

Nonylphenol Promotes the Differentiation of 3T3-L1 Preadipocytes

Ian Tang

Zunyi Medical University <https://orcid.org/0000-0001-9333-7574>

Jie Xu

Zunyi Medical University

Jie Yu (✉ 737717694@qq.com)

Zunyi Medical University <https://orcid.org/0000-0002-4796-7177>

Mei Wen Li

Zunyi Medical University

Research

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Abstract

Background

Nonylphenol (NP) induces obesity, we elucidated the influence of NP on the differentiation of 3T3-L1 preadipocytes and characterize the key stages in the underlying mechanism.

Methods

3T3-L1 preadipocytes were cultured until contact inhibition occurred and subsequently induced to differentiate using the MDI (methylisobutylxanthine, dexamethasone, and insulin) induction protocol. The cells were exposed to NP during the middle and late stages of the MDI-induced differentiation. After 24 h of exposure to NP, cell growth and differentiation were evaluated under an inverted phase-contrast microscope, lipid deposition was assessed by Oil Red O (ORO) staining, and the levels of the lipid-metabolism-related proteins CCAAT/enhancer-binding protein α (C/EBP α), fatty acid synthesis (FAS), peroxisome proliferator-activated receptor γ (PPAR γ), and sterol regulatory element binding protein 1 (SREBP1) were determined by western blotting.

Results

1) Compared with the control group, the lipid droplets in the NP-treated cells were significantly more abundant and bigger, and the levels of C/EBP α , FAS, PPAR γ and SREBP1 were significantly higher ($P < .001$). 2) The intensity of ORO staining was stronger and there were more intensely stained lipid droplets in the NP-treated cells during the middle stages than the late stage—the levels of the C/EBP α , FAS, PPAR γ and SREBP1 during the middle stages were approximately higher than those during the late stage ($P < .001$).

Conclusion

NP promotes proliferation, differentiation, and lipid accumulation in 3T3-L1 preadipocytes, and increases the expression of the C/EBP α , FAS, PPAR γ and SREBP1—and NP mainly promotes the proliferation and differentiation of 3T3-L1 preadipocytes during the middle stages of the MDI-induced differentiation.

Highlights

1. NP increased the expressions of lipid-metabolism-related proteins in 3T3-L1.
2. NP promoted the proliferation, differentiation, lipid accumulation of 3T3-L1.
3. Middle stage of MDI-induced differentiation was the key period of NP's effects.

Background

In recent years, the prevalence of obesity has rapidly increased worldwide, especially in young individuals[1]. According to the obesity and overweight data reported by the World Health Organization

(WHO)[2], approximately 2 billion adults were overweight in 2016 and among these, 650 million individuals were obese. It is estimated that 2.7 billion adults will be overweight by 2025, with 1 billion of them considered obese. Although it is commonly believed that obesity is mainly related to lifestyle, dietary habits, and genetic factors[3], studies have suggested that the obesity epidemic is correlated with the increased production of chemicals after World War II [4]. This has led to the proposal of the “environmental obesogen” hypothesis in recent years. Endocrine-disrupting chemicals (EDCs) are a key class of presumptive environmental obesogens; in particular, certain EDCs such as nonylphenol (NP) [5], bisphenol A[6], and phthalates[7] at various exposure levels have been proven to promote weight increase and obesity in animal models or human.

NP is a typical lipophilic EDC capable of influencing secretion of hormones[8], development of the reproductive system[9], airway inflammation[10], and functions of the nervous system[11]. NP may enter the human body through the use of household products and consumption of contaminated foods and drinks, such as tap water[12] or even infant formula powders [13]. Zhang[14] and Chang[15] studies have found that perinatal exposure to NP increases the body weight, fat mass, and total cholesterol level in the serum. Furthermore, in utero or neonatal exposure to NP causes hyperadrenalism and metabolic syndrome in the F1 generation. We have also shown that NP can induce diabetes mellitus[16] and non-alcoholic fatty liver disease[17], thereby resulting in metabolic syndrome[18]. The findings described above indicate that NP may be a potential chemical stressor for obesity and obesity-related diseases. However, the key period and mechanism underlying the effect of NP are currently unclear, and investigation of adipose tissue expansion, especially adipocyte differentiation, is critical to the understanding of obesity.

