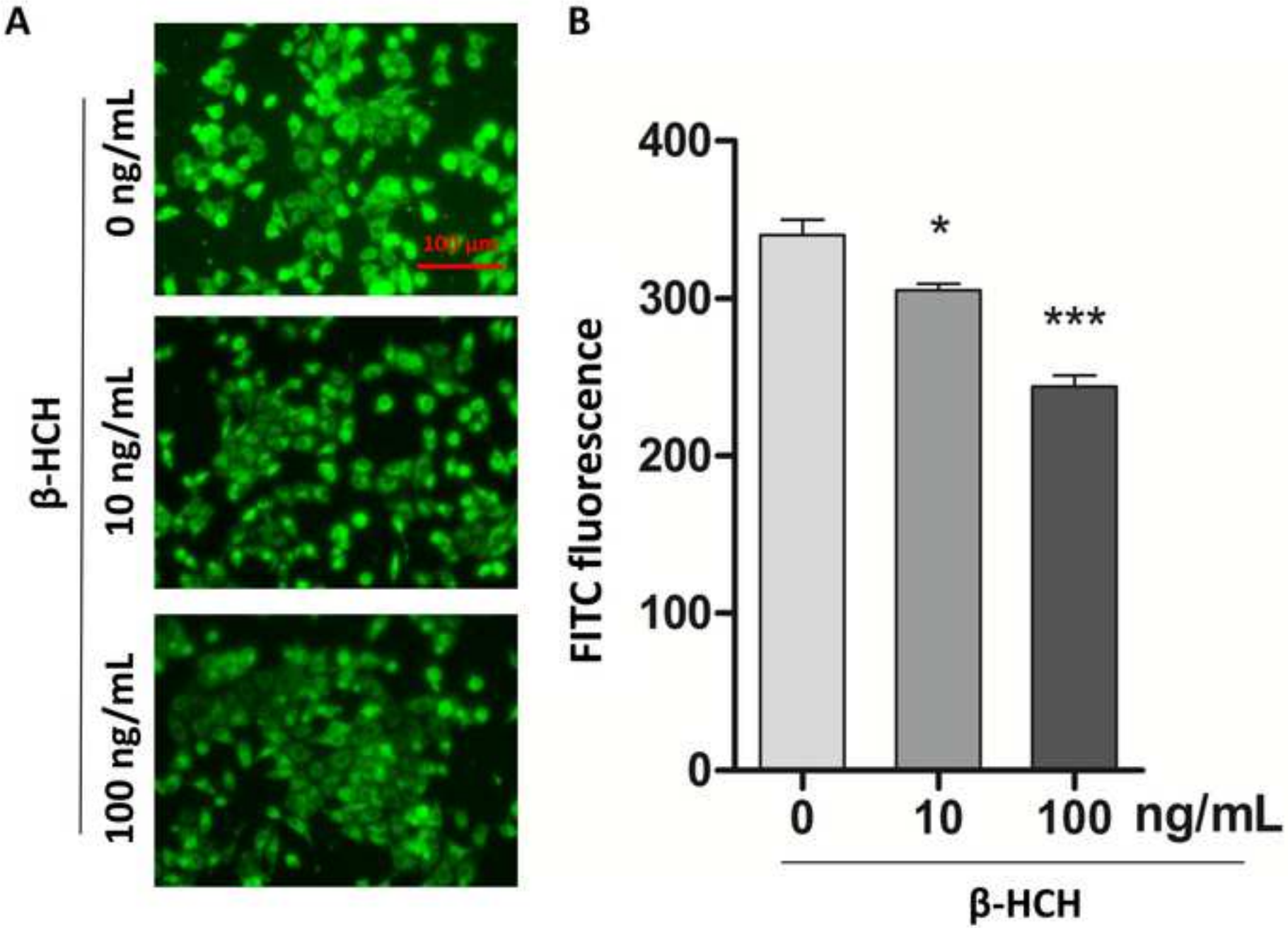

