



## Autophagy perturbation upon acute pyrethroid treatment impacts adipogenic commitment of mesenchymal stem cells

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### ABSTRACT

Environmental chemical exposure can cause dysregulation in adipogenesis that can result in metabolic syndrome, which includes insulin resistance, type 2 diabetes, cardiovascular disease, as well as excessive body weight. The role of autophagy in adipocyte differentiation is debatable since both positive and negative effects have been reported. Type-I and type-II synthetic pyrethroids α-cypermethrin (CPM) and permethrin (PER), respectively, are reported to increase adipogenesis *in vitro* and *in vivo*. However, it is not known how these pyrethroids affect mesenchymal stem cells (MSCs). Thus, this study focused on evaluating the effect of pyrethroids (CPM and PER) pre-treatment (24 h) on MSC commitment and the regulatory role of autophagy in adipogenic lineage commitment. The formation of adipocytes was observed through nile red staining, perilipin expression by immunofluorescence, and adipogenic markers PPAR $\gamma$ , C/EBP $\alpha$ , and FABP4 by western blotting. It was found that the adipogenic differentiation ability of MSCs was significantly increased upon CPM or PER pre-treatment at 100  $\mu$ M concentration as evident by lipid accumulation and enhanced expression of adipogenic markers. To assess the involvement of autophagy, the expression of p62 and LC3II were evaluated following pre-treatment. Immunoblotting results revealed an increased expression of p62 and LC3II in CPM or PER pretreated MSCs suggesting CPM and PER mediated inhibition of autophagy at 24 h. Further, an increase was observed in adipogenesis upon CPM or PER pre-treatment in combination with chloroquine, while use of rapamycin during pre-treatment abrogated the effect of CPM and PER. Thus, this study concludes that CPM or PER pre-treatment increases the adipogenic differentiation of MSCs. Since chloroquine also demonstrated similar adipogenic response, it further highlights that 24 h pre-treatment with autophagy modulators to inhibit basal autophagy primes MSCs towards adipogenic lineage.

### 1. Introduction

Obesity and weight gain have emerged as one of the most concerning human health problems in recent times. The global prevalence of obesity has risen nearly three times since 1975 according to the World Health Organization (<https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight>). Evidence suggests a link between obesity and adipogenesis, the process of differentiation of mesenchymal stem cell (MSCs) into mature adipocytes (Rosen and MacDougald, 2006). Recently the involvement of autophagy, a catabolic process for the breakdown of bulk cytoplasmic contents and subcellular organelles, has been investigated in adipogenesis (Sekar and Thirumurugan, 2022).

Adipogenesis and obesity have been functionally related to autophagy. Obese individuals and animals show enhanced autophagy and autophagic flux in adipose tissues (Jansen et al., 2012; Kovsan et al., 2011; Nunez et al., 2013). Several reports also suggest that elevated level of autophagy promoted MSC differentiation towards adipogenesis (Song et al., 2015; Spalletta et al., 2018; Yu et al., 2021). However, suppression of adipogenic conversion was also observed with increased autophagy (Zhao et al., 2014). A study demonstrated that MSCs have a sensitive phenotype, with amassed autophagosomes, which are on the verge of autophagic breakdown. The autophagy balance, thus, appears to be crucial for MSC differentiation and function (Nuschke et al., 2014). These fascinating findings highlight the functional significance of

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autophagy in adipogenesis.

Apart from autophagy, the tremendous rise in overweight and obesity rates around the globe has moved attention to other potential environmental contributors as well, like pesticides. Studies have also established the connection of pesticide exposure with higher risk of obesity, including pyrethroids (Zuo et al., 2022; Wang et al., 2021; Jiang et al., 2022). Permethrin (a type-I pyrethroid) and  $\alpha$ -cypermethrin (a type-II pyrethroid) have structural similarities to natural pyrethrins, and represent the two classes of pyrethroids (Suppl. Fig. 1). Due to their widespread use in agriculture and domestic pest control, exposure of humans is highly likely. Occupational exposure to farmers and industrial workers has led to blood serum concentrations up to 32  $\mu$ g/ml (~77  $\mu$ M) of CPM (Azmi et al., 2006). CPM and its metabolite, 3-phenoxybenzoic acid, have also been detected in blood and urine, respectively, of humans (Silver et al., 2015; Dewailly et al., 2014). Both pyrethroids have been recently reported to increase preadipocyte 3T3-L1 differentiation towards adipocytes (He et al., 2020; Kim et al., 2014). In addition, permethrin and deltamethrin have also been shown to cause fat accumulation in mice and *C. elegans*, respectively (Xiao et al., 2018; Shen et al., 2017). Studies have also reported the role of oxidative stress in pyrethroid mediated toxicity (Lu et al., 2019; Wang et al., 2016; Romero et al., 2017; Afolabi et al., 2019). This is important as oxidative stress results in increased adipogenic differentiation of MSCs (Denu and Hematti, 2016) and also modulates autophagy (Kiffin et al., 2006; Yun et al., 2020).

Most of the studies have focused on evaluating the effect of pyrethroid exposure on adipocyte differentiation using the well-established 3T3-L1 cell line. However, 3T3-L1 is a type of committed pre-adipocyte that has lost its stem cell properties, and undergo post-confluent mitotic expansion unlike primary adipogenic precursor cells (Zhao et al., 2014). None of the reported studies reflect upon whether short-term exposure to pyrethroids impacts MSC differentiation potential. Since MSCs are the precursors of preadipocytes, it is worthwhile looking at if cells are affected before becoming committed to adipogenic lineage. Hence, this study was undertaken to assess how acute CPM and PER pre-treatment of MSCs affects their adipogenic commitment, and does autophagy play any role.

