Mitochondria structural integrity is essential for the protection of mitochondrial transplantation against UV-induced cell death

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Research

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Abstract

Background: Mitochondrial transplantation (MT) is a new technology developed in recent years, which injects healthy mitochondria directly into damaged tissues or blood vessels to play a therapeutic role. It has been studied in many animal models of various diseases, such as myocardial ischemia, cerebral stroke, liver and lung injury, and even has been successfully used in the treatment of childhood heart disease. MT can quickly improve tissue function within a few minutes after injection. This fast response is what is frequently questioned, for it is hard to understand how the whole mitochondria transport to the damaged sites, enter cells and function within such a short period of time. Are there any small molecules of mitochondrial component rather than the whole mitochondria that being responsible for the function of MT? To test this hypothesis, we established a much simple ultra-violet (UV)-irradiated HeLa cell model instead of the more complex animal models.

Results: The results of colony formation, sulforhodamine B (SRB), and Hoechst 33342/PI double staining assay strongly indicated that MT exhibited a significant protective effect on UV irradiation damage. The UV irradiation-induced cell cycle arrest at S phase, apoptosis, mitochondrial membrane potential (MMP) decreasing, and the related apoptosis signalling factors p-IKKα, p-p65, I-κB and the activation of caspase3 were all reversed by MT treatments to some extent. Furthermore, mechanisms of MT were evaluated through comparing the effect of thermal inactivation, ultrasonic crushing, and repeated freezing and thawing treatments on MT function. The results denied the above hypothesis that mitochondrial component may function to MT, excluded the function of ATP, mtDNA and other small molecules, and indicated that the mitochondria structural integrity is essential.

Conclusions: Our data support a potent anti-UV irradiation effect of MT, and structural integrity of mitochondria is critical for its function.

1. Introduction

Mitochondrial transplantation (MT) refers to a procedure for introducing isolated mitochondria into a damaged area of the heart or other organs[1]. The mitochondria can be delivered either through directly injected into the damaged area[2], or by vascular perfusion through the coronary arteries[3] or intravenous system[4].They all can get an obvious therapeutic result. This technique was first proposed in 2009 by McCully et al.[2], who reported that directly injected of healthy mitochondria isolated from normal tissues into the ischemic area of rabbit heart can enhance the functional recovery after myocardial ischemia. Since then, the therapeutic function of MT has been evaluated in many animal models, such as rabbit[2], mouse[5], rat[6] and pig[7] et al., in the multiple organ systems, including heart[2, 6], lung[5], kidney[7], neurological tissue[8], liver[9], and limb[10], as well as in sepsis[11]. Most astonishingly, the clinical trials of MT has also been reported by McCully's group in pediatric patients with cardiogenic shock following ischemia-reperfusion injury in 2017[12] and 2020[13]. Not surprisingly, it received widespread attention, comments, and questionings from peer scholars immediately[14-18].

Most commentaries questioned about the postulated mechanisms of MT by McCully and colleagues, that is the exogenous mitochondria penetrate into cardiomyocytes, retaining functional activity, and compensate for impaired energy output of endogenous mitochondria. The concerns are mainly focusing on three questions. First, mitochondria cannot survive calcium overload during MT, which occurs both in the blood and the extracellular fluid in the interstitium[1, 16, 19]. This was confirmed recently with isolated skeletal muscle mitochondria from mice[20]. Second, many studies found that there were a very small number of mitochondria eventually internalized by cells, much less than 10%, scholars wondered if and how these very few mitochondria can help and so quickly [14, 16]. Third, the function of MT appeared within minutes, while integration of mitochondria into cardiomyocytes took more time, and even much more time will be needed if delivering through vascular perfusion. It is hard to understand that MT functions so fast. Thus, some new postulations are suggested. Such as, the contents of permeabilized mitochondria (eg, peptides, glutathione, adenosine di- or triphosphate, mitochondrial DNA) might account for the beneficial effects in a paracrine fashion rather than the mitochondria directly contributing to respiration[20], or the benefits of MT may depend on specific components or the secretome contained within the mitochondria, not necessarily on the intact, complex microstructure per se[18]. In other words, does the function of MT rely on intact mitochondria or some more easily transport component of mitochondria? The wondering about whether mitochondria or its components are responsible for the benefits of MT are still under debate[18, 20].

In view of the complexity of animal heart ischemia-reperfusion model, it is not a convenient model to illustrate the details of MT and the precise mechanism of its action. So, in the present study, we suggest an ultraviolet (UV) irradiated cell model and incubated with isolated healthy mitochondria, to simplify MT and try to answer whether intact mitochondria or their components are responsible for the function of MT.

2. Material And Methods

2.1 Chemicals and reagents

Sulforhodamine B (SRB), propidium iodide (PI), adenosine triphosphate (ATP), and the cell culture medium RPMI-1640 were purchased from Sigma (USA). Neonatal bovine serum was purchased from Lanzhou Minhai Bio-engineering Co., Ltd. (Lanzhou, China).

2.2 Cell culture

HeLa cells were cultured in 10% serum RPMI 1640 (Sigma), containing penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified incubator with 5% CO_2. The exponentially growing cells were used to the experiments mentioned below.

2.3 UV irradiation

Short wavelength ultraviolet C (UVC) was selected as the UV irradiation method. The UVC irradiation dosage was measured with a UV Digital Radiometer (Handy®; Sanpometer, Shenzhen, China).

2.4 Mitochondria isolation and mitochondrial transplantation

Healthy mitochondria were isolated through differential centrifugation method as described previously[21]. Briefly, exponentially growing cells were rinsed with the isolation buffer (Medium I, MI: 100 mM KCl, 50 mM Tris –HCl, 1 mM K_2HPO_4, 50 mM EGTA, and 0.2% BSA, pH 7.4), scraped with a cell scraper and collected. Then the cells were homogenized in ice-cold MI, centrifuged at 600 g at 4 °C for 10 min, the supernatants were aspirated and centrifuged at 10 000 g at 4°C for 10 min, the pellets were resuspended in MI and centrifuged at 6 000 g at 4 °C for 10 min to collect the mitochondria in the pellet, and resuspended in the preservation buffer (Medium II, MII: 225 mM mannitol, 75 mM sucrose, 10 mM Tris –HCl, and 0.1 mM EDTA, pH 7.4) for MT.

MT was performed as adding 5 μl isolated mitochondria directly to the cell culture medium to get a 6.25 μg/mL or the indicated final concentrations and incubating as well. The same volume of mitochondria preservation buffer (MII) was added as a loading control.

2.5 Colony formation assay

Cells were seeded in a 96-well plate (1×10^4 cells/well) and cultured for 24 h, then treated with UV irradiation. After incubated with isolated mitochondria for another 24 h, cells were harvested and seeded at lower density (the control group was diluted 25 times, the irradiated group was diluted 5 times) in 6-well plates. After 12 days cultivation, the colony formation rate was calculated.

2.6 SRB assay

The SRB assay was performed as described previously [22] , with some modifications. Briefly, Cells were seeded in a 96-well plate in a final volume of 100 μl (1×10^4 cells/well). After a 24 h incubation, cells were irradiated with UVC. Then cells were incubated with the isolated mitochondria for 48 h, and the routine procedure of SRB assay was performed.