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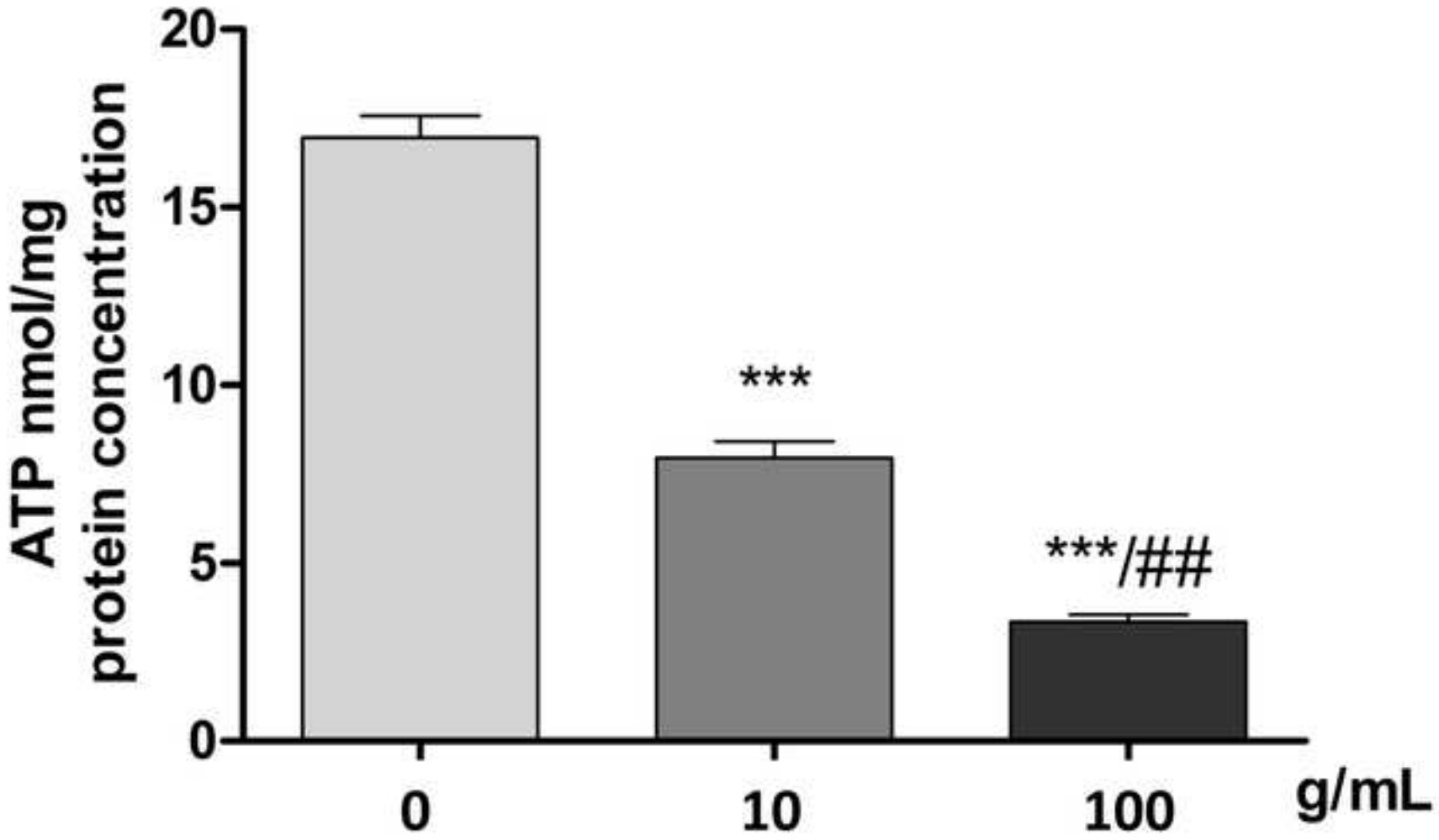

