

# The Effect of CCCP on Mitophagy in CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells in Human Peripheral Blood

**Running title: CCCP effect on Tregs mitophagy**

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**ABSTRACT:** **Objective:** To investigate the effects and mechanisms of different concentrations of CCCP on mitophagy in human peripheral blood regulatory T cells. **Methods:** Tregs were isolated, identified and then grouped, treating with CCCP at a concentration of 2.5  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M and 40  $\mu$ M for 24h in an incubator. Flow cytometry detected the reactive oxygen species (ROS), mitochondrial membrane potential (MMP), mitochondrial quality, and fluorescence microscopy observed the co-localization of mitochondria and lysosomes in each group. **Results:** The purity of CD4<sup>+</sup>CD25<sup>+</sup>Tregs was (93.36  $\pm$  1.87) %. With the increase of CCCP concentration, the level of ROS gradually increased, while the MMP decreased gradually. About the mitochondria and lysosome fusion, the fluorescence intensity of orange (yellow) was the highest when the concentration of CCCP was in the range of 5-10  $\mu$ M while decreased with the CCCP concentration continually increasing. The mitochondrial quality decreased with the increase of CCCP concentration. However, there was no significant difference between groups C, D and E. The mitochondrial quality of groups F and G were significantly lower than that of group E. **Conclusions:** With

the concentration of CCCP gradually increased, the level of ROS in Treg cells increased, and MMP decreased, which promoted the mitophagy, mitochondrial quality maintains homeostasis; When ROS accumulated, and MMP decreased significantly, the mitophagy was inhibited, and the mitochondrial quality was significantly decreased.

**KEYWORDS:** CCCP; Mitophagy; Regulatory T cells; Flow cytometry; fluorescence microscopy

## 1 INTRODUCTION

Mitochondria are essential sites for eukaryotic cells to undergo bio-oxidation and energy conversion. Mitochondrial autophagy (Mitophagy) is a process of selectively scavenging excess or damaged mitochondria, which plays an essential role in regulating the number of mitochondria, maintaining mitochondrial function and the stability of the intracellular environment<sup>[1]</sup>. Mitophagy abnormalities can cause a variety of diseases such as cancer, autoimmune diseases, cardiovascular diseases, metabolic syndrome.<sup>[2]</sup> The carbonyl cyanide m-chlorophenylhydrazone (CCCP) is an uncoupler of the mitochondrial oxidative respiratory chain, which can eliminate the transmembrane proton concentration difference on both sides of mitochondrial intima and slow down the process of oxidative phosphorylation driven by proton concentration difference and hinders the normal production of adenosine triphosphate (ATP). It can make mitochondrial depolarisation to alter MMP and is thought to be induced parkin-dependent mitophagy to remove depolarised mitochondria, to maintain the stability of quality and quantity of mitochondria<sup>[3-4]</sup>. The treating time of CCCP is generally 24h, but for different cells, the optimal concentration for promoting autophagy is different<sup>[5]</sup>. Regulatory T cells (Tregs) are CD4<sup>+</sup> effector T cell subsets with immunosuppressive function; their phenotype is CD4<sup>+</sup>CD25<sup>+</sup>, Tregs can inhibit the activation of autoreactive T cells and autoreactive B cells, which is the key to maintaining immune balance and autoimmune tolerance<sup>[6]</sup>. The use of immunosuppressive Tregs for therapeutic purposes is of great interest to researchers because of their great potential in the treatment of autoimmune diseases, prevention of transplant rejection and tumour immunotherapy<sup>[7-9]</sup>. Previous research in our research group showed that the abnormal mitophagy in peripheral blood Treg cells in patients with myasthenia gravis (MG)<sup>[10]</sup>. In this research, the autophagy state of mitochondria was

changed In Vitro by using different concentrations of CCCP in Tregs. The intracellular ROS, MMP, mitochondrial fusion, lysosome fusion, and mitochondrial quality level were observed to explore the effect and mechanism of CCCP on the mitophagy in Treg cells to find the optimal concentration of CCCP to promote mitophagy in Treg cells. Which can lay the foundation for our group to use CCCP In Vitro to change the mitophagy status in Tregs of MG patient; and to observe the level of autophagy and metabolism, and then to explore the immune metabolic mechanism of abnormal mitophagy involved in MG in the future.