Previous studies[19] have reported that 3T3-L1 preadipocytes have the potential to differentiate from fibroblasts into adipocytes, and mature adipocytes differentiated from 3T3-L1 cells by using the MDI (methylisobutylxanthine, dexamethasone, insulin) induction protocol are ideal for the establishment of adipocyte models. The commonly used standard procedure for the MDI-induced differentiation of 3T3-L1 preadipocytes is as follows: First, 3T3-L1 preadipocytes with proliferative ability are cultured until contact inhibition occurs (i.e., the cells enter the G0 phase of the cell cycle and stop dividing). These contact-inhibited preadipocytes are then subjected to MDI (methylisobutylxanthine, dexamethasone and insulin) induction using the hormones methylisobutylxanthine, dexamethasone, and insulin. Under the stimulation of MDI, preadipocytes enter a specific cell division phase known as clonal expansion and ultimately differentiate into mature adipocytes. Therefore, 3T3-L1 preadipocytes were selected for this study. After 24 h of exposure, lipid deposition in the preadipocytes was examined by Oil Red O (ORO) staining, and the levels of lipid-metabolism-related proteins C/EBP α , FAS, PPAR γ and SREBP1 were determined by western blotting to determine the key period and underlying mechanism of the pro-obesity effect of NP. The results of this study can provide a theoretical basis for early and precise intervention of obesity and obesity-related diseases.

Materials And Methods

1.1 Reagents

The NP were purchased from West Asia Chemical Technology Co., Ltd (Shangdong, China). Fetal bovine serum (FBS), newborn bovine serum (NBS), Dulbecco's Modified Eagle Medium (DMEM) high-glucose were purchased from Gibco (New York, USA). 0.25 % trypsin were purchased from Sigma Aldrich Shanghai Trading Co., Ltd (Shanghai, China). Phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), dexamethasone (Dex), Oil Red O solution Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). All other chemicals were commercially available.

1.2 Induction of cell differentiation, staging, and NP exposure

1.2.1 Induction of cell differentiation

3T3-L1 preadipocytes were inoculated onto cell culture plates (6-well plates, 12-well plates, and 10-cm culture dishes) and cultured at 37 °C with 5% CO₂ in medium containing 10% NBS. Differentiation into mature adipocytes was induced using the as described below:

3T3-L1 preadipocytes at the stage of contact inhibition were cultured for 48 h in the MDI[20]induction medium composed of 0.5 mM 3-isobutyl-1-methyl-7H-xanthine (IBMX), 1 μM dexamethasone (Dex), 0.1 μM insulin (I), and 10% fetal bovine serum (FBS) in medium[21]. Afterward, the cells were cultured in induction medium B (0.1 μM insulin and 10% FBS in medium) for 48 h. The medium was replenished every 48 h with 10% FBS in medium without insulin until the maturation was achieved.

1.2.2 Staging and NP exposure

3T3-L1 preadipocytes at the state of contact inhibition were treated with NP (0, 40 pM, 40 nM, or 40 μM) for 24 h during the following stages while undergoing MDI-induced differentiation: middle stage, and late stage, according to the following Figure 1.

(1) Middle stage: NP working solution was prepared using the MDI induction medium described in Figure 1, and cells were concurrently exposed to NP for 24 h while induced to differentiate in the MDI induction medium. Subsequently, all other steps of the MDI induction protocol in Figure 1 were followed, and routine sampling was performed.

(2) Late stage: After inducing differentiation using the procedure described in Figure 1, the cells were exposed to the NP working solution for 48 h on Day 10 of induced differentiation, and routine sampling was performed.

1.3 Assessment of lipid deposition by Oil Red O staining

Oil Red O (ORO) staining was performed on successfully differentiated preadipocytes. The lipid droplets in the cells on each stained slide were observed and photographed under an optical microscope at 50× and 200× magnification[22].

1.4 Assessment of the levels of lipid-metabolism–related proteins in 3T3-L1 preadipocytes

Western blotting (WB) was performed to measure the levels of lipid-metabolism–related proteins C/EBP α [23], FAS, PPAR γ [24] and SREBP1 in 3T3-L1–derived preadipocytes[25]. These proteins play key roles in the proliferation, differentiation, maturation, and lipid metabolism of adipocytes.