2.7 Hoechst 33342 / PI double staining assay

Hoechst 33342 is a cell membrane permeable fluorescence dye, which can stain both live and dead cells, while PI can only stain the dead or membrane damaged cell. Thus, the cell viability can be expressed as the percentage of PI negative cells. Cells were seeded into 12-well plates (2.5×10^4 cells/well), and after

treatment, cells were stained with 1 μg/mL Hoechst 33342 for 10 min at 37°C in the dark, then incubated with 1 μg/mL PI staining buffer for 2 min. After observed under fluorescence microscopy and recorded the fields, the images were analyzed with ImageJ software.

2.8 Cell cycle analysis

After MT for 24 h, cell cycle was assessed with PI staining and flow cytometry assay as described previously[22].

2.9 Detection of mitochondrial membrane potential (MMP)

MMP was detected with Mitochondrial Membrane Potential Assay Kit with JC-1 (C2006, Beyotime, Shanghai, China) according to the instruction. Briefly, cells were incubated with 0.5 ml of JC-1 working solution at 37°C for 20 min, washed twice with JC-1 buffer, and then resuspended cells in the 0.5 mL JC-1 buffer. MMP was assessed by flow cytometry.

2.10 Apoptosis analysis

Annexin V–FITC/PI staining assay was used to detect cell apoptosis as described previously[23]. Cells were seeded in 6-well plates (1×10^5 cells/well) and incubated for 24 h, then treated with UVC. After another 24 h incubation with the isolated mitochondria, cell apoptosis was evaluated with an Annexin V-FITC Apoptosis Detection Kit purchased from eBioscience Inc. (San Diego, CA, USA) using a flow cytometer (BD LSRFortessa).

2.11 Western blot

To evaluate the involvement of apoptosis pathways in the effect of MT on UVC induced apoptosis, the expressions of related cell factors were detected with western blot assay following the conventional protocol. The antibodies of GAPDH and HRP-conjugated secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA); Bcl-2 rabbit ploYclonal antibody, Phospho-NF-κB p65(Ser536) rabbit polyclonal antibody were purchased from Beyotime (Shanghai, China); IKKα mouse monoclonal antibody was purchased from Santa Cruz (Dallas, Texas, USA); NF-κB p65 mouse monoclonal antibody was purchased from TransGen Biotech (Beijing, China); active + pro Caspase-3 rabbit monoclonal antibody were purchased from Abcam (Cambridge, UK). Western blot results were shown as “Supplementary data”.

2.12 ATP content detection

ATP contents were detected with the Enhanced ATP Assay Kit (S0027), purchased from Beyotime (Shanghai, China), according to the instruction.

2.13 Mitochondrial DNA isolation and analysis

Mitochondrial DNA (mtDNA) was isolated from the isolated mitochondria with a Tissue Genomic DNA kit purchased from MOIBIO (Lanzhou, China). mtDNA was then analyzed with agarose electrophoresis.

2.14 Malate dehydrogenase (MDH) activity

MDH activity was detected with the Micro NADP-Malate Dehydrogenase（NAD-MDH）Assay Kit (BC1055), purchased from Solarbio (Beijing, China), following the instruction.

2.15 TEM sample preparation

Mitochondria morphology was captured with Transmission Electron Microscopy (TEM). The TEM samples were prepared as follows. After treatments, mitochondria were centrifuged at 15000 g for 10 min, the supernatant was discarded, and 400 μl of electron microscope fixative (2.5% glutaraldehyde) was added. Then the precipitate was slowly suspended with the needle of a micro syringe in the fixative and kept at 4 °C overnight. After washing twice with PBS, the samples were dehydrated and fixed in the electron microscope sample preparation instrument. The processed samples were placed in epoxy resin and polymerized in an oven at 75 °C for 24 h, followed by polymerization at 90 °C for 24 h. Trimming the embedded sample, using an ultra-thin microtome for fine trimming and sectioning, the section thickness was 50 nm, and the appropriate section was transferred to a 230-mesh copper net with a film ring. The samples were then stained with heavy metal salts and observed on the microscope.

2.16 Statistical analysis

Data are presented as means ± SD from at least three independent experiments. Statistical analysis was conducted using Microsoft Excel 2010. Statistical differences were determined using the two-tailed Student's t-test, and the statistically significant differences are indicated * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

3. Result

3.1 MT promotes cell survival after UV-irradiation

The protective effect of MT on UV-irradiated cells was found through cell morphology observation in the beginning. The phase-contrast micrographs showed that cell morphology of MT group (UV+M, 25 μg/mL)

are much better than UV irradiation group (UV, 21 J/m^2) and mitochondria preservation buffer II group (UV+MII) at 24 and 48 h (Figure 1a). Therefore, we further confirmed this protective effect with the colony formation assay (Figure 1b, 1c). Because the mitochondrial concentration (25 μg/mL) used above needed to culture too much cells, it is inconvenient for the following experiments. So, we evaluated the effect of mitochondrial concentration on the function of MT with SRB assay. The results indicated that 6.25 μg/mL (M2, Figure 1d) is an optimal mitochondrial concentration for MT. This optimal concentration was further tested with colony formation assay, and the protective effect was found under both 21 and 42 J/m^2 UV-irradiation (Figure 1e). Thus, 6.25 μg/mL mitochondrial concentration was used in the following experiments.

It must be pointed out that although 25 μg/mL mitochondrial concentration exhibited potent protective effect on UV-irradiation when detected with colony formation assay (Figure 1b, 1c), this effect cannot be found with SRB method (Figure 1d). The cell morphology results exhibited clearly that 25 μg/mL mitochondria MT potently alleviated UV-induced cell death in both 24 and 48 h (Figure 1a), while the SRB results cannot exhibit this protective effect at the same treatment (Figure 1d). This may be due to the difference between the methods. Colony formation is the “gold standard” for cell viability evaluation, it reproduced the protective effect shown in the morphology study very well, but the “12 days” colony formation period is not convenient for evaluating the effect of MT, and morphology observation cannot be quantitatively analyzed easily, this pushed us to look for another more suitable method.

3.2 Hoechst 33342/PI double staining assay detects the protective effect of MT

Hoechst 33342 and PI are two fluorescence dyes, the former can stain the nucleus of all cells, while PI can only stain the nucleus of dead or membrane damaged cells. The Hoechst 33342/PI double staining assay only needs stain cells for about 12 min, and then captures the micrographs with a fluorescent microscope, analyzes with Image J software. It does not need to wait for 12 days as colony formation assay, and no need to fixation and washing cells as the SRB assay, so, it much convenient than colony formation assay and more accurate than SRB assay. Figure 2a showed the double-stained cell micrographs after 21 J/m^2 UV-irradiation and MT for 24 h. Figure 2b is a group of representative merged graphs showing the effect of MT on cells under 7 and 21 J/m^2 UV irradiation, and Figure 2c is the statistical results of Figure 2b, which indicates that MT exhibited a significant protective effect at 24 h under both 7 and 21 J/m^2 UV doses.