## 2 MATERIALS AND METHODS

### 2.1 Materials

Choosing twenty healthy volunteers in medical examination centre in our hospital, 10 males, 10 females, aged 24.3 - 49.6 years, mean age ( $35.6 \pm 9.1$ ) years old, collecting 30ml peripheral blood from them for separating Tregs, the hospital ethics committee has reviewed the program, and volunteers have signed informed consent. Lymphocyte separation liquid was purchased from a Biotechnology Co., Ltd in China. Human CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T cell sorting kit, LD column, and MS column were purchased from German company Miltemyi Biotec. Labelled antibodies FITC-CD4, PE-CD25 Purchased from BD Biosciences, USA, and DCFH-DA probe purchased from Sigma USA. JC-10 probe purchased from Wuhan Plandor Biotechnology Company; Mito Tracker@Green, Lysotracker@Red purchased from Life Technologies, USA; RPMI-1640 culture medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco and used a CytoFLEX analytical flow cytometer from Beckman Co., Ltd. to detect, a fluorescence microscope of Olympus Corporation to observe.

### 2.2 Methods

#### 2.2.1 Isolation of PBMC and Treg Cells and Identification of Tregs

(1) PBMC: Use a 50 ml's heparinised sterile syringe to collect 30 ml peripheral blood, diluted to 90 ml in a biological safety cabinet, and then added 15 ml of lymphocyte separation solution to each of three sterile 50 ml centrifuge tubes. After that, 30 ml of diluted peripheral blood was slowly added along the wall of the tube and centrifuged at 2000 rpm/minutes for

20 minutes. Cells from the white film layer were harvested and washed with PBS twice to obtain PBMC.

(2) CD4<sup>+</sup>CD25<sup>+</sup> Tregs: PBMCs were resuspended in 2 ml PBS and the cell count was approximately (4-5) x 10<sup>7</sup> cells/ml. The cells were centrifuged at 1200 rpm/minutes for 8 minutes, and the supernatant was removed. After resuspension with buffer, appropriate amounts of CD4<sup>+</sup> T-cell biotinylated cocktail antibodies were added. After mixing, the cells were incubated at 4 °C in the dark for 5 minutes, and then the anti-biotin labelled beads were added and mixed. After incubation at 4 °C in the dark for 10 minutes, the cell suspension was passed through the LD column, and CD4<sup>+</sup>T cells were isolated. Resuspended the obtained cells, add an appropriate amount of anti-CD25 magnetic beads antibody, mixed and incubated at 4 °C in the dark for 15 minutes, washed the cells with buffer, centrifuged, removed the supernatant, and resuspended. The cell suspension was passed through the MS column to obtain CD4<sup>+</sup>CD25<sup>+</sup> Tregs. Antibodies FITC-CD4, PE-CD25 labelled Tregs, the purity of which was detected by flow cytometry, and the cells were treated with CCCP at a concentration of 2.5 µM, 5 µM, 10 µM, 20 µM, 40 µM respectively, and the cells of each group were incubated for 24h in a cell incubator at 37 °C, 5% CO<sub>2</sub>.

### ***2.2.2 Measurement of ROS***

Cells were collected in each group, and the blank group was resuspended with added 1 ml serum-free cultures were, healthy control group and CCCP groups with different concentrations were added 1ml 10 µM DCFH-DA active oxygen probe diluted in serum-free medium, incubated at 37 °C in the dark for 20 minutes. Mixed every 3 minutes. Washed the cells three times with serum-free medium, resuspended in 200 µl PBS, and measured by flow cytometry.

### ***2.2.3 MMP in Treg Cell***

The Treg cells in each group were collected, centrifuged, and the supernatant was removed and resuspended in 0.5 ml serum-free medium. 1 ml of serum-free medium was added to the blank group, and 1 ml of diluted JC-10 MMP fluorescent probe was added to the healthy control group and CCCP groups. Incubated at 37 °C in the dark for 20 minutes, centrifuge,

remove the supernatant, wash with PBS twice, resuspended in 200  $\mu$ l of serum-free medium, and measured by flow cytometry.

#### ***2.2.4 Observe the fusion of mitochondria and lysosomes***

Take 3  $\mu$ l mitochondrial probe (1 $\mu$ M) and 6  $\mu$ l lysosomal probe (1 $\mu$ M) into 6 ml serum-free medium respectively, and pre-heat the probes at 37 °C for 30 minutes. The cells in each group were collected, centrifuged, and the supernatant was removed, add 1 ml of pre-heat mitochondrial probe working solution and 1 ml of pre-heat lysosome probe working solution to the healthy control group and CCCP groups. , mixed, incubated at 37 °C in the dark for 40 minutes, centrifuged, and removed the supernatant and then washed with PBS twice, resuspended with 50  $\mu$ l medium and dropped it onto a slide, added coverslip and observed immediately under a fluorescence microscope. The images of mitochondrial green fluorescence and lysosomal red fluorescence were taken under the same magnification and the same field of vision. After Merging, the fusion of mitochondria and lysosomes was analysed using Image J software.