1.5 Statistical analysis

Statistical analysis of all the data was performed using SPSS 18.0 with a significance level of $\alpha = 0.05$. One-way analysis of variance (ANOVA) was used to compare the differences among groups, and the least significant difference (LSD) test was adopted for pairwise comparisons when intragroup differences were observed. All the data are expressed as mean \pm SD.

Results

1.1 Culturing and differentiating 3T3-L1 preadipocytes

Figure 2 shows the morphologies of the 3T3-L1 cells at different stages of the experiment. 3T3-L1 preadipocytes exhibited a fibroblast morphology with a long fusiform shape [Figures 2(a) and (b)]. When the contact inhibition state was reached, the cells showed a long and slender morphology and a disorderly arrangement [Figure 2(c)]. After culturing in the MDI induction medium for 24 h, the cells became plump, and sporadic lipid droplets could be seen in the cytosol of a small sub-set of the cells [Figure 2(d)]. As the cells continued to be cultured in the induction medium, a few lipid droplets exhibiting a small spherical shape and scattered cellular distribution gradually appeared in the cells [Figure 2(e)]. Ultimately, these small lipid droplets gradually merged to form big droplets, and the cells differentiated into round mature preadipocytes with a smooth surface and various sizes. These cells aggregated into clusters with time [Figure 2(f)]

1.2 Influence of NP on the viability of 3T3-L1 preadipocytes

1.2.1 Growth curve of 3T3-L1 preadipocytes

Figure 3 shows the growth curve of 3T3-L1 cells obtained by fitting the relevant data to a growth equation in Graphpad 6.0. From the curve, it can be seen that the optical density (OD) values of the 3T3-L1 mouse embryonic fibroblasts gradually increased with time. The cells entered the logarithmic phase after 36 h and reached the contact inhibition state with the formation of a confluent monolayer after 48 h.

1.2.2 Influence of DMSO on the viability of 3T3-L1 preadipocytes and basis for the selection of DMSO concentration

To determine if DMSO exposure affected the viability of 3T3-L1 cells, the MTT assay was performed (Figure. 4). The OD (wavelength: 490 nm) of the cells cultured with 0.1% or 0.01% DMSO was not significantly different from that of the control group, which indicates that DMSO at these concentrations did not influence cell viability. However, as the difference in cell viability between the

0.1% DMSO-treated cells and control group was somewhat bigger than that caused by 0.01% DMSO, the latter DMSO concentration was selected for further experiments (Figure 4).

1.3 Influence of NP exposure on the differentiation of 3T3-L1 preadipocytes

The intensity of ORO staining in 3T3-L1 preadipocytes increased in correlation with the NP dose during all the two stages of the induced differentiation. Compared with the cells during the late stage, mid-stage cells had a denser distribution, higher staining intensity, and higher degree of differentiation (Figure 5)

1.4 Influence of NP exposure on the expression of lipid-metabolism–related proteins in 3T3-L1 preadipocytes during their differentiation

1.4.1 Influence of NP exposure on lipid-metabolism–related proteins (C/EBP α , FAS, PPAR γ , and SREBP1) in 3T3-L1 preadipocytes during the middle and late stages of induced differentiation During the middle stage, the levels of FAS, PPAR γ , and SREBP1 in the NP-exposed group were higher than those in the control group ($F_{C/EBP\alpha} = 539.103$, $F_{FAS} = 715.740$, $F_{PPAR\gamma} = 114.783$, and $F_{SREBP1} = 139.600$; $P < .001$). During the middle stage, the levels of FAS, PPAR γ , and SREBP1 in the

NP-exposed group were higher than those in the control group ($F_{C/EBP\alpha} = 539.103$, $F_{FAS} = 715.740$, $F_{PPAR\gamma} = 114.783$, and $F_{SREBP1} = 139.600$; $P < .001$). During the late stage, the level of C/EBP α was more than that in the control group ($F_{C/EBP\alpha} = 29.727$, $P < .001$). Under 40 μ M NP exposure, FAS protein level slightly increased ($F_{FAS} = 20.609$, $P < .001$). The protein levels of PPAR γ and SREBP1 in the mature preadipocytes exposed to 40 μ M NP were slightly higher than those in the control group ($F_{PPAR\gamma} = 330.951$ and $F_{SREBP1} = 27.093$; $P < .001$, Figure 6).