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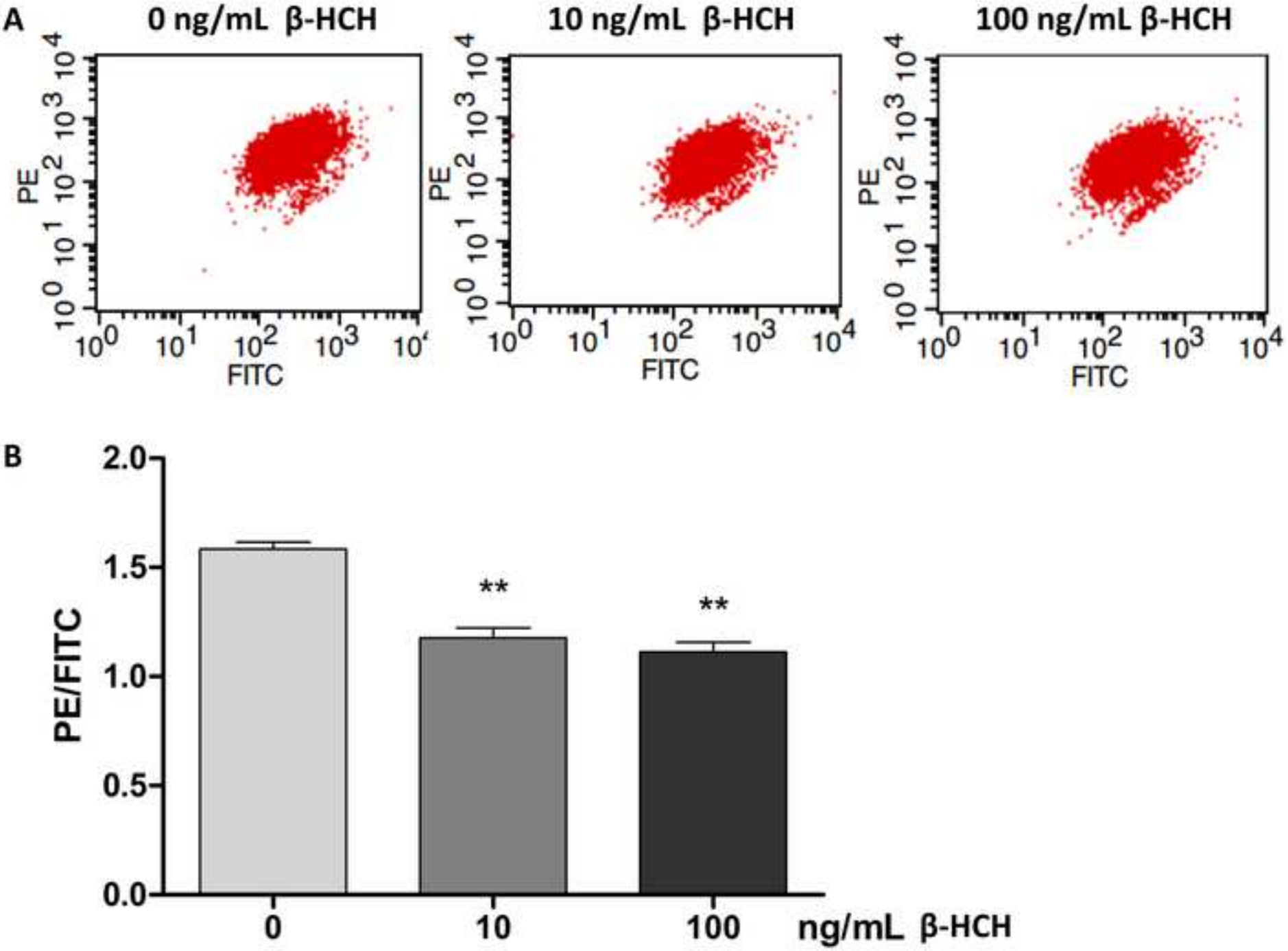