## 2. Materials and methods

### 2.1. Chemicals/Reagents

Cypermethrin ( $\alpha$ -cypermethrin) (36,128, CAS No. 52315-07-8), (molecular formula  $C_{22}H_{19}Cl_2NO_3$ ; >90% purity) and Permethrin (45,614, CAS No. 52645-53-1) (molecular formula  $C_{21}H_{20}Cl_2O_3$ ; >90% purity) were procured from Sigma-Aldrich (USA). ProLong<sup>TM</sup> Diamond Antifade Mountant (P36970) mounting media was purchased from ThermoFisher Scientific (USA). Minimum Essential Medium alpha ( $\alpha$ -MEM) (12000–022), Fetal Bovine Serum (FBS) (10270–106) and Antibiotic- antimycotic solution (100 $\times$ ) (15240062) were purchased from Gibco (USA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (475989), dexamethasone (D4902), 3-Isobutyl-1-methylxanthine (IBMX) (I5879), Indomethacin (I7378), Insulin solution Human (I9278), Bovine Serum Albumin (A3608), Sodium bicarbonate (S5761), Ethylenediaminetetraacetic acid (EDTA) (E6758), Neutral Red (N4638), 4',6-diamidino-2-phenylindole (DAPI) (D9542), Trypsin (T4799) and Chloroquine diphosphate salt (C6628) were purchased from Sigma-Aldrich (USA). Western Chemiluminescent HRP Substrate (1705062) was purchased from Bio-Rad (USA). Rapamycin (sc-3504) was purchased from Santa Cruz Biotechnology (USA). Triton X-100 (64518) was purchased from Sisco Research Laboratories (SRL) (India). Primary antibodies rabbit anti-PPAR $\gamma$  (2435S), rabbit anti-CEBP $\alpha$  (8178S), rabbit anti-FABP4 (3544S), rabbit anti-perilipin-1 (9349S) and secondary antibodies horse anti-mouse IgG (7076P2), goat anti-rabbit IgG (7074P2) were procured from Cell Signaling Technology (USA). Mouse Anti-SQSTM1/p62 (ab56416) and Goat Anti-

rabbit IgG H&L (Alexa Fluor<sup>®</sup> 488) (ab150077) were purchased from abcam (USA). Rabbit anti-LC3 antibody (L8918) was obtained from Sigma-Aldrich (USA). Mouse anti- $\beta$ -Actin (MA5-15739) was purchased from Thermo Scientific (USA).

### 2.2. Primary culture of bone marrow mesenchymal stem cells

Adult male and female BALB/c mice (8–10 weeks) were obtained from animal breeding colony of CSIR-IITR. Mice were fed with pellet diet and water *ad libitum* and maintained under controlled standard temperature and humidity environment. The study was approved by the Institutional Animal Ethics committee (IITR/IAEC/03/2016; IITR/IAEC/16/20) and animals were handled during the experiment according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Government of India. Mouse was euthanized using a single high dose of Ketamine (100 mg/kg b.wt.) and xylazine (2 mg/kg b.wt.) intraperitoneally. Skin was cleaned by 70% ethanol, followed by fur and skin removal. Incisions were made around the connection between hind limbs and trunk. The leg was detached from the joint and washed with 70% ethanol then immersed in ice cold phosphate buffered saline (PBS). Inside the laminar hood the muscles, ligaments, and tendons were removed and the bones were transferred into PBS containing antibiotics. Tibia and femur bones were separated from the joint using dissecting scissors and surgical scalpel and cells were flushed out from the bone cavities with  $\alpha$ -MEM medium using needle until the bones become pale. The cell suspension was centrifuged at 300g for 5 min and the pellet was resuspended in fresh complete  $\alpha$ -MEM Medium, supplemented with 20% FBS and 1% antibiotic-antimycotic solution (100 $\times$ ). Cells were plated in two 25 cm<sup>2</sup> cell culture flasks (Corning, USA) and were incubated in CO<sub>2</sub> incubator (5% CO<sub>2</sub>) at 37°C. Medium was replaced with fresh complete  $\alpha$ -MEM medium after 24 h to remove unattached cells. Cells were passaged after 4–6 days using 0.25% trypsin-EDTA. MSCs were characterized and used in further experiments after five passages to ensure using pure population of MSCs for experiments.

### 2.3. MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a colorimetric assay which is used to detect the metabolic state and survival of cells. MSCs were plated in 96-well plate (5000 cells/well) and treated with various concentrations of CPM and PER (1, 10 and 100  $\mu$ M). After 24 h treatment, MTT (0.5 mg/ml in PBS) was added to each well containing treatment media and incubated for 2 h in dark at 37°C. Formation of purple coloured insoluble formazan crystals was observed under the microscope after 2 h. Once the crystals were formed, media was removed and cells were washed once with PBS. 100  $\mu$ l DMSO was added to dissolve formazan crystals. Colorimetric absorbance was taken at 570 nm and 630 nm using spectrophotometer (Biotek PowerWave XS Microplate Reader, USA).

### 2.4. Neutral red uptake (NRU) assay

Neutral red is a cationic dye which has a net charge close to zero. This dye accumulates in lysosomes and becomes charged which makes it unable to leave lysosome. If cell dies or lysosomal proton gradient is disturbed neutral red cannot stay inside the lysosome and freely passes out. For neutral red assay, cells were seeded into 96-well plates (5000 cells/well) and treated with different concentrations of CPM and PER (1, 10 and 100  $\mu$ M) for 24 h. Media was replaced with neutral red containing media after 24 h and incubated for 2 h at 37°C. Cells were washed with PBS followed by dye extraction from each well using 100  $\mu$ l neutral red destain solution (50% ethanol, 49% deionized water and 1% glacial acetic acid) per well. Absorbance was read at 540 nm using spectrophotometer (Biotek PowerWave XS Microplate Reader, USA).