1.4.2 Comparison of the influence of NP exposure on lipid-metabolism–related protein levels (C/EBP α , FAS, PPAR γ , and SREBP1) in 3T3-L1 preadipocytes during the late stage of the differentiation with during the middle stages

When the two stages of induced differentiation were compared, significant differences in the expression levels of the lipid-metabolism–related proteins (C/EBP α , FAS, PPAR γ , and SREBP1) were found between the two stages, when the NP dose was 40 nM ($F_{C/EBP\alpha} = 248.299$, $F_{FAS} = 38.386$, $F_{PPAR\gamma} = 9.565$, and $F_{SREBP1} = 82.378$; $P < .001$). In particular, the levels of PPAR γ and SREBP1 were higher during the middle stages than during the late stage. Under 40 μ M NP treatment, the levels of C/EBP α , FAS and PPAR γ significantly changed between the two stages of the differentiation ($F_{C/EBP\alpha} = 81.254$; $F_{FAS} = 52.526$, $F_{PPAR\gamma} = 220.286$, $F_{SREBP1} = 25.858$; $P < .001$), with the levels being higher during the middle stages than during the late stage (with the exception of SREBP1 level, figure 7).

Discussion

The results of ORO staining indicate that NP exposure promoted lipid deposition in 3T3-L1 preadipocytes. ORO staining, which involves the use of Oil Red O, a liposoluble dye that readily dissolves in fat and specifically stains lipids within tissues, is the most commonly used method for the detection of abnormalities in lipid droplets[26, 27]. Wada et al. [28] have found that NP exposure significantly promotes the accumulation of triglycerides after the differentiation of 3T3-L1 preadipocytes into mature adipocytes, and thus their results are consistent with the results of the present study. Another study by Masuno et al. [29] showed that NP could promote the proliferation of fully differentiated 3T3-L1 cells. We[16, 17]have also identified NP exposure as a risk factor for the onset of type II diabetes mellitus (DM) and non-alcoholic fatty liver disease (NAFLD). Furthermore, studies have proven that obesity is also a risk factor for the onset of type II DM and NAFLD [30, 31]. In view of these findings, it can be deduced that NP exposure can promote the proliferation and differentiation of 3T3-L1 preadipocytes and lipid accumulation in cells, thereby leading to obesity. This may be the mechanism by which NP causes the onset of type II DM and NAFLD.

Results of the measurement of lipid metabolism-related protein levels revealed that NP exposure promoted the expression of the lipid-metabolism-related proteins C/EBP α , FAS, PPAR γ , and SREBP1 in 3T3-L1 preadipocytes[32, 33]. These proteins play key roles in the proliferation, differentiation, maturation, and lipid metabolism of adipocytes[34]. Specifically, PPAR γ is pivotal in lipid utilisation and storage, lipoprotein metabolism, and adipokine expression; C/EBP α is essential for the survival of mature adipocytes as high C/EBP α expression can trigger and accelerate adipocyte differentiation; SREBP1 is the main transcriptional mediator of lipid homeostasis as it can activate genes related to fatty acid synthesis, including the FAS and acetyl-CoA carboxylase genes. FAS is a key regulatory enzyme that guides the de novo synthesis of fats from fatty acids and plays an important role in the synthesis and deposition of lipids in animal cells and participates in PPAR γ activation in mouse embryonic fibroblasts[35]. Therefore, a large number of studies have regarded the increase in the expression levels of PPAR γ , C/EBP α , SREBP1, and FAS as an indicator of lipid formation. For instance, the differentiation process of 3T3-L1 mouse preadipocytes is accompanied by upregulation of C/EBP α , PPAR γ , and SREBP1 levels[6, 32]. In contrast, hypaphorine (an indole alkaloid) blocks the differentiation and lipid accumulation of 3T3-L1 adipocytes by inhibiting the expression of the PPAR γ , C/EBP α , SREBP1, and FAS genes during the differentiation process[36]. Hao[5]and Zhang[14] have found that perinatal NP exposure leads to a significant increase in the mRNA levels of PPAR γ , SREBP1, FAS, and lipoprotein lipase (LPL) in the adipose tissues of F1 and F2 rats, and these observations are consistent with the results of the present study. In view of the results described above, we deduce that NP may promote proliferation, differentiation, and lipid deposition in 3T3-L1 preadipocytes by increasing the expression of the lipid-metabolism-related proteins C/EBP α , FAS, PPAR γ , and SREBP1. It is possible that NP shows the same effect in vivo, ultimately resulting in the onset of obesity.