3.3 MT alleviates UV-irradiation affected cell cycle, apoptosis, and MMP

It is reported that UV-irradiation usually causes cell cycle arrest[24, 25], mitochondrial membrane potential depletion[25], and apoptosis[24, 25]. So, we evaluated that if the protective effect of MT in our model are

correlated with these UV-related mechanisms. The results indicated that UV irradiation caused a S phase cell cycle arrest (Figure 3a, 3b), MMP depletion (Figure 3c, 3d), and apoptosis (Figure 3e, 3f), while MT significantly improved these phenomena (Figure 3a-3f), respectively. In view that a paper even reported that apoptosis is the only known cell death mechanism induced by UVB irradiation, after they have evaluated the implication of apoptosis, necroptosis, ferroptosis and parthanatos in UVB-induced cell death in human diploid dermal fibroblasts[26], we further evaluated the involvement of apoptosis-related signaling pathways with western blot assay (supplementary data). The results indicated that MT alleviated UV-induced apoptosis through the NF-κB pathway. Compared with UV treated groups (UV+MII), MT resulted in the decrease of p-IKKα and p-p65, and the increase of I-κB levels, especially at 4 and 8 h (Figure S1). MT also inhibited the UV-induced caspase 3 activation (cleaved) at 8 and 24 h, while Bcl-2 decreased in a time-dependent manner but the effect of MT was not found (Figure S1).

So far, we have demonstrated that the protective effect of MT on UV-induced cell death with morphology observation, colony formation, SRB, and double staining assay, the cell cycle, apoptosis, and MMP related mechanisms have also been evaluated. The next question is how can MT exhibit this UV protection function? In other words, is it any mitochondrial components that responsible for it, or MT function is really because of transplanting mitochondria? To answer this question, we treated mitochondria with heating, sonicating and, repeated freezing and thawing before MT.

3.4 MT function totally lost after mitochondria being heated or sonicated, while still partly preserved after being repeated freezing and thawing

Heat-inactivated mitochondria (HM) were boiled for 10 min under boiling water to inactivate the heat sensitive components of mitochondria. Ultrasonicated mitochondria (UM) were intermittently ultrasonicated for 10 s every 10 s in an ice-water bath, to reach a total period of 10 min under the power of 1670 W. Repeated freeze-thaw treated mitochondria (FM) were treated with liquid nitrogen and 37 °C water for 30 s each, and 3 times in all. The effect of different treatments on MT was evaluated with both Hoechst 33342/PI double staining assay and colony formation assay. The results indicated that HM and UM lost the protective function of MT, while FM still kept part of that function (Figure 4). Because both ultrasonicating (UM) and repeated freeze-thaw (FM) are the methods to break cells and release the components, the different effect on MT of them make it is hard to conclude that there is any mitochondrial components which may be responsible for the function of MT. As ATP and mtDNA are the energy product and the specific genetic material of mitochondria, respectively, we further evaluated the role of them on the function of MT.

3.5 ATP is not an effective mitochondrial component in transplantation

The main function of mitochondria is to produce ATP and provide energy for cells. Therefore, the role of ATP in MT is considered first. We first measured the ATP content of the mitochondria treatment groups (M, HM, UM, FM). The results indicated that compared to the untreated group (M), ATP content in group HM and FM decreased significantly while there is almost no change in UM (Figure 5a). As ATP is easily decomposed when being heated, the decrease of ATP content in HM group is reasonable. Both M and UM were kept in ice, so they can preserve almost the same content of ATP. The significant decrease content of ATP in FM group may be because that part of the ATP being consumed by mitochondria when thawing at 37 °C. We even tested the effect of ATP (14.28 pM, same content as the normal M group) on UV-induced cell death, and the results indicated that there is no difference between ATP group and MII group (Figure 5b, 5c). Which means that there is no effect of ATP on MT.

3.6 mtDNA is not an effective component in transplantation

The role of mtDNA in MT was further analyzed. After different treatments, mtDNA was isolated (Figure 6a) and electrophoresed with agarose gel (Figure 6b). The results indicated that no mtDNA can be found in the sample HM, and the fact that mtDNA can be destroyed by heating was further confirmed by the result of mtDNA isolated from sample M and then heated (M+H, Figure 6b). More interestingly, although the contents of mtDNA of UM and FM are both preserved and lower than M (Figure 6b, 6c), there was only FM which have some function of MT while UM not (Figure 4). The loss of MT function of UM, which still has mtDNA, indicated that mtDNA is not the effective component of MT.

When comparing UM and FM, both are broken mitochondria samples, both are treated under lower temperature to preserve the biological activity of mitochondrial components, and both preserved mtDNA (Figure 6b, 6c), why it is only FM preserved a degree of MT function (Figure 4)? So, we further separated FM sample with centrifugation (6 000 g, 10 min), and tested the MT function of the supernatant (FMs) and precipitation (FMp). The results indicated that FMp exhibited the MT activity, while FMs didn't (Figure 6d, 6e). This means that the MT activity of FM can be centrifuged down. What is in the precipitation? If considering the differences between ultrasonicating and repeated freeze-thaw, the latter is much gently. Thus, it is most likely that FM is not thoroughly destroyed as UM, and the integrated mitochondria in FM may be responsible for its MT activity. The structural integrity of mitochondria was then evaluated.

3.7 Mitochondrial structural integrity is essential for MT

The mitochondrial structural integrity was evaluated with malate dehydrogenase (MDH) and transmission electron microscopy (TEM). As a marker enzyme of the mitochondrial matrix, MDH will be released to the medium if mitochondria were broken. Because the integrated mitochondria can be precipitated with centrifugation (6 000 g, 10 min), the MDH activity in supernatant represents broken mitochondria, while what in the precipitant represents the structural integrated mitochondria. Thus, through evaluating the MDH activity in supernatant and precipitation, the mitochondrial structural integrity can be evaluated. The results showed that MDH activity in supernatant and precipitation of HM

were both very low (background amount), which indicated that MDH protein enzyme were denatured by 10 min boiling. The results bar looked totally opposite between M and UM groups, M exhibited that almost all the MDH activity was in precipitation, while UM's MDH activity was almost all in the supernatant. Which indicated that almost all the M groups preserved integrated mitochondria, while all the mitochondria in UM group were broken. Most interestingly, the MDH of FM looks distributed almost half in the supernatant and half in the precipitation (Figure 7a). Which indicated that almost half of the FM were structural integrated mitochondria. These results have also been confirmed by the ultrastructural observation with TEM. There are much more integrated mitochondria in FM compared with UM, and the morphology of mitochondria are more integrated in M than the other groups (Figure 7b).

Discussion

In the present study, we demonstrate that MT is able to protect HeLa cells from UV-induced cell death, and the mitochondrial structural integrity is essential for the protective effect. Our results also testified that MT is indeed the transplantation of mitochondria, but not the component of them.

The protective effect of MT on UV-induced cell death was demonstrated by cell morphology observation, colony formation, SRB assay, and Hoechst 33342/PI double staining assay. Among them, colony formation is the "gold standard" for cell viability measuring but is more time-consuming with the long experimental period up to 12 days. Another method, Hoechst 33342/PI double staining, is more applicable and the results can be easily analyzed and quantified with ImageJ software. It must be pointed out that, although Hoechst 33342/PI double staining method is more convenient and efficient than colony formation assay, there are still some differences between the results derived from them. For example, the results between M and FM showed significant difference in Figure 4d but not in 4b. We also used SRB assay to evaluate how mitochondria concentration affect the protective effect of MT. It is worth noting that the fixing and washing steps in SRB assay are prone to make more deviations between each repeat, for the reason that mitochondria added to the culture system can also be fixed. Given these considerations, we chose the Hoechst 33342/PI double staining method to evaluate the protective of MT.