#### ***2.2.5 Determination of the mitochondrial quality level***

Take 3  $\mu$ l mitochondrial probe (1 mM) into 6 ml of serum-free medium. Preheat at 37 °C for 30 minutes. Collect cells from each group, centrifuge, and the supernatant was removed, add 1 ml of pre-heat mitochondrial probe working solution to each group. Mixed, incubated at 37 °C in the dark for 40 minutes, centrifuged, and washed with PBS twice, resuspended in 200  $\mu$ l of serum-free medium, and measured by flow cytometry.

### **2.3 Data Analysis**

SPSS 17.0 software was used for statistical analysis. Experimental data were presented as means  $\pm$  standard error of mean (SEM). Statistical comparisons were performed by using one-way analysis of variance (ANOVA).  $P < 0.05$  or less were considered to be of statistical significance.

### 3 RESULTS

#### 3.1 Identification of Treg Cells

The Treg cells separated by magnetic beads were labelled with FITC-CD4 and PE-CD25 antibodies, and the purity was  $(93.36 \pm 1.87)\%$  (see figure 1).

#### 3.2 ROS level in Treg Cells

The DCFH-DA probe has no fluorescence and can pass through the cell membrane freely. After entering the cell, it is hydrolysed into DCFH by intracellular esterase, while the DCFH cannot cross the cell membrane so that the probe can be easily loaded into the cell. ROS can oxidise non-fluorescent DCFH to fluorescent DCF, so the fluorescence of DCF can reflect the level of ROS in cells, the higher the fluorescence of DCF, the higher the level of ROS. Analysis of variance showed a significant difference between groups,  $F = 19.66$ ,  $P < 0.05$ , ROS levels in each group were not equal; the linear effect between groups,  $F = 94.21$ ,  $P < 0.05$ . Therefore, the level of ROS increased after CCCP treatment, and the level of ROS gradually increased as the concentration of CCCP increased,  $P < 0.05$ , the difference was statistically significant (see figure 2).

#### 3.3 MMP in Treg Cell

When the MMP is high, JC-10 accumulates in the matrix of mitochondria to form a polymer that can produce red fluorescence; when the MMP is low, JC-10 cannot accumulate in the matrix of mitochondria, at this time JC-10 is a monomer that produces green fluorescence. Therefore, FITC and PE channels can be set for flow cytometry to represent green fluorescence and red fluorescence. The cross-sectional door is divided the two-dimensional scatter plot into four quadrants. The lower right quadrant represents that JC-10 exists as a monomer in the cell and emits green fluorescence. Comparing the proportion of cells in the lower right quadrant can reflect the level of MMP. The higher the proportion of cells in the lower right quadrant, the lower the MMP. Analysis of variance used for comparison between groups,  $F = 24.56$ ,  $P < 0.05$ , the difference was statistically significant; the total intergroup effect was broken down into linear and quadratic terms, the linear part  $P < 0.05$ , statistically significant, quadratic item  $P = 0.711$ , not statistically

significant. Therefore, as the concentration of CCCP gradually increased, the mitochondrial membrane potential gradually decreased (see figure 3).

### 3.4 Fusion of Mitochondria and Lysosomes in Treg Cells

The mitochondria and lysosomes were labelled with probes, observed under the fluorescence microscope, the mitochondria showed green fluorescence, and the lysosomes showed red fluorescence. The mitochondrial autophagic lysosomes showed orange (or yellow) fluorescence after the fusion. The level of orange (yellow) fluorescence can reflect the level of mitochondrial autophagy in Treg cells. The stronger the orange (yellow) fluorescence, the higher the mitochondrial autophagy level. Analysis of variance used for comparison between groups,  $F = 12.79$ ,  $P < 0.05$ , the difference was statistically significant; the fluorescence levels of cells in each group were different. The results of multiple comparisons showed that the fluorescence levels of the B, C, F, and G groups were lower than those of the E group, and the difference was statistically significant. Compared with the G group, the fluorescence levels of the B, C, D, E, and F groups were higher. The difference was statistically significant, so we found that when the CCCP concentration was between 5-10  $\mu\text{M}$ , the orange (yellow) fluorescence level was the strongest, and as the CCCP concentration continued to increase, the orange (yellow) fluorescence level decreased,  $P < 0.05$  (see figure 4).