## 2.5. Differentiation assay

MSCs were plated in 96-well plate with 5000 cells/well. Post confluence, cells were treated with different doses of CPM and PER (1, 10 and 100  $\mu$ M) for 24 h. For adipogenic differentiation, treatment media was replaced with differentiation media after 24 h without washing the cells. Cells were then cultured for one to two weeks in adipogenic differentiation medium (complete  $\alpha$ -MEM media supplemented with 1  $\mu$ M dexamethasone, 0.5  $\mu$ M 3-isobutyl-1-methylxanthine, 50  $\mu$ M indomethacin and 1  $\mu$ g/ml Insulin) with medium changed every alternate day. Cells were also treated with chloroquine (CQ) (10  $\mu$ M) and rapamycin (200 nM) alone and in combination with PER and CPM for 24 h.

## 2.6. Nile red staining

Nile red staining is used to stain intracellular lipid droplets. To analyze the lipid droplets in adipocytes, wells were washed with PBS and fixed with 4% PFA for 15 min at room temperature. After fixation, cells were washed twice with PBS, followed by incubation in DAPI (0.5  $\mu$ g/ml) - nile red stain (200 ng/ml). The plate was incubated in dark for 5 min at room temperature. Cells were washed with PBS and images were captured. Five microscopic fields per well for each individual experimental group ( $n = 3$  in triplicate) were captured using Nikon ECLIPSE Ti-S inverted microscope (Nikon, Japan). Image processing and quantification was done using particle analysing tool of Image J (NIH, USA). Nuclei count was similar for all treatment groups and signals of nile red were normalized to the number of nuclei during analysis. A representative image is represented in the results. Details of image quantification and nuclei count are given in supplementary information (Suppl. Fig. 2A, B, 3A, B).

## 2.7. Western blotting

For western blotting, cells were washed with PBS followed by direct lysis of cells with hot laemmlie buffer, and denatured at 95°C for 5 min. Proteins were separated by SDS-PAGE, and transferred onto PVDF membrane using wet transfer. Membranes were blocked in 3% BSA in Tris-buffered saline-Tween 20 (TBST) and thereafter, incubated with primary antibody for p62 (1:1000), LC3 (1:2000), PPAR $\gamma$  (1:1000), CEBP $\alpha$  (1:1000) and FABP4 (1:2000) overnight at 4°C. Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG 1:10000, and anti-rabbit IgG 1:5000) for 1 h at room temperature. Bands were developed using chemiluminescent HRP substrate, and image was captured through Amersham Imager 600 (GE Healthcare Life Sciences, USA). The bands were quantified using Quantity One software (Bio-Rad, USA).  $\beta$ -actin was used as a house keeping gene for protein loading control.

## 2.8. Perilipin staining

Perilipin is a protein which coats the lipid droplets in adipocytes. To analyze perilipin protein expression, cells grown on coverslips were fixed with 4% PFA for 15 min at room temperature, and permeabilized with 0.2% Triton X- 100 + 1% BSA solution. Cells were washed twice with PBS, followed by incubation with rabbit anti-perilipin antibody (1:200) overnight at 4°C. Secondary goat anti-rabbit IgG Alexa Fluor 488 (1:500) was used against perilipin antibody. Cell nuclei were stained with DAPI (0.5  $\mu$ g/ml). Cells were washed with PBS and images were captured using Nikon ECLIPSE Ti-S inverted microscope, Japan. Image processing and quantification was done using Image J (NIH, USA). Nuclei count was similar in each experimental group. Signal of perilipin was normalized to the number of nuclei while analysing the results. A representative image set is shown in the results. Details of image quantification and nuclei count are given in supplementary information (Suppl. Fig. 2C, D, 3C, D).

## 2.9. Statistical analysis

All data are expressed as the mean  $\pm$  standard error of mean of atleast three independent experiments run in duplicate or triplicate. Statistical analysis of the data was done by using Prism5 GraphPad software. Comparison between two groups was performed by Student's t-test. Comparison between multiple groups was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's (when comparing all the groups to control) or Tukey's (when comparing all the groups with each other) multiple comparison test.  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. CPM and PER do not cause cell death

MSCs cultures established from mouse bone marrow were characterized. The cells expressed Sca-1, had no expression of CD11b (monocytic marker), and differentiated into adipocytes and osteoblasts (data not shown). Using these cells, firstly, the effect of different concentrations (1, 10 and 100  $\mu$ M) of CPM and PER on viability was assessed by MTT and NRU assays. Treatment of MSCs for 24 h did not result in any significant loss of cell viability upto 100  $\mu$ M concentration of CPM and PER (Fig. 1).