A lot of new studies have reported the MT function in the recent two years, while most of them aimed to expand the utility of MT in various biological context, like in diabetic hearts[6], ex-situ—perfused hearts donated after circulatory death [27], acute limb ischemia [10], brain in Sepsis [28], lung injury [5], acute kidney injury [7], aging-related hair loss[29] etc., and even a new clinical study in pediatric patients undergoing cardiogenic shock [13]. However, the mechanisms of MT still kept on being questioned [18, 19], especially considering that mitochondria cannot survive under calcium overload during transplantation [20]. The authors further questioned that whether the contents of permeabilized mitochondria (eg, peptides, glutathione, adenosine di- or triphosphate, mitochondrial DNA) account for the beneficial effects of MT in a paracrine fashion rather than the mitochondria directly contributing to respiration [20]. Our results answered this question directly. That is, MT functions rely on the transplantation of mitochondria, but not the component inside mitochondria. To verify this issue, we first established the MT protected UV irradiated HeLa cell model, then evaluated the MT function of heat-

inactivated mitochondria (HM), ultrasonicated mitochondria (UM), and repeated freeze-thaw treated mitochondria (FM). Moreover, we analyzed the level of ATP and mtDNA, measured the activity of MDH, observed the mitochondria structure with TEM. We concluded that the structural integrity of mitochondria is critical for the protective effect of MT on UV-induced cell damage. In fact, McCully and colleagues have ever reported that mitochondrial components or mitochondrial DNA + RNA provided no cardioprotection in their early study [2]. They used New Zealand White rabbits as animal model instead of cultured cell models. Several studies have evaluated the mitochondria incorporation of MT with cell model [30-33] and the results revealed that mitochondria were internalized by macropinocytosis [30, 31] or endocytosis [33], and the incorporation process happened within minutes [32]. One of these studies reported the significant role of mitochondrial outer membrane during this process, with the digitonin treatment, they found the integrity of the mitochondrial outer membrane is essential for mitochondrial transformation into cells [30]; In conclusion, these results support our findings that the structural integrity of mitochondria is essential for MT.

Looking forward, three directions need to be considered. First, keeping on expanding the application of MT in other kinds of diseases; second, exploring the mitochondria preservation methods; and third, deepening the MT mechanisms study.

Declarations

* Ethics approval and consent to participate

Not applicable.

* Consent for publication

The authors declare no different opinions.

* Availability of data and material

The data and material both are true and reliable, and the data are repeatable.

* Competing interests

Not applicable.

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* Authors' contributions

Chun-Ming Wang designed and instructed all the experiments; Shan-Shan Hu carried out the experiments and wrote the paper; Ruo-Yun Li, Xin-Hui Cao, Jing-Jing Liu, Mu-Lin Yang and Li-Ming Hu helped to analyse the results and figures; Zhen-Hua Wang and Chang-Jun Lin helped to design and write the paper.

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Figures

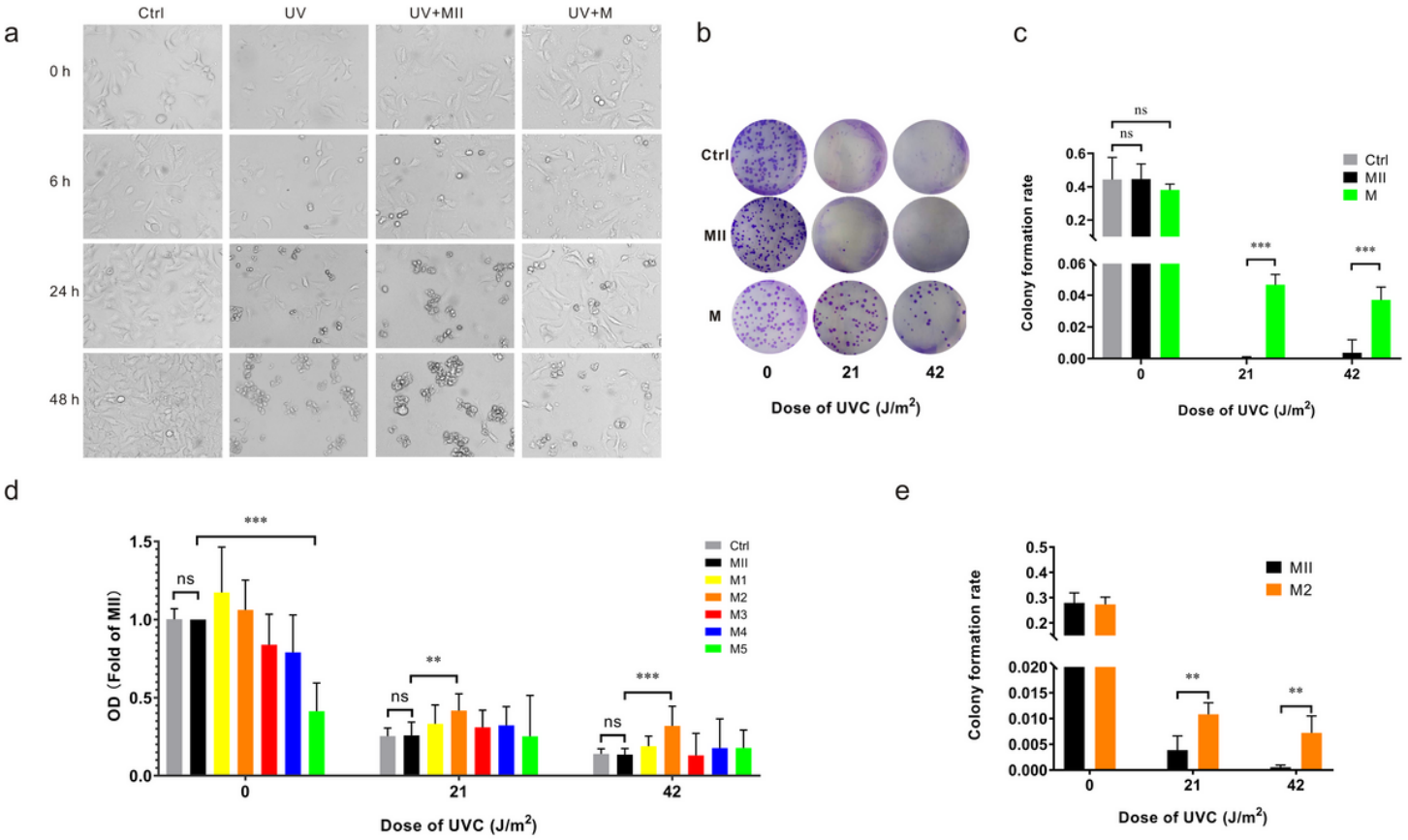

Figure 1

MT promotes cell survival after UV-irradiation. (a) The effect of MT on HeLa cell morphology after UV-irradiation (21 J/m2) at 0, 6, 24, and 48 h. Ctrl: the control group without UV-irradiation; UV: the UV-irradiation group; UV+MII: the UV-irradiation plus mitochondria preservation buffer II (MII) group, UV+M: the UV-irradiation plus mitochondria (25 μg/mL) group. (b) Representative experimental diagrams of colony formation in 6-well plates after cultured for 12 days. Cells were treated in 96-well plates first for MT 24 h before colony formation test. mitochondria concentration 25 μg/mL. (c) Statistical results of colony formation rate (*** $p < 0.001$; $n = 3$). (d) Evaluating the effect of mitochondria concentration on MT function with SRB assay to select the optimal concentration. MT for 48 h ($n = 3$). Ctrl: control group; MII: Mitochondria preservation buffer II; M1-5: different concentrations of mitochondria. M1: 3.13 μg/mL; M2: 6.25 μg/mL; M3: 9.375 μg/mL; M4: 12.5 μg/mL; M5: 25 μg/mL. (e) Verifying the optimal mitochondria concentration (6.25 μg/mL) selected by SRB study with colony formation assay ($n = 3$). Statistical significance (** $p < 0.01$, *** $p < 0.001$) was obtained by the Student's t-test.