### 3.5 Mitochondrial Quality in Treg Cell

The mitochondrial probe is a cellular osmotic structure derivative of carbonyl anthocyanin and is a bright green fluorescent probe. It almost does not fluoresce in aqueous solution, but only when it is in the lipid environment of mitochondria, and its ability to locate in the mitochondria is not affected by the MMP, and once it is ingested into the cells, they are not easy to be washed away. When the mitochondrial quality is impaired, the level of mitochondrial green fluorescence decreases. By standardising cell number, dye concentration and incubation time, semi-quantitative detection of mitochondrial quality can be achieved. In figure 5, the proportion of cells with standard mitochondrial quality on the V1R side, while the V1L side is the proportion of cells with impaired mitochondrial quality. The proportion of cells on the V1L side of each group was compared using analysis of variance (ANOVA),

$F=94.36$ ,  $P<0.05$ , and the difference was statistically significant, it means the mitochondrial quality of each group was not equal. The results of multiple comparisons showed that there was no significant difference between C, D and E groups,  $P> 0.05$ . Which means the mitochondrial quality of C, D and E groups maintained a dynamic balance; compared with group E, the proportion of cells on the V1L side in group B was lower, while in the group F and the group G increased, the difference was statistically significant ( $P<0.05$ ) (see figure 5).

#### 4 DISCUSSIONS

Mitochondria are organelles coated by two membranes that are present in most cells and have a diameter of about 0.5 to 10  $\mu\text{m}$ . Mitochondria are the primary sites for intracellular oxidative phosphorylation and synthesis of adenosine triphosphate (ATP), and 95% of the energy required for cellular vitality comes from mitochondria. Therefore, it is also called "power plant" for cells. In addition to supplying energy to cells, mitochondria also participate in processes such as cell differentiation, cell information transmission, and apoptosis, and has the ability to regulate cell growth and cell cycle<sup>[11]</sup>. Oxidative phosphorylation of mitochondria continues to provide energy support to the body, but ROS are produced during the process. Under the low or normal level, ROS represents essential cell signalling molecules and has a positive physiological effect, but high levels of ROS can cause oxidative damage. When ROS increase, mitophagy can selectively remove damaged mitochondria, reduce the release of some pro-apoptotic factors, prevent the intensification of oxidative stress, and maintain the stability of the intracellular environment<sup>[12]</sup>. The mechanism may be: when the mTOR- dependent pathway sensed the increased environmental signal of ROS, its inhibitory effect on the ULK1-Atg13-FIP200 complex was weakened. And the complex was activated and transported from the cytoplasm to the specific site of the damaged mitochondrial membrane structure to form autophagy pronucleus, recruitment of downstream PI3K, LC3 and other autophagy-related proteins to form the autophagic vacuoles. Which was then followed by nucleation, autophagic body formation, and fused with the lysosome and then mitophagy was completed, abnormal mitochondria were removed timely, cell survival was promoted<sup>[13]</sup>. Another mechanism that has been researched at present is the HIF-1 $\alpha$ /BNIP3 / BNIP3L pathway, the increase of ROS promote the expression of HIF-1 $\alpha$

and HIF- 1 $\alpha$  activates the autophagy signal of downstream BNIP3/BNIP3L gene transcription, and the expression of autophagy genes such as Beclin-1 and Atg5 are upregulated, eventually promoting mitophagy [14]. The mitochondrial permeability transition pore (mPTP) is a voltage-gated ion channel across the mitochondrial inner and outer membranes that consists of multiple subunits, it can regulate the permeability of the mitochondrial inner membrane. The increase of ROS level can promote the opening of mPTP, which activates mitophagy [15].

Mitochondria are both major sources of oxidative stress and target organelles for oxidative stress. Mitochondria can sensitively sense the changes in oxygen concentration in the body. CCCP can affect the function of the electron transport chain during mitochondrial oxidative phosphorylation and inhibit ATP production, produce a lot of ROS. The accumulation of ROS can induce oxidative stress in cells by directly inducing single-stranded, and double-stranded DNA breaks, or by oxidising fatty acids, amino acids in proteins, or enzymatic cofactors, and can directly attack mitochondria. Which is causing swelling of mitochondria and mitochondrial membranes permeability increases, MMP decreases, and apoptosis-related proteins such as cytochrome C and apoptosis-inducing factor are released, which affect cell function and induce apoptosis. [16]. Studies have found that when the ROS produced by damaged mitochondria are released into the cytoplasm, which can damage the normal mitochondria around, and positive feedback produces a large amount of ROS that unbalances the mitochondrial oxidative phosphorylation and eventually leads to extensive mitochondrial damage [17].