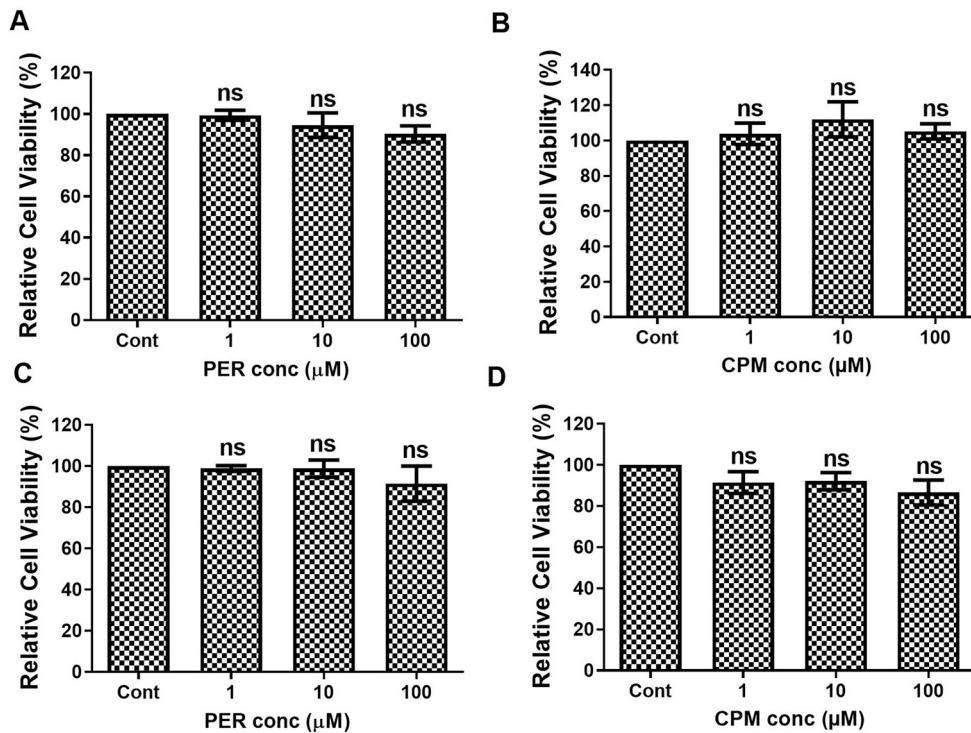
### 3.2. CPM and PER pretreatment makes MSCs more vulnerable towards adipogenic differentiation

Since no cytotoxicity was observed, the impact of 24 h pre-treatment of different concentrations of CPM and PER on adipogenic commitment of MSCs was evaluated. CPM and PER pretreated MSCs were subjected to adipogenic stimulus and differentiation was monitored by staining lipid droplets using nile red stain. Nile red staining showed that acute pre-exposure of CPM and PER increased the adipogenic differentiation potential of MSCs in a dose-dependent manner compared to control (Fig. 2A, C, E, G). This result was further validated by staining for perilipin whose expression was found to be increased with increase in concentration of CPM and PER (Fig. 2B, D, F, H). Maximum differentiation was observed at 100  $\mu$ M concentration which exhibited highest number of lipid-loaded cells. Thus, 100  $\mu$ M concentration of CPM and PER was chosen for further validation and mechanistic studies.

Adipogenesis is regulated by various transcription factors and proteins. PPAR $\gamma$  and C/EBP $\alpha$  are regarded as major transcription factors required for adipogenic differentiation as demonstrated through loss-of-function studies *in vivo* and *in vitro*. (Rosen et al., 1999; Rosen et al., 2002). Study has also reported expression of FABP4 during adipogenesis and its transcriptional regulation by PPAR $\gamma$  agonists, insulin, fatty acids (FAs) and dexamethasone (Furuhashi et al., 2014). Therefore, the expression of these markers was assessed through western blotting for evaluating adipogenic differentiation. It was found that the expression of these markers was significantly increased by ~36%, ~46% and ~30% in CPM pretreated cells (Fig. 2J) and ~90%, ~85% and ~43% in PER pretreated cells as compared to control (Fig. 2I). These results suggest that CPM and PER pre-exposure makes MSCs more prone towards adipogenic lineage without significantly affecting the viability of cells. It was also observed that PER pre-treatment causes slightly higher degree of adipogenic differentiation compared to CPM.

### 3.3. Autophagy inhibition induced by CPM and PER

As autophagy is one of the major mechanisms reported to regulate adipogenesis, therefore perturbation of basal autophagy levels in MSCs was assessed upon CPM and PER exposure to evaluate the effect at the stem cell level. MSCs were treated with CPM or PER for 24 h and the levels of crucial autophagic markers (LC3II and p62) were observed through immunoblotting. Western blot data showed significant



**Fig. 1.** PER and CPM mediated alteration in cell viability of MSCs. Concentration-dependent changes in the viability of MSCs were observed on exposure of MSCs to different concentration of PER and CPM (1, 10 and 100 μM) for 24 h through MTT assay (A, B) and NRU assay (C, D). Data are represented as mean ± SEM of three independent experiments performed in triplicate. ns = non-significant. Comparison between groups was done by one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

accumulation of LC3II as well as p62 upon CPM or PER treatment compared to control (Fig. 3A, B). This suggested CPM and PER mediated inhibition of autophagy.

Further, the rate of autophagic clearance, *i.e.* flux, was assessed using chloroquine (CQ) (an autophagy inhibitor) and rapamycin (an autophagy activator). Co-treatment of CPM or PER with CQ significantly upregulated LC3II and p62 levels compared to CPM or PER alone (Fig. 3C, D). However, the levels of LC3II and p62 were significantly decreased in cells treated with CPM or PER in combination with rapamycin (Fig. 3C, D). These results confirmed the inhibition of autophagy in MSCs upon CPM or PER treatment.

### 3.4. Inhibition of autophagy enhances adipogenic commitment of MSCs

To ascertain if inhibition of autophagy before the induction of adipogenic differentiation can result in enhanced adipogenesis, MSCs were pretreated with CQ for 24 h followed by adipogenic stimulus. This resulted in significant increase in adipogenesis (lipid droplets) compared to control (Fig. 4A, C, E, G). Combined pretreatment of CPM or PER with CQ resulted in further increase in the number of lipid-loaded cells as compared to CPM or PER alone (Fig. 4A, C, E, G). This increase in adipogenic differentiation was also confirmed through immunofluorescence staining for perilipin (Fig. 4B, D, F, H). The expression of adipogenic markers PPAR $\gamma$ , C/EBP $\alpha$  and FABP4 was also significantly increased in CQ treated group compared to control (Fig. 4I, J). These results suggest that reduced level of basal autophagy in mesenchymal stem cells is one of the mechanisms responsible for enhanced adipogenic commitment.