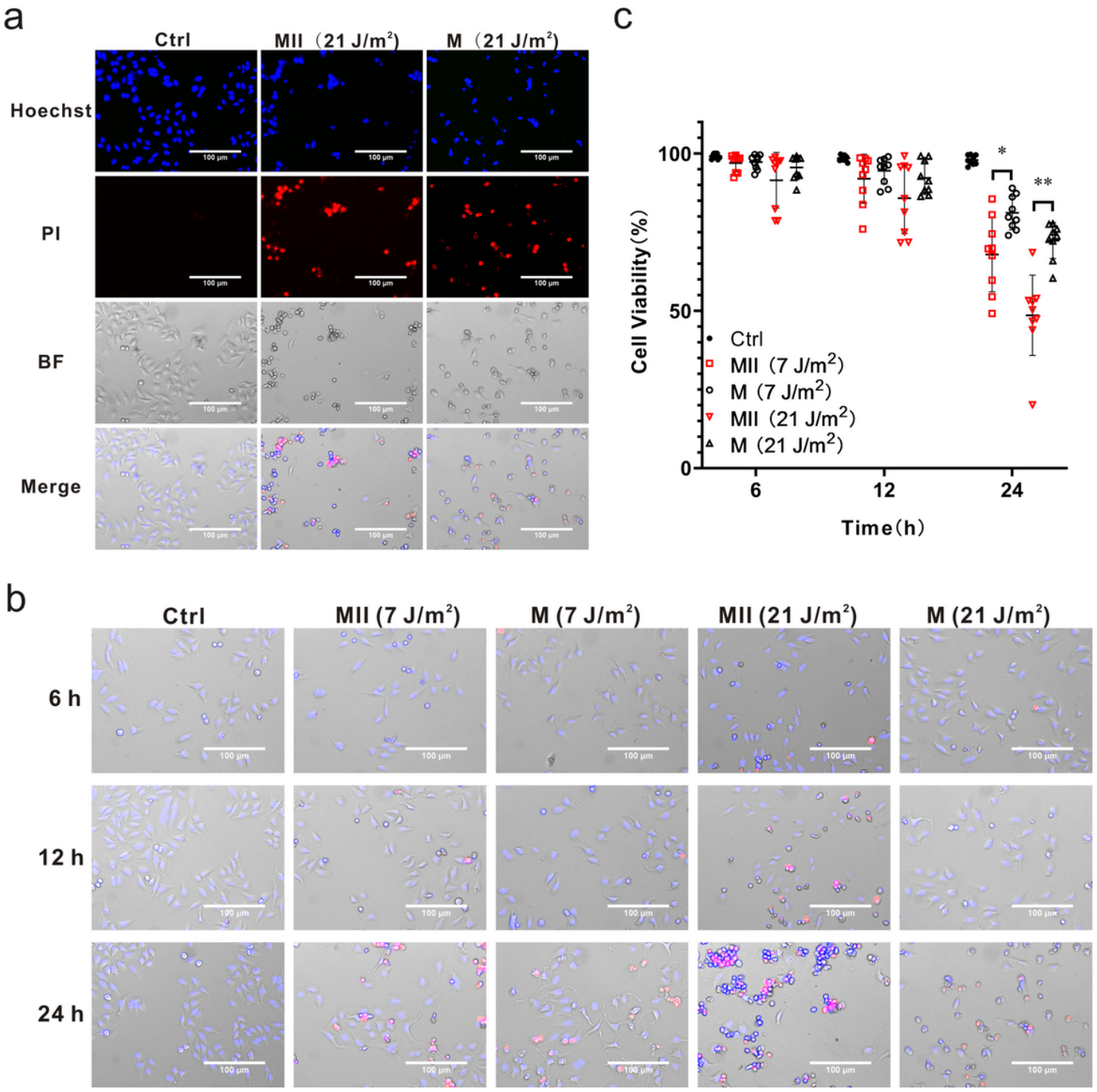

Figure 2

Using Hoechst 33342/PI double staining assay to evaluate the protection of MT against UV-irradiation. (a) Representative photos of the Hoechst 33342/PI double staining assay after MT for 24 h (mitochondria concentration, 6.25 μg/mL). Hoechst 33342 can enter both the live and dead cells to stain nucleus and mark all cells. PI can only pass through dead cell membranes or damaged cell membranes with increased permeability to mark dead cells or membrane damaged cells. (b) Merged photos showed the Hoechst 33342/PI double staining results of MT on UV-irradiated cells after 6, 12, and 24 h. (c) Statistical results of the groups shown in (b) analyzed with Image J software. The formula of Cell

Viability = (1 - PI/Hoechst 33342) ×100%, n = 3, * $p < 0.05$ and ** $p < 0.01$. Ctrl: control group; MII: Mitochondria preservation buffer II group; M: MT group. The number in parentheses is the UV dose.