In the process of mitophagy, autophagy body can wrap up damaged mitochondria in the early, and in the mid-term, mitochondrial autophagosomes fuse with lysosomes to form mature mitochondrial autophagy lysosomes [18]. Mitochondrial and lysosomes were labelled with Mito Tracker@Green and Lysotracker@Red, respectively. Under fluorescence microscopy, mitochondria showed green fluorescence, lysosomes showed red fluorescence, and mitochondrial autolysosomes showed orange (or yellow) fluorescence. Observing the level of orange (yellow) fluorescence can reflect the level of mitophagy in Treg cells. We found that when the concentration of CCCP was between 5-10  $\mu$ M, the orange (yellow) fluorescence level was the strongest, and as the CCCP concentration continued to increase,

the orange (yellow) fluorescence level decreased. Which is indicating that the concentration of CCCP was at 5-10  $\mu$ M, the level of mitophagy was the highest, and as the concentration of CCCP continued to increase, the level of mitophagy decreased.

Mitochondria have a high degree of plasticity. Under normal circumstances, the number, morphology, and function of mitochondria are always maintained in a relatively stable state. When these conditions are disturbed, the energy supply of the cells and the life activities of the organism are bound to be affected. In order to deal with this damage, the cells have evolved a system of selective removal of damaged mitochondria - mitochondrial quality control (MQC), and mitophagy plays an essential role in the maintenance of mitochondrial quantity and quality by removing damaged mitochondria [19]. As can be seen from the above experiments, compared with the healthy control group, the mitochondrial quality decreased as the concentration of CCCP increased. But the multiple comparisons between the groups found that there was no significant difference between the groups of C, D, E, and compared with the group E, the mitochondrial quality in group F and G decreased significantly. We can conclude that when the concentration of CCCP is between 2. 5-10  $\mu$ M, mitophagy can remove damaged mitochondria and synthesise new mitochondria through the reuse of degradation components. Mitochondrial quality maintained in a dynamic balance, but when the concentration of CCCP reaches 20  $\mu$ M and above, mitophagy cannot remove the damaged mitochondria adequately, mitochondrial quality decreased significantly.

## 5 CONCLUSIONS

As the concentration of CCCP gradually increases, the ROS level in Treg cells gradually increases and the MMP gradually decreases, but only when the ROS level slightly increases, and the MMP slightly decreases, mitophagy is promoting, mitochondrial quality maintains a dynamic balance. When ROS accumulates, and MMP decreases significantly, a large number of mitochondria are damaged, the level of mitophagy decreased, and mitochondrial quality is severely damaged. Mitochondria are the most critical organelle involved in energy metabolism in Treg cells, the proliferation and differentiation of Treg cells are mainly dependent on mitochondrial lipid oxidation to supply energy and the proportion occupied by aerobic glycolysis a little [20]. When the mitochondrial quality of Treg cells is severely

damaged, the energy metabolism of Treg cells may be disturbed, the signal transduction of cells and the production of free radicals are abnormal, which may induce cell apoptosis, the specific mechanisms need to be further study.

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### Declaration of Conflicting Interests

The authors report no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Author Contributions

All authors contributed equally to this work in different phases of this project.