Figure5

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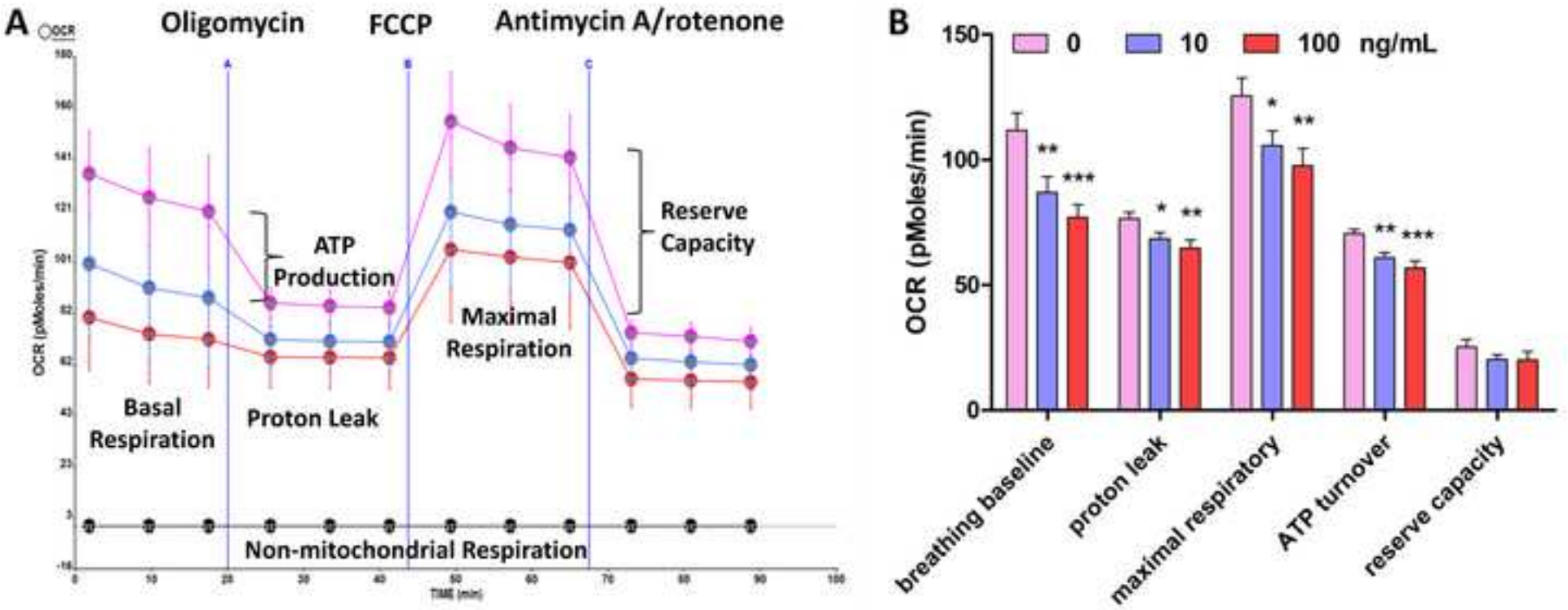

Materials Table

Click here to download Materials Table Material table.xlsx

Name of Material/ Equipment	Company	Catalog Number
Transmission electron microscope	FEI	Tecnai G2 Spirit Bio TWIN
Mito-Tracker Green	Beyotime	C1048
Laser scanning confocal microscope	Zeiss	700B
Enhanced ATP Assay Kit	Beyotime	S0027
Luminometer	Berthold	Centro LB 960
BCA Protein Assay Kit	Beyotime	P0012
Multimode reader	TECAN	InfiniteM200

Comments/Description
High-contrast, high-resolution imaging
Low-dose observation and imaging
Low-temperature observation
Outstanding analytical performance
Automation for convenience and performance
Mito-Tracker Green is a mitochondrial green fluorescent probe that can be used for live cell mitochondrial-specific fluorescent staining.
The design is compact, stable, light path is the shortest, high light precision, creative technology and sophisticated scanning technology together to produce a perfect 3-dimensional specimen image.
Enhanced ATP Assay Kit can be used to detect ATP (adenosine 5'-triphosphate) levels in common solutions, cells or tissues. Cells and tissue samples can be split to complete the sample preparation, detection sensitivity up to 0.1nmol / L, chemiluminescence can be sustained for 30 minutes.
Luminometer is chemiluminescence detector, the test sample itself can be light, do not need to stimulate. Luminometer is the instrument that detects chemiluminescence.
BCA Protein Assay Kit is one of the most commonly used methods for detecting protein concentrations.
Multimode reader be used to detect protein consentration.

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Title of Article: Experimental protocol for detecting mitochondrial function in hepatocytes exposed to organochlorine pesticides

Author(s): Qian Liu, Zhaoyan Jiang, Aihua Gu

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Article Title: Experimental protocol for detecting mitochondrial function in hepatocytes exposed to organochlorine pesticides

Signature: Zhaoyan Jiang Date: 2017-6-16

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Best regards,

Aihua Gu MD, PhD

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Response:
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2. For protocol steps, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Here I just list two examples (Please make sure all steps are clear):
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