### 3.5. Activation of autophagy mitigates CPM and PER effect

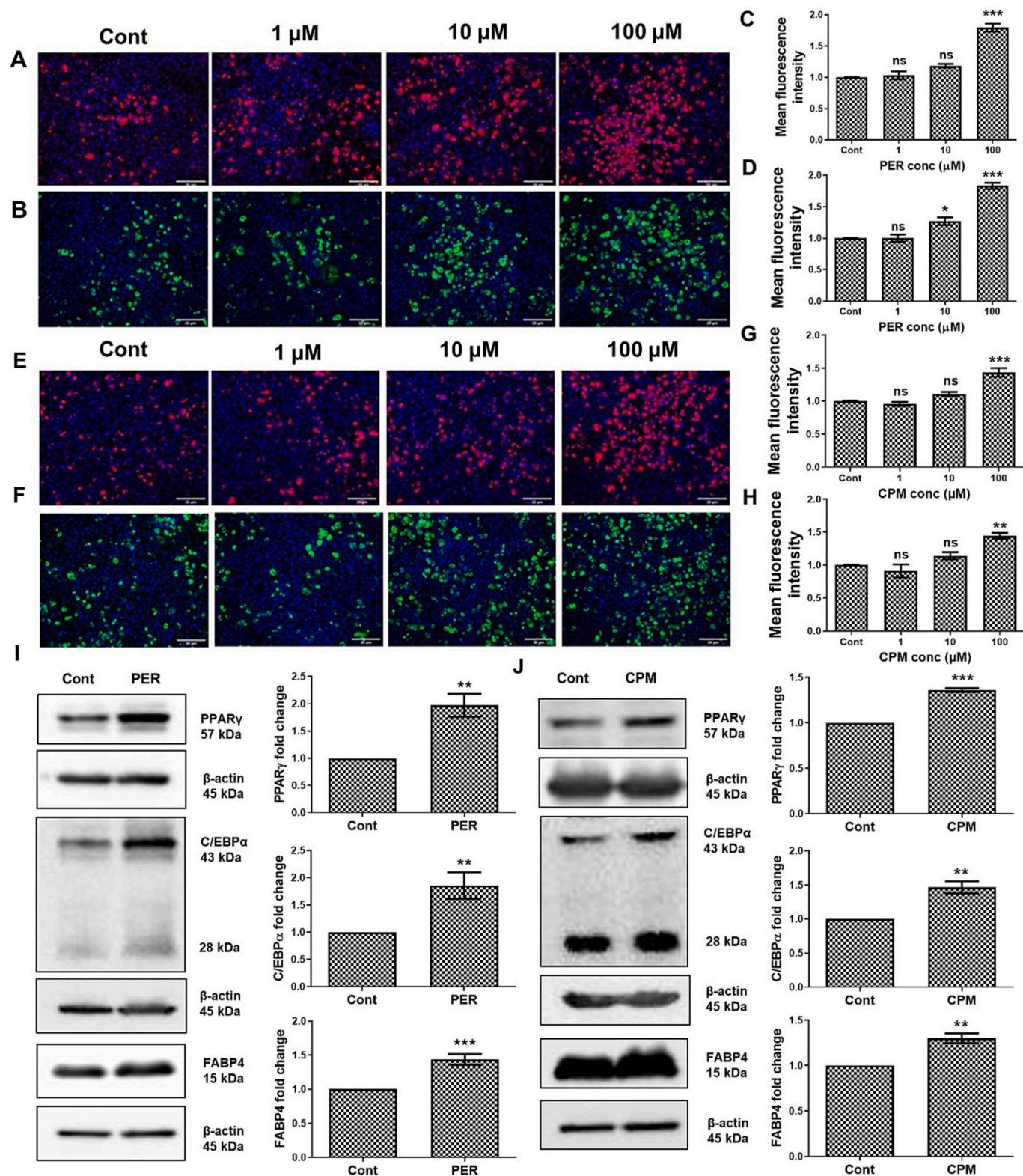
To reconfirm whether CPM and PER mediated autophagy inhibition was responsible for the enhancement of adipogenic differentiation of MSCs, cells were treated with rapamycin for 24 h prior to the induction of adipogenic differentiation. Rapamycin treatment resulted in significant reduction (~65%) in the number of adipocytes as compared to control (Fig. 4A, C, E, G). Pretreatment with CPM or PER in combination

with rapamycin lead to ~36% and ~44% decrease in adipocyte number compared to CPM or PER alone, but an increase in the number of adipocytes compared to rapamycin alone (Fig. 4A, C, E, G). Immunofluorescence staining also showed ~42% and ~54% reduction in perilipin expression upon pretreatment with CPM or PER in combination with rapamycin as compared to CPM or PER alone while an increase in perilipin expression compared to rapamycin alone (70% and 80% in case of CPM and PER, respectively) (Fig. 4B, D, F, H). Similar results were observed upon western blotting for adipogenic markers (Fig. 4I, J). This reduction in adipogenesis is due to enhanced autophagy as a result of rapamycin co-treatment. This confirms that increased autophagy mitigates CPM and PER effect on lineage commitment towards adipogenesis. Thus, suggesting an antagonist action of pyrethroids and rapamycin.