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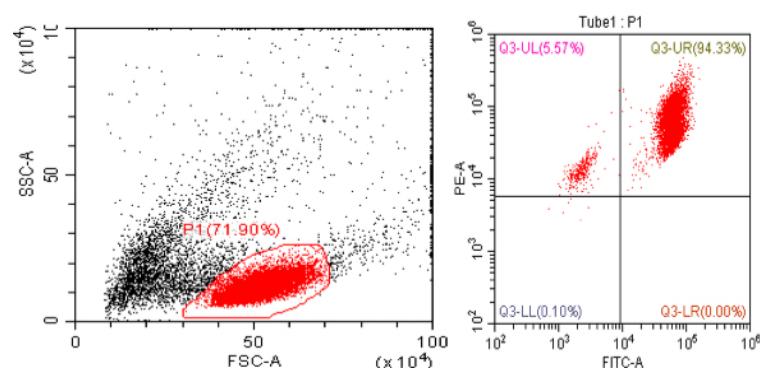
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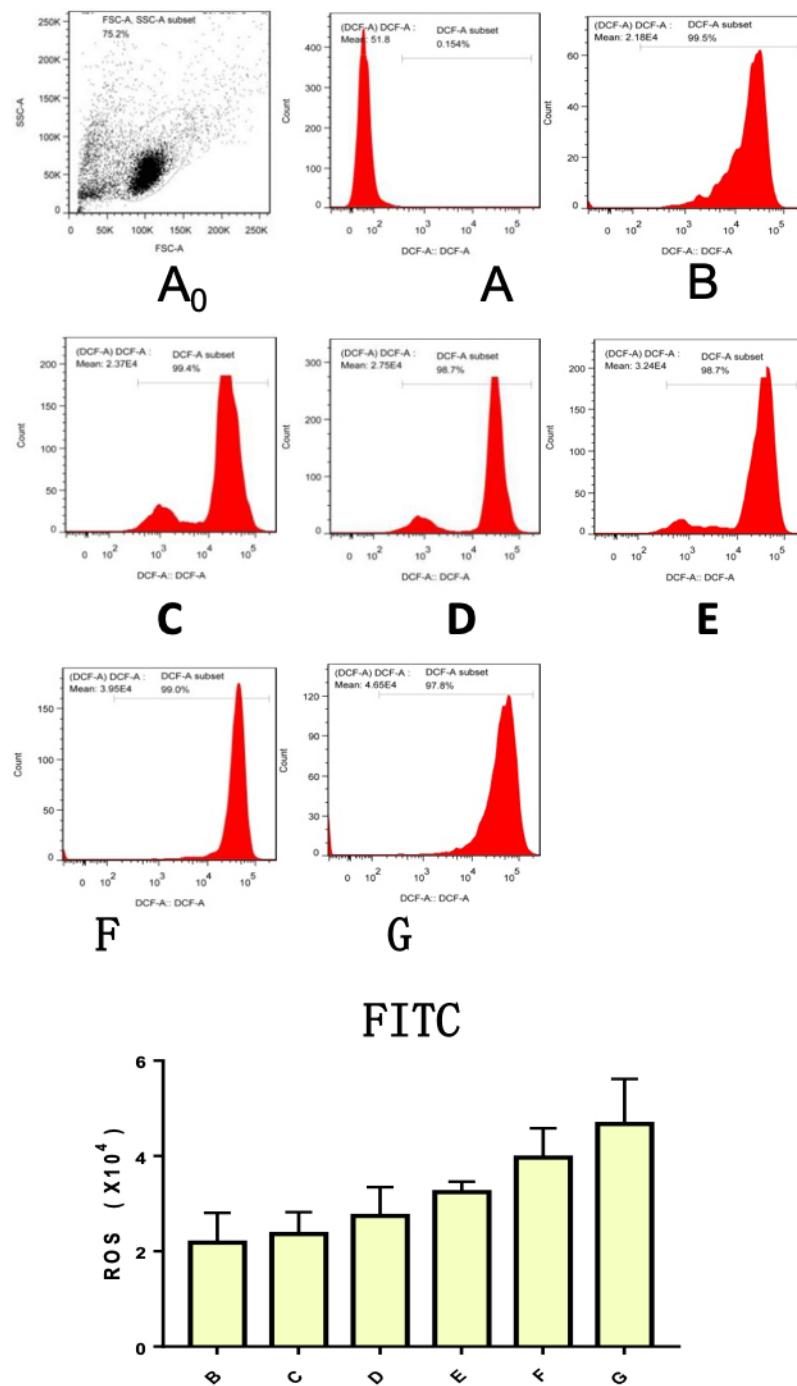
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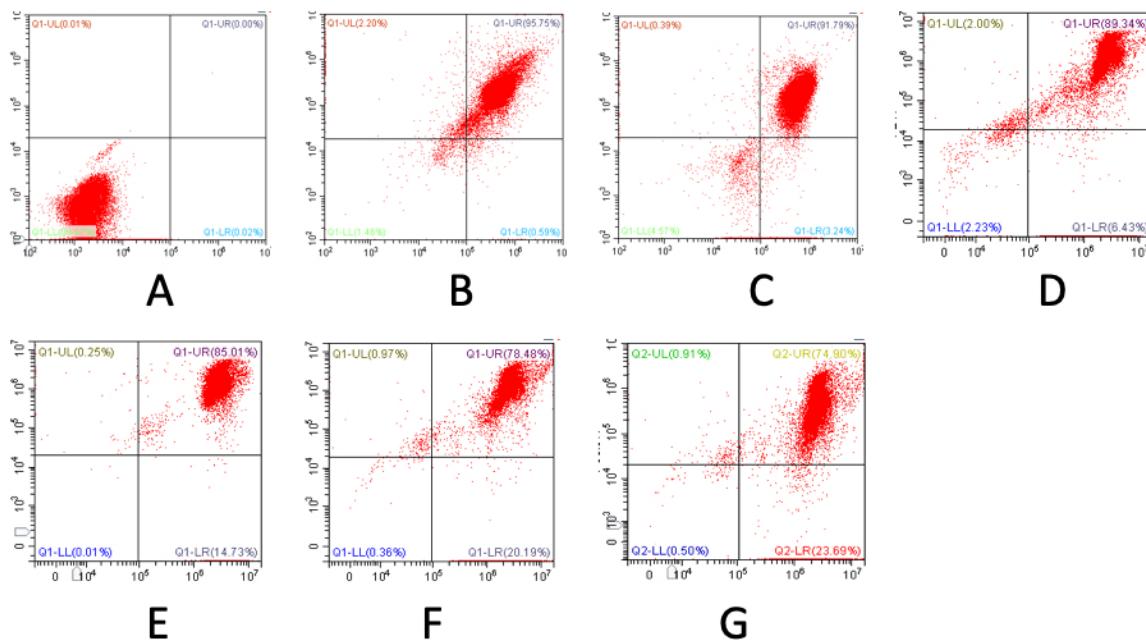
## FIGURES