Although a large number of studies have proven that EDCs can influence the proliferation and differentiation of preadipocytes, the key periods of influence of EDCs on adipocytes during the differentiation of preadipocytes into mature adipocytes have rarely been reported. Therefore, the investigation of the key period of influence of NP exposure on preadipocyte proliferation and differentiation is of great significance. If the proliferation and differentiation of preadipocytes during the

key period of influence can be inhibited by chemical or physical methods, the incidence of obesity and obesity-related diseases caused by EDCs, such as NP, can be effectively reduced. In the present study, preadipocytes exposed to NP had a higher distribution density and intensity of staining during the middle stages of induced differentiation compared with the levels during the late stage. In addition, the levels of the lipid-metabolism-related proteins C/EBP α , FAS, PPAR γ , and SREBP1 in the medium- and high-NP dose groups were also significantly higher during the middle stages of the differentiation than during the late stage. Therefore, it can be deduced that the key periods of the NP influence on 3T3-L1 preadipocyte differentiation may be the middle stages of MDI-induced differentiation .

Conclusion

NP increases the expression of lipid-metabolism-related proteins, thereby promoting the proliferation, differentiation, and intracellular lipid accumulation of 3T3-L1 preadipocytes, with the possible key periods of influence being the middle stages of MDI-induced differentiation. The results of this study can provide a theoretical basis for early and precise intervention of obesity and obesity-related diseases.

Abbreviations

NP: Nonylphenol; MDI: Methylisobutylxanthine, Dexamethasone, and insulin; Oil Red O staining: ORO staining; EDCs: Endocrine-disrupting chemicals; C/EBP α : CCAAT/enhancer-binding Protein α ; FAS: Fatty Acid Synthesis; PPAR γ : Peroxisome Proliferator-activated Receptor γ ; SREBP1: Sterol Regulatory Element Binding Protein 1

Declarations

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Authors' Contributions

JX and JY designed the study. WML, LT, JLZ, DLZ, GHL, JX and JY analyzed and interpreted the data. WML conducted the laboratory work. WML, JX participated in the sample collection. JY and LT wrote the manuscript, JX revised the manuscript. All the authors read and approved this paper.

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Availability of data and materials

The data used during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of Biomedical Ethics Committee of the Zunyi Medical University. Informed consent was obtained from all individual participants included in the study.

Consent for publication

Written informed consent for publication was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

Author details

¹School of Public Health, Zunyi Medical University, 6 Xuefu West Road, Xipu District, Zunyi City, Guizhou Province, P.R. of China.

²Department of Laboratory Medicine, Honghuagang district People's Hospital, 185 Wanli Road, Honghua District, Zunyi, Guizhou, P.R. of China.

³Morphological laboratory, School of Basic Medicine, Zunyi Medical University, 6 Xuefu West Road, Xipu District, Zunyi City, Guizhou Province, P.R. of China.