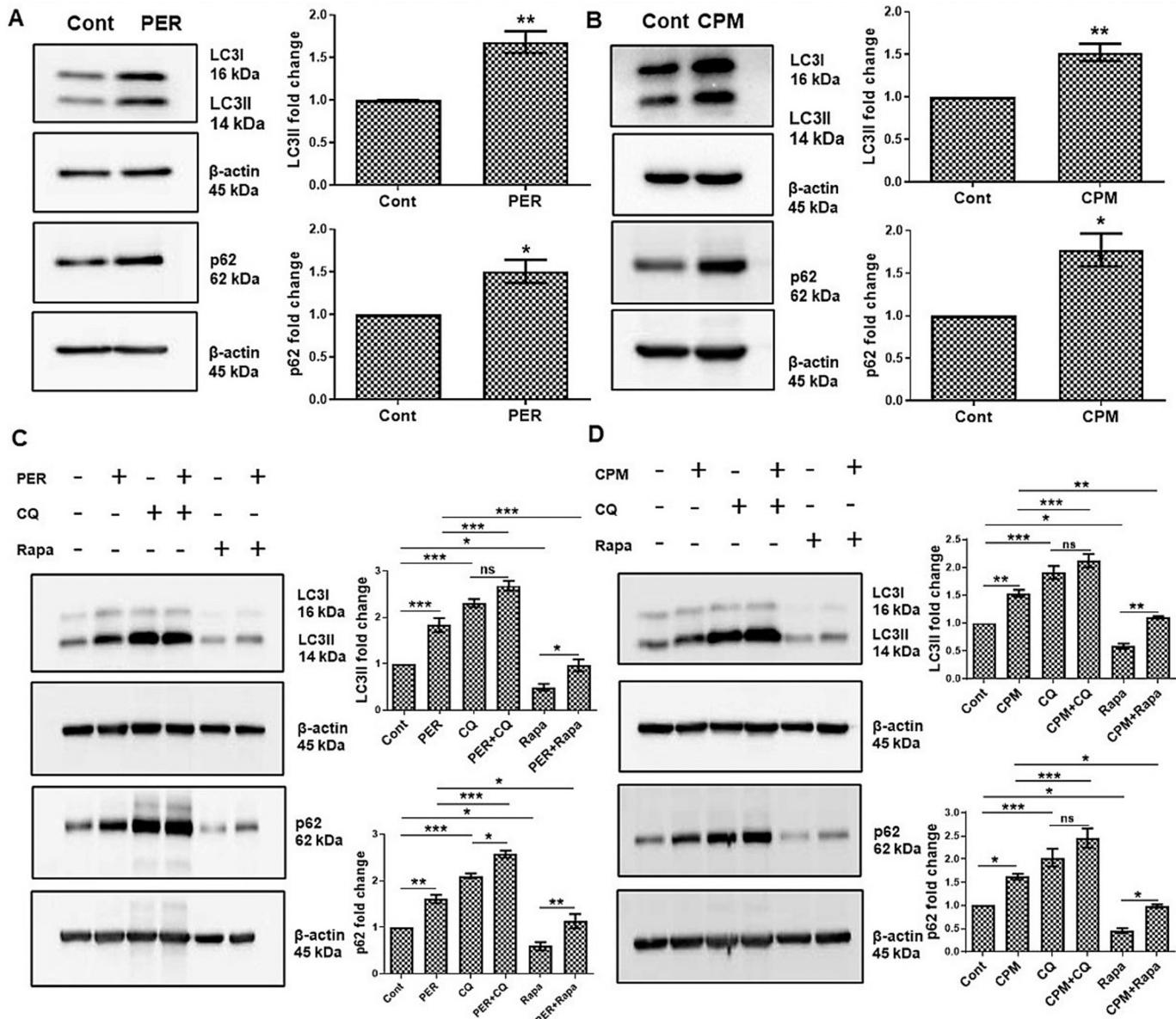
## 4. Discussion

With ever increasing global burden of metabolic disorders including obesity, the significance of mesenchymal stem cells as precursors of adipocytes cannot be neglected. Literature is replete with studies linking adipogenesis to pyrethroids (Zuo et al., 2022; Sacks et al., 2018) (Xiao et al., 2018; Shen et al., 2017). However, the impact of pyrethroids on MSC commitment has not been addressed as most studies on adipogenesis have utilized the standard 3T3-L1 cells, which are pre-adipocytes, and do not retain stem cell properties. Hence, they are not suitable to study MSC commitment. Thus, this study was undertaken to assess whether CPM and PER can affect adipogenic lineage commitment of MSCs, and if autophagy is involved in this process since it is shown to regulate MSC function. To the best of our knowledge, this is the first study demonstrating CPM and PER pre-treatment induced enhancement of adipogenic commitment of MSCs. The study also shows inhibition/reduction of autophagy by these pyrethroids in MSCs.

Our results show that both pyrethroids at 100 μM concentration affect MSC commitment without affecting cell viability. Although 100 μM seems high concentration but CYP levels upto 77 μM have been reported in blood samples of farmers, and 100 μM has been used in *in vitro* studies (He et al., 2020; He et al., 2019; Puvula et al., 2022). Numerous studies have shown cytotoxic and non-cytotoxic effects of these



**Fig. 2.** PER and CPM exposure increases adipogenic differentiation of MSCs. MSCs were treated with various concentrations of PER and CPM (1, 10 and 100  $\mu\text{M}$ ) for 24 h followed by adipogenic stimulus for 7 and 14 days. Adipogenesis was measured through fluorescence microscopy by nile red staining on 7th day (A, C, E, G) and perilipin on 14th day (B, D, F, H) (10 $\times$  magnification). Scale bar = 20  $\mu\text{m}$ . Data are represented as mean  $\pm$  SEM of three independent experiment performed in triplicate. \*\*\* $P < 0.0001$ , \*\* $P < 0.001$ , \* $P < 0.01$  and ns = non- significant. Expression of adipogenic markers PPAR $\gamma$ , C/EBP $\alpha$ , and FABP4 were evaluated upon PER and CPM exposure (100  $\mu\text{M}$ ) through western blotting (I, J). Data are represented as mean  $\pm$  SEM of three independent experiments performed in duplicate. \*\*\* $P < 0.0001$ , \*\* $P < 0.001$  and ns = non- significant. Comparison between groups was done by Student's *t*-test and one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