**Figure 1:** FITC-CD4, PE-CD25 labelled Tregs (target cells in P1, a proportion of CD4+CD25+ cell in the Q3-UR quadrant).

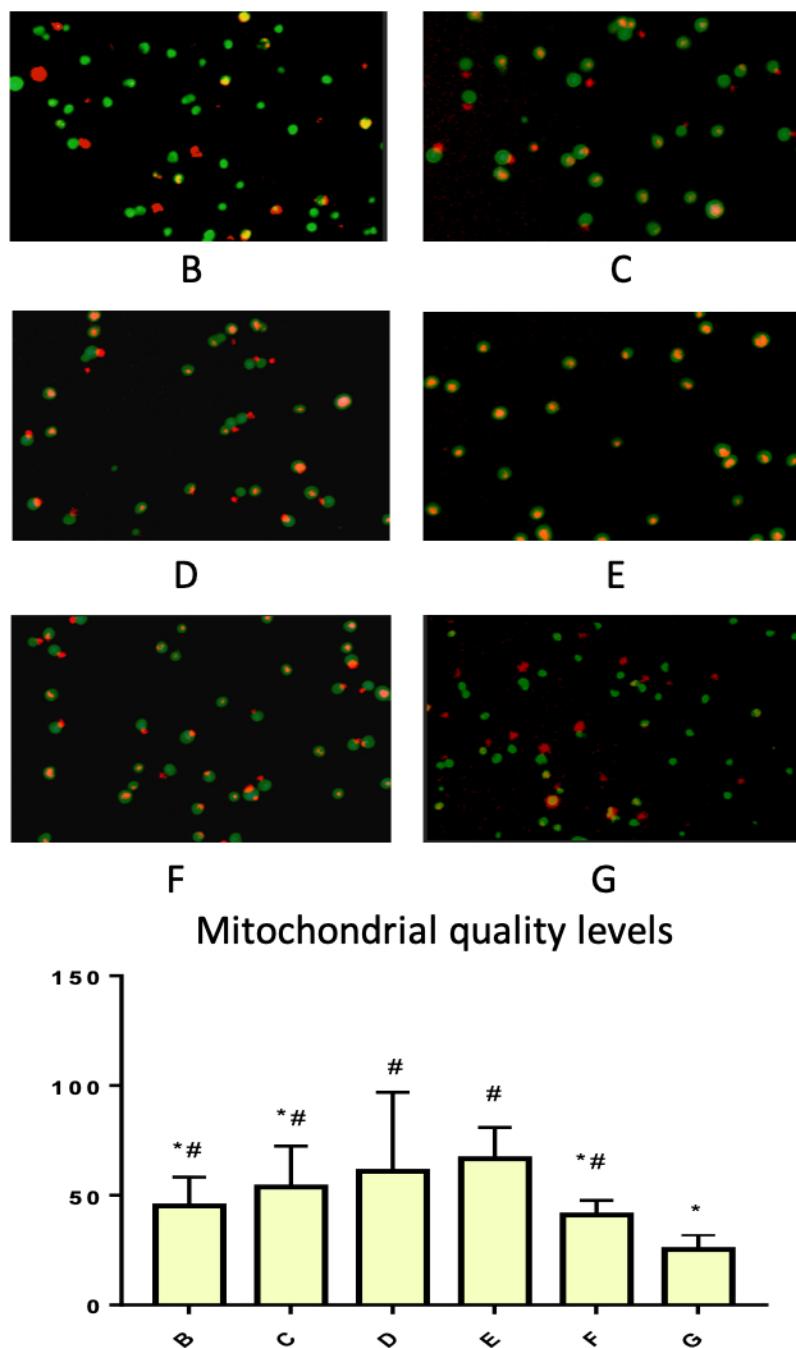


**Figure 2:** Detection of ROS level in each group by DCFH-DA probe, the average fluorescence intensity of DCF represents ROS levels (A. blank group, unstained Tregs; B. healthy control group; C. CCCP 2.5  $\mu$ M; D. CCCP 5.0  $\mu$ M; E. CCCP 10  $\mu$ M; F. CCCP 20  $\mu$ M; G. CCCP 40  $\mu$ M).