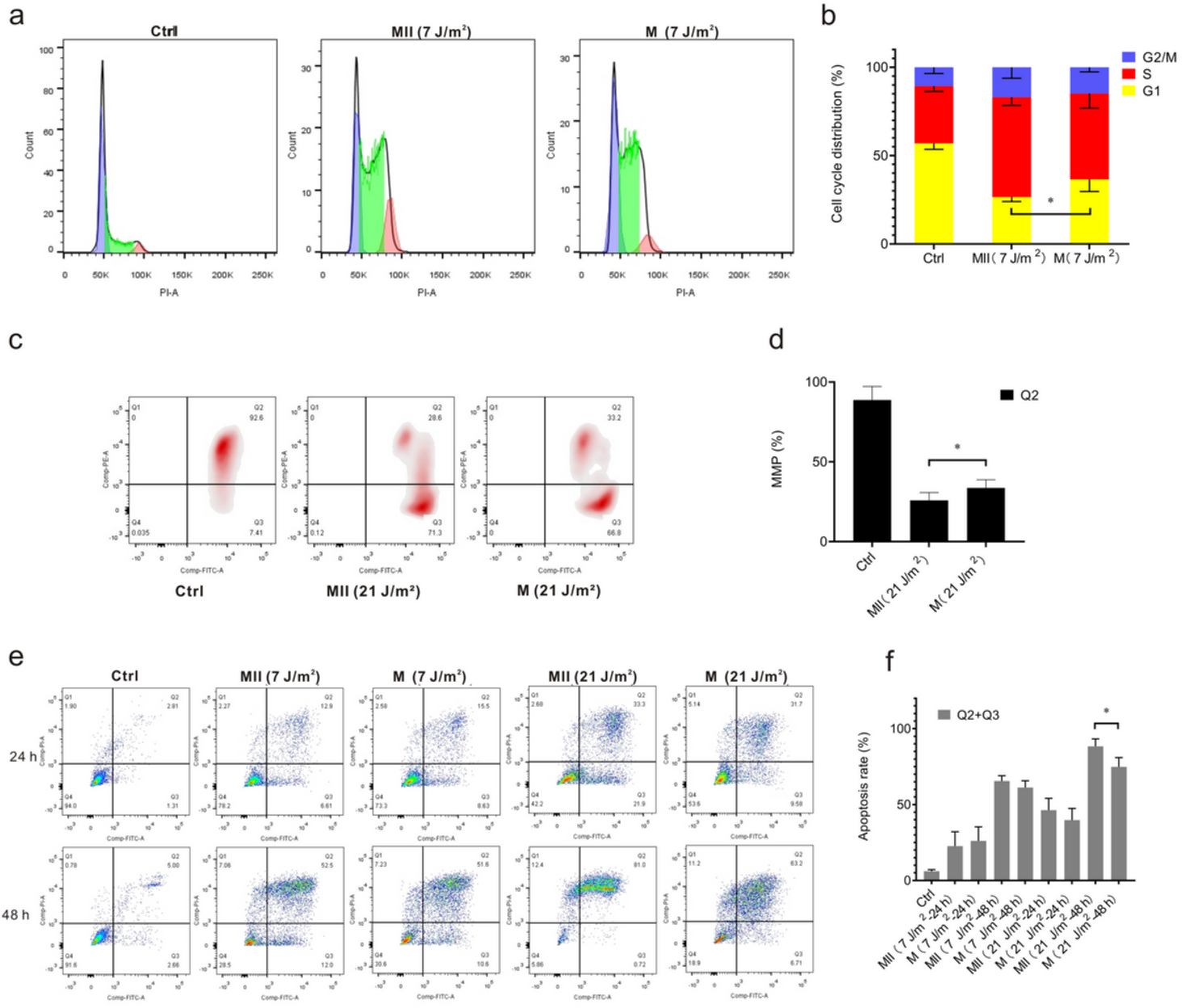

Figure 3

MT improves cell cycle and reduces apoptosis affected by UV irradiation. (a) Representative flow cytometry histograms showed the cell cycle distribution. MT for 24 h. (b) Statistical results of cell cycle distribution, * $p < 0.05$, n = 3. (c) Representative density graph results of MMP detected with flow cytometry. MT for 24 h. (d) Statistical results of MMP, * $p < 0.05$, n = 5. (e) Representative scatter plot graphs of apoptosis detected with flow cytometry. (f) Statistical results of apoptosis, * $p < 0.05$, n = 3.

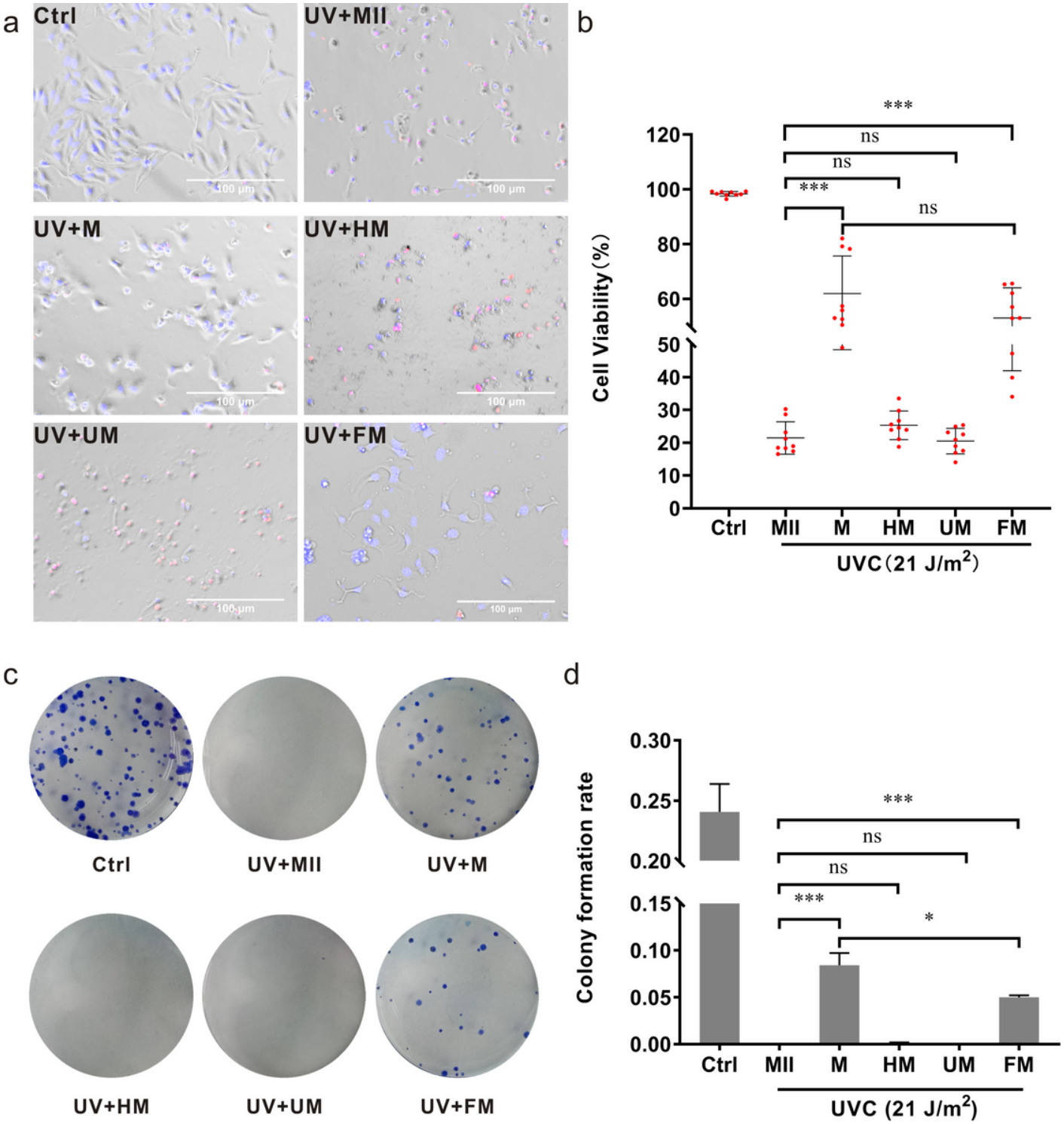

Figure 4

Effects of different treatments against mitochondria on the benefit of MT. The effect of Heat-inactivated mitochondria (HM), ultrasonicated mitochondria (UM), and repeated freeze-thaw treated mitochondria (FM) on UV-irradiated HeLa cells were evaluated. (a) Representative cell images obtained from Hoechst 33342/PI double staining assay. MII: Medium II; M: Mitochondria. MT for 24 h. (b) Statistical results of cell viability analyzed with Image J software. Formula: Cell Viability = (1- PI/Hoechst33342) × 100%, n =

3. (c) Representative colony formation images. (d) Statistical results of colony formation rate, n = 3. Statistical significance * p < 0.05, *** p < 0.001.