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Figures

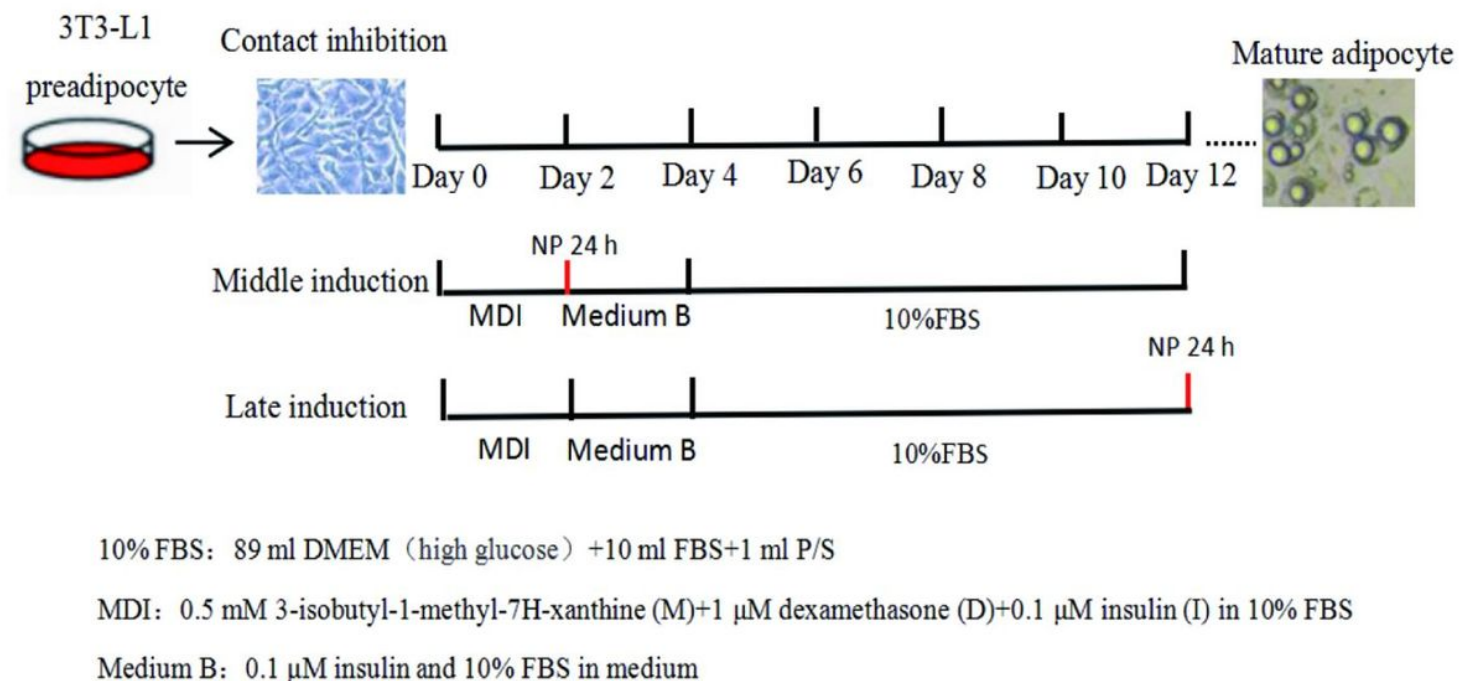
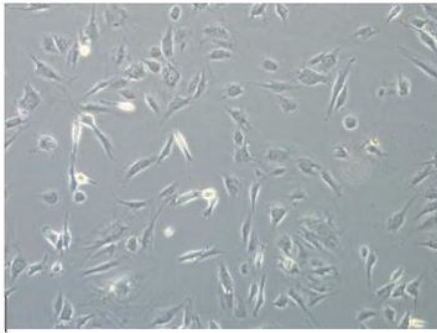
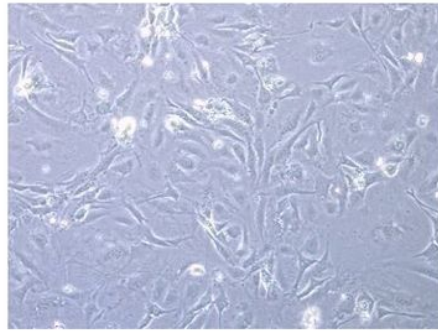


Figure 1

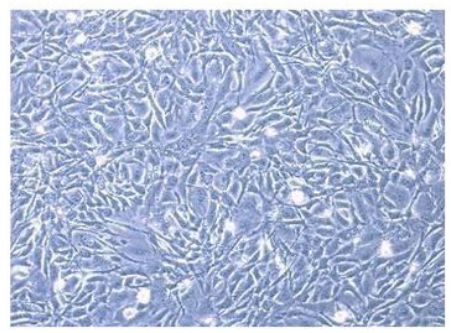
MDI induction protocol and nonylphenol exposure period