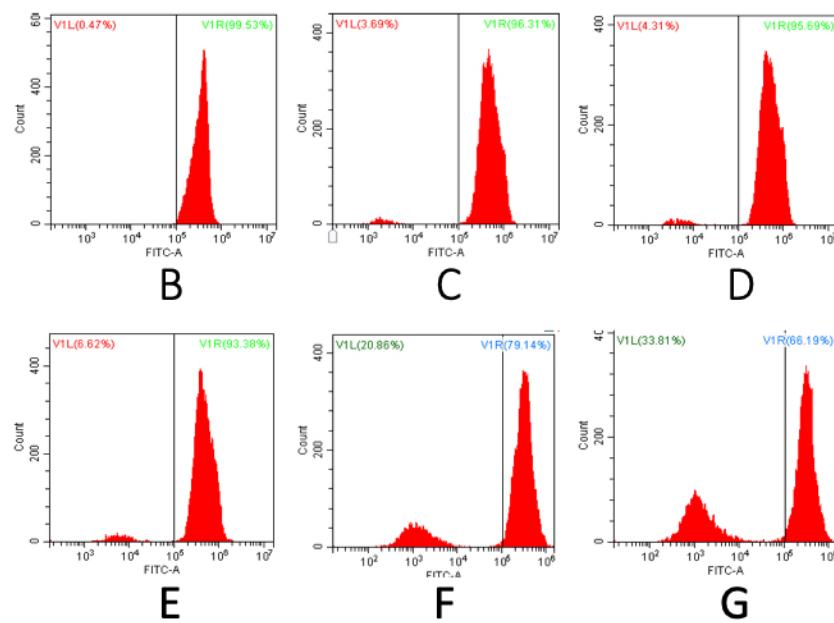


Group	Right lower quadrant cell ratio (%)		F Value	P Value
B	0.68±0.55	Cluster	24.56	0.000
C	3.32±2.10	linear items comparison	119.80	0.000
D	7.10±2.78	Deviation	0.749	0.564
E	14.35±6.39	Second items comparison	0.139	0.711
F	20.15±6.78	Deviation	0.952	0.424
G	22.39±7.70			

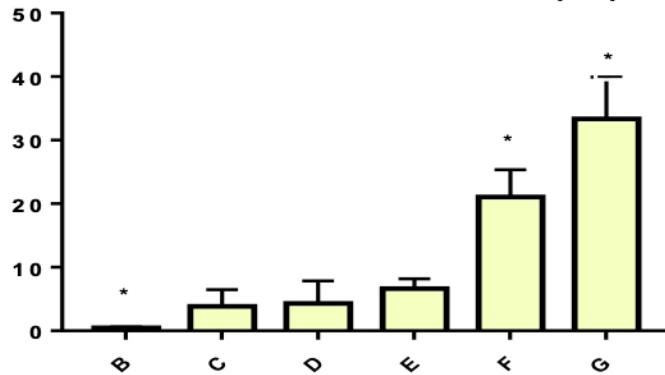
**Figure 3:** Detection of MMP level in each group by JC-10 probe, the proportion of cells in the lower right quadrant indicates the level of MMP (A. blank group, unstained Tregs; B healthy control group; C. CCCP 2.5  $\mu$ M; D. CCCP 5.0  $\mu$ M; E. CCCP 10  $\mu$ M; F. CCCP 20  $\mu$ M; G. CCCP 40  $\mu$ M).



**Figure 4:** mitochondria and lysosomes were labelled with probes, observed the yellow (orange) fluorescence levels in each group (the fluorescence level was analyzed with Image J software, magnification was: 200X; B. healthy control group; C. CCCP 2.5  $\mu$ M; D. CCCP 5.0  $\mu$ M; E. CCCP 10  $\mu$ M; F. CCCP 20  $\mu$ M; G. CCCP 40  $\mu$ M, compared with CCCP 10  $\mu$ M group, \* $P<0.05$ ; compared with CCCP 40  $\mu$ M group, #  $P<0.05$ ).



### V1L side cell ratio (%)



**Figure 5:** Probe labelled mitochondria, flow cytometry detected mitochondrial quality levels in each group (B. healthy control group; C. CCCP 2.5  $\mu$ M; D. CCCP 5.0  $\mu$ M; E. CCCP 10  $\mu$ M; F. CCCP 20  $\mu$ M; G. CCCP 40  $\mu$ M; compared with CCCP 10  $\mu$ M group, \*P<0.05).