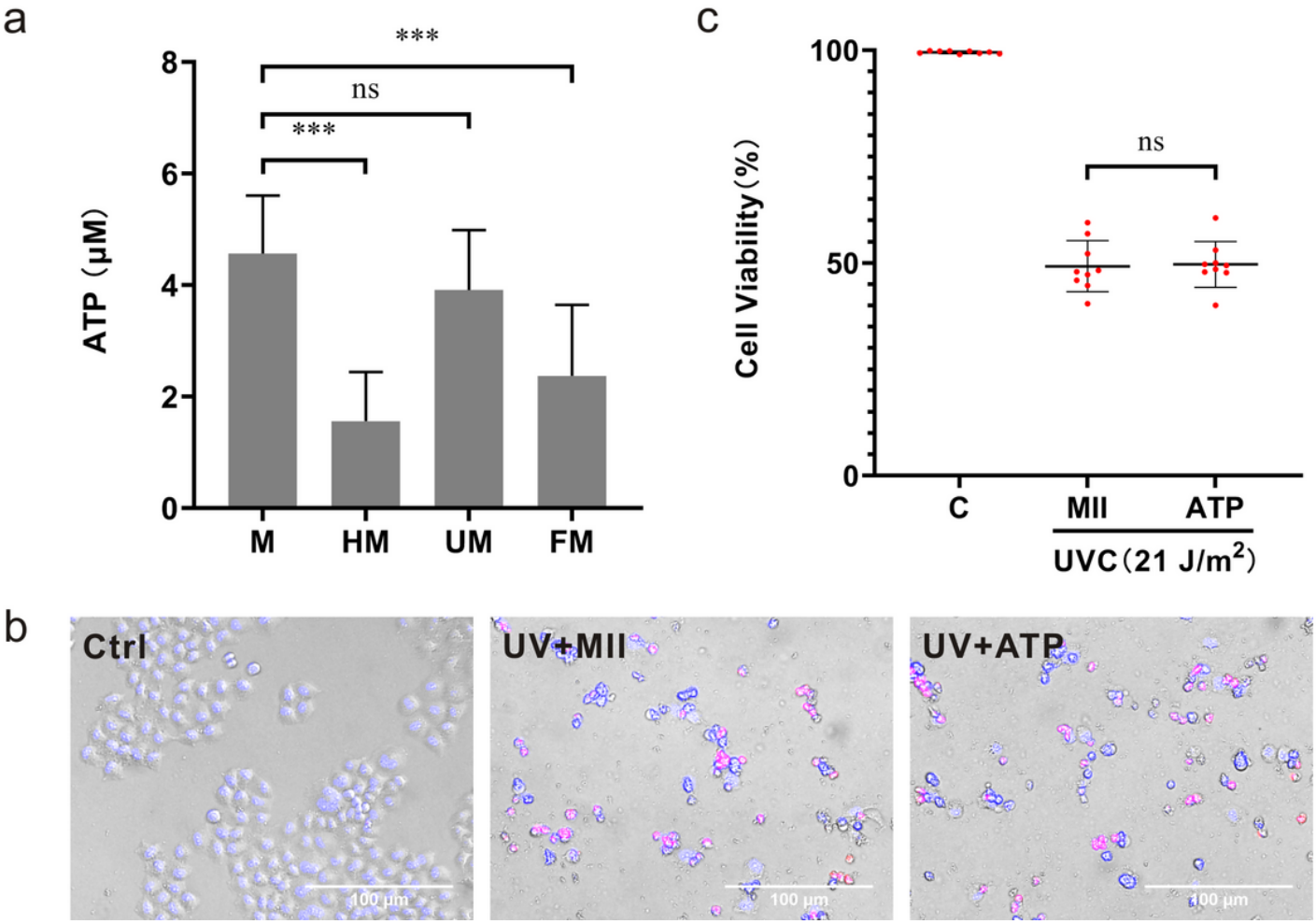

Figure 5

The role of ATP in MT. (a) ATP content of 2 mg/ml mitochondria samples under different treatments (***p < 0.001). M: Mitochondria; HM: Heat-inactivated Mitochondria; UM: Ultrasonicated Mitochondria; FM: repeated Freeze-thaw treated Mitochondria. (b) Representative merged photos of Hoechst 33342/PI double staining assay to show the effect of ATP (14.28 pM, calculated according to 6.25 μg/ml mitochondria used for MT) on UV-irradiated cell morphologies and viabilities after 24 h incubation. (c) Statistical results of the double staining assay analyzed with Image J software. Formula: Cell Viability = (1 - PI/Hoechst 33342) ×100%, n = 3.