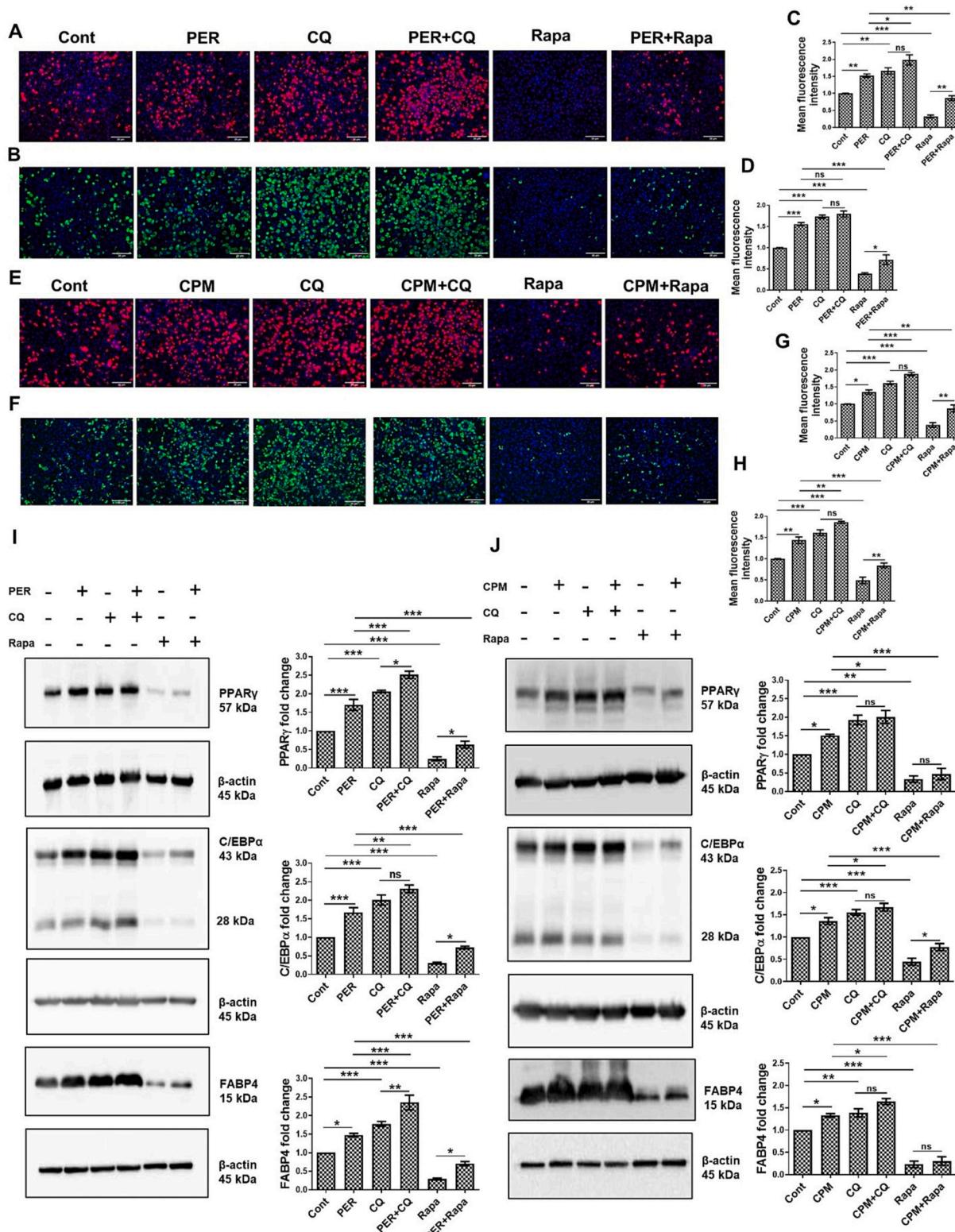
**Fig. 3.** Adipogenic differentiation of MSCs is associated with inhibition of autophagy in response to PER and CPM treatment. MSCs were treated with 100 μM of PER and CPM for 24 h. Autophagy level in MSCs was monitored through western blotting of major autophagy proteins (LC3II and p62) (A, B). Autophagy flux assessment was done by treating PER and CPM, with or without CQ and rapamycin (Rapa). Levels of LC3II and p62 were measured by western blotting (C, D). Data are represented as mean ± SEM of three independent experiments performed in duplicate. \*\*\*P < 0.0001, \*\*P < 0.001, \*P < 0.01 and ns = non-significant. Comparison between groups was done by Student's t-test and one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test.

pyrethroids in different cell types (Raszewski et al., 2016; AlKahtane et al., 2018; Huang et al., 2016). The variation in response is due to differences in concentration, treatment schedule, and the inherent physiology of different cells.

Pre-treatment (24 h) of MSCs with CPM and PER before giving adipogenic stimulus was adapted as the approach in order to study the effect on MSC, which is different from published reports of PER and CPM effect on adipogenesis (He et al., 2020; Kim et al., 2014). Enhanced ability of MSCs to give rise to adipocytes upon pyrethroid pre-treatment is observed in our study. Broadly, this result is in line with previous reports demonstrating increased adipogenesis in cell line, mainly 3T3-L1 (He et al., 2020; Yuan et al., 2019; Xiao et al., 2017), and in *in vivo* models like *C. elegans* (Yuan et al., 2019) and mouse (Yuan et al., 2019), upon pyrethroid treatment. The study by Xiao et al. also highlights the fact that fat accumulation in PER exposed mice takes place when mice are fed high fat diet (Xiao et al., 2018). These studies differ from ours as

they have used 3T3-L1 cells (a preadipocyte cell line), lower concentration of pyrethroids and longer duration of treatment compared to 24 h pre-treatment in this study. So in those studies the impact was monitored on the overall adipogenesis process and not specifically on adipogenic commitment.

Autophagy has been reported to regulate adipogenesis (Sbrana et al., 2016; Sekar and Thirumurugan, 2022; Kaushik et al., 2022). Our data shows autophagy inhibiting activity of CPM and PER in MSCs as their treatment resulted in accumulation of autophagic substrate p62 and autophagosome marker LC3II. CPM mediated autophagy inhibition and activation has been reported in neuroblastoma SH-SY5Y cells (Mishra et al., 2018) and 3T3-L1 cells (He et al., 2020), at 15 μM and 100 μM concentration, respectively. In murine macrophage RAW 264.7 cell line, short treatment (upto 24 h) of CPM (100 μM) activated autophagy while longer treatment impaired autophagy (He et al., 2019). Natural pyrethrins have been shown to induce autophagy in HepG2 cells (Yang et al.,



**Fig. 4.** PER and CPM mediated inhibition of autophagy increases adipogenic differentiation. MSCs were treated with 100  $\mu$ M of PER or CPM for 24 h, alone or in combination with CQ and rapamycin (Rapa). Adipogenic differentiation was observed and quantified by nile red imaging on 7th day (A, C, E, G) and perilipin on 14th day (B, D, F, H) (10 $\times$  magnification). Scale bar = 20  $\mu$ m. Data are represented as mean  $\pm$  SEM of three independent experiments performed in triplicate. \*\*\*P < 0.0001, \*\*P < 0.001, \*P < 0.01 and ns = non-significant. Expression of adipogenic markers was evaluated by western blotting (I, J). Data are represented as mean  $\pm$  SEM of three independent experiments performed in duplicate. \*\*\*P < 0.0001, \*\*P < 0.001, \*P < 0.01 and ns = non-significant. Comparison between different groups was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2018), while allethrin (100 µM) and prallethrin (100 µM) induced autophagy in human PBMCs (Puvula et al., 2022). Thus, pyrethroids, in general, seem to affect autophagy but the response varies among different cell types. However, we did not come across any report on PER mediated autophagic alteration. Different modes of action of different pyrethroids involving AMPK (Yang et al., 2018), ERK1/2 (Puvula et al., 2022), oxidative stress (He et al., 2020; Wang et al., 2016; Lu et al., 2019), lysosome dysfunction (Mishra et al., 2018), etc. are involved in autophagic perturbation. In this study, we have not looked at the mode of action of pyrethroids since our interest was to assess whether autophagic modulation was involved in CPM and PER mediated perturbation in adipogenic commitment of MSCs.

An exciting observation was that CQ (an established autophagy inhibitor) pre-treatment of MSCs showed enhanced generation of adipocytes, similar to CPM and PER, and rapamycin, an autophagy activator, resulted in opposite effect. This observation strengthened our hypothesis that autophagy can affect MSC lineage commitment. Similar findings have been reported by Nuschke et al. upon rapamycin and baflomycin treatment of human MSCs for 3 h during the initiation of differentiation (Nuschke et al., 2014). Low levels of autophagy in MSCs from aged mice also exhibited higher adipogenic potential, and 3-methyladenine (autophagy inhibitor) treatment of MSCs from young mice also resulted in an aged state with high adipogenic differentiation capacity (Ma et al., 2018). γ-tocotrienol mediated activation of autophagy as well suppressed adipogenesis in adipose derived stem cells (Zhao et al., 2014). In contrast, inhibiting autophagy by knocking down Atg5 (Zhang et al., 2013) or Atg7 (Nuschke et al., 2014) in 3T3-L1 cells, or *in vivo* by systemic chloroquine administration (Zhang et al., 2013) or adipocyte specific Atg7 knockdown (Nuschke et al., 2014) reduced adipogenic differentiation. These reports suggest differing role of autophagy at different stages of adipogenesis. Focusing at the stem cell level, the published reports along with our observations highlight a potent role of basal autophagic level of MSCs on adipogenic differentiation in response to stimulus. However, the data presented in this study is based on acute pre-treatment. It remains to be determined how chronic exposure is likely to affect MSCs.

All these data raise an interesting question how changes in autophagy level in MSCs regulates adipogenic commitment, which remains unaddressed. Possibilities include autophagy mediated regulation of ROS (García-Prat et al., 2016) since ROS promotes adipogenesis (Atashi et al., 2015), repression of proteasome-dependent PPARγ2 degradation (Zhang et al., 2013), and selective degradation of FABP3, which promotes adipogenesis, via autophagy receptor optineurin (Liu et al., 2021). A recent study has described the role of chaperone-mediated autophagy in adipocyte differentiation, but this work focused on early adipogenic differentiation and not adipogenic commitment of MSCs (Kaushik et al., 2022). Thus, the exact mechanism of autophagic regulation of adipogenic commitment needs further investigation. In this direction, we are undertaking proteomics approach to decipher autophagic control in MSC lineage commitment.

## 5. Conclusion

In conclusion, this study provides evidence for the tolerant nature of MSCs not showing cytotoxicity to CPM and PER, and the autophagy inhibiting capability of these pyrethroids upon acute exposure of 24 h. It also demonstrates that pyrethroids, CPM and PER, as well as CQ program MSCs, by reducing basal autophagy, into a pro-adipogenic state exhibiting enhanced adipogenic potential during long-term differentiation. Therefore, targeting basal autophagy could be an interesting approach to regulate MSC adipogenic commitment. It also points to the fact that exposure to these pyrethroids coupled with unhealthy diet, such as high fat diet, might lead to fat accumulation in the body.

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## CRediT authorship contribution statement

**Julee Verma:** Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft. **Ajit Kumar Rai:** Writing – original draft. **Neeraj Kumar Satija:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing – review & editing.

## Declaration of Competing Interest

The authors declare no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pestbp.2023.105566>.

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