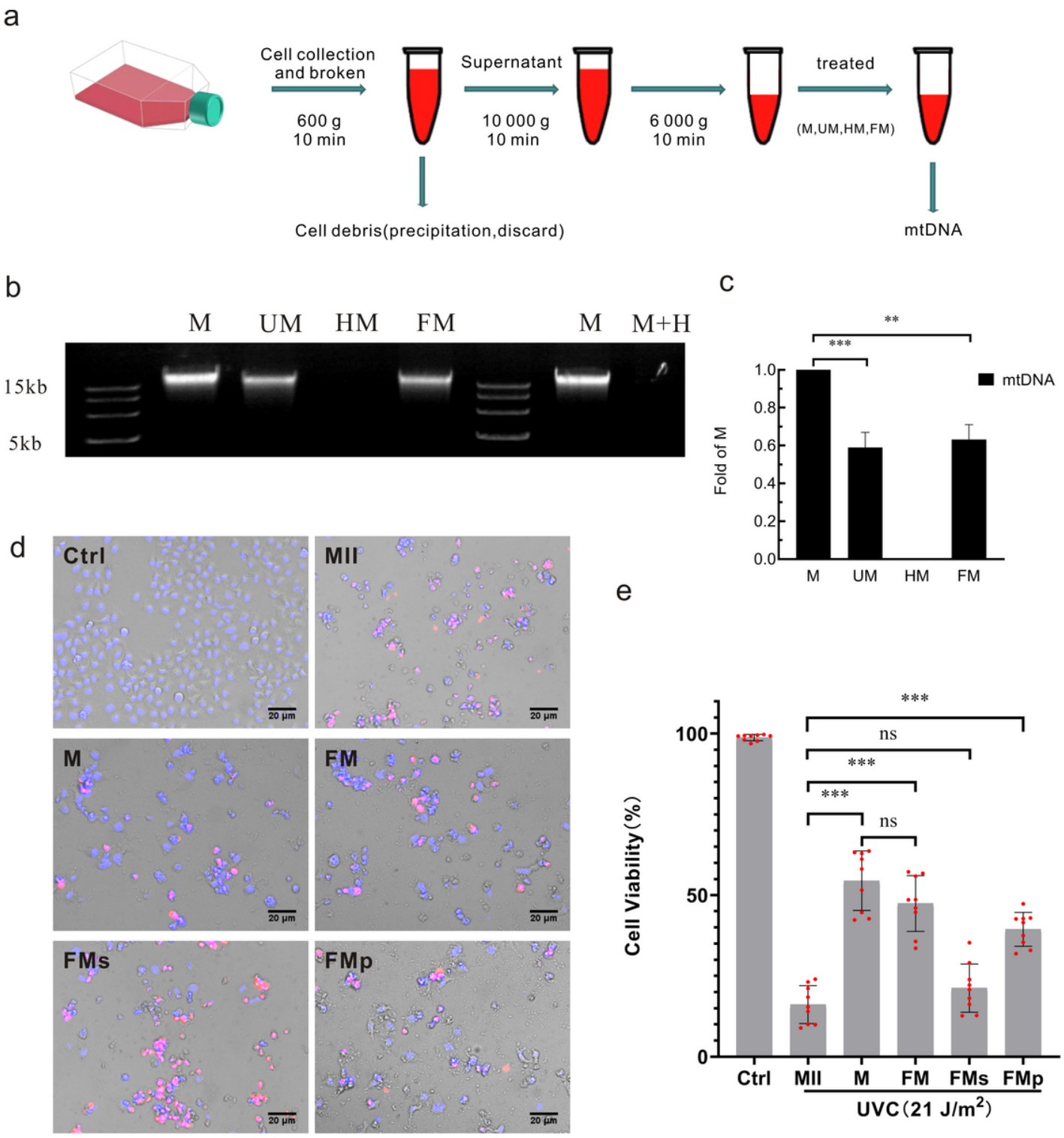

Figure 6

The role of mtDNA in MT. (a) Schematic diagram of mtDNA extraction process. mtDNA represents DNA isolated from the whole mitochondria samples of M, UM, HM and FM. (b) Representative agarose electrophoresis analysis of mtDNA samples. M: untreated Mitochondria; UM: Ultrasonicated Mitochondria; HM: Heat-inactivated Mitochondria; FM: Repeated Freeze-thaw treated Mitochondria; M+H: mtDNA obtained from M group (untreated mitochondria) first and, then boiling 10 min. (c) Statistical results of mtDNA content of (b). ** $p < 0.01$, *** $p < 0.001$. (d) Representative merged photos of Hoechst

33342/PI double stained HeLa cells under MT (24 h) with different treatments. Ctrl: control cells without UV-irradiation; UV+MII: UV-irradiation plus Medium II; UV+M: UV-irradiation plus mitochondria; UV+FM: UV-irradiation plus repeated freeze-thaw treated mitochondria; UV+FMs: UV-irradiation plus supernatant of the repeated freeze-thaw treated mitochondria; UV+FMp: UV-irradiation plus precipitate of the repeated freeze-thaw treated mitochondria. (e) Statistical results of cell viability analyzed with Image J software. Formula: Cell Viability = (1- PI/Hoechst 33342) × 100%, n = 3. Statistical significance *** p < 0.001.

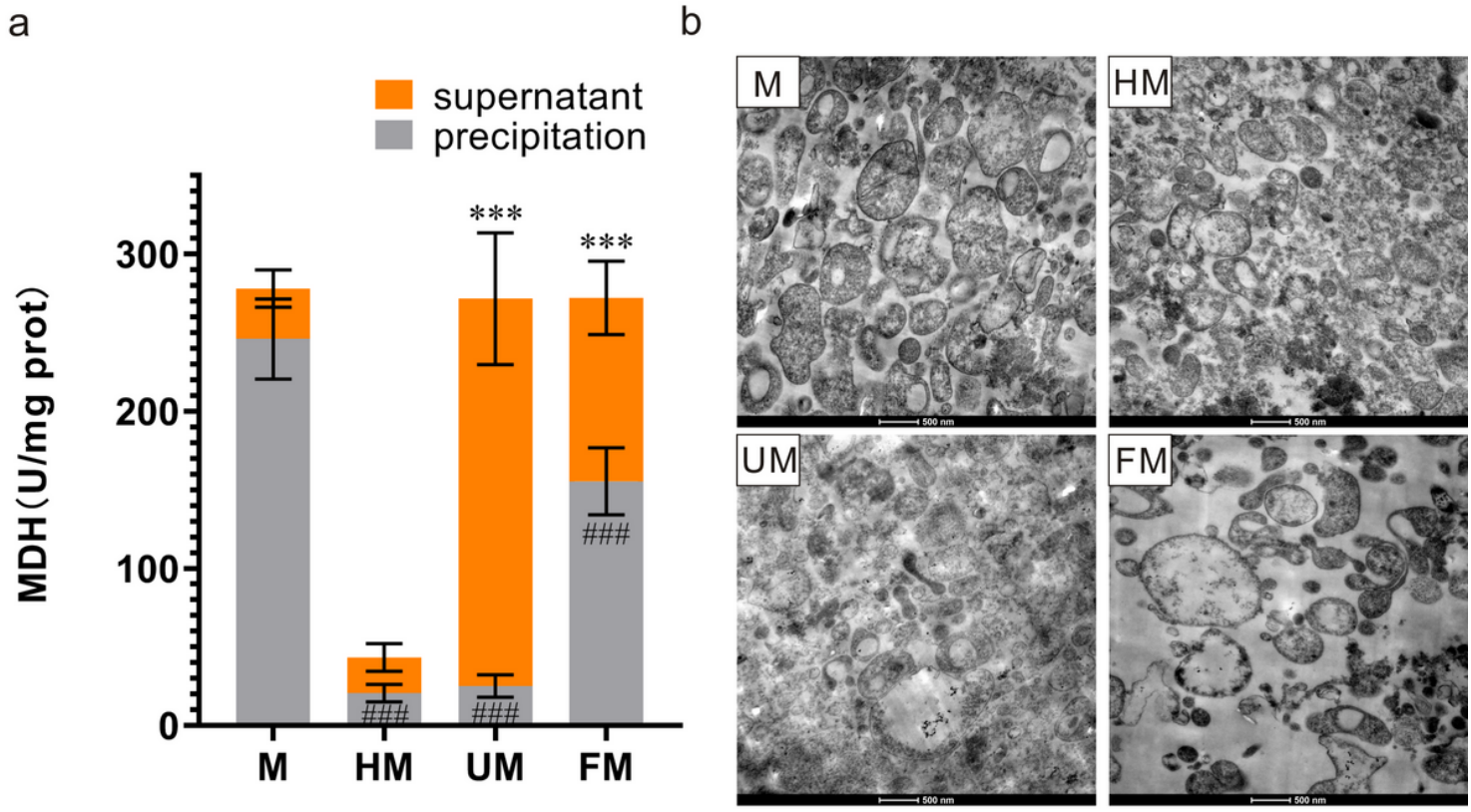

Figure 7

Mitochondria structural integrity studied with MDH activity and TEM. (a) MDH content in mitochondria supernatant and precipitation of different treatment groups, n = 3. (*** p < 0.001 vs. M supernatant; ### p < 0.001 vs. M precipitation). (b) Representative TEM photos of mitochondria of different treatment groups. M: untreated mitochondria; HM: Heat-inactivated Mitochondria; UM: Ultrasonicated Mitochondria; FM: repeated Freeze-thaw treated mitochondria.

Supplementary Files

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- FigureS1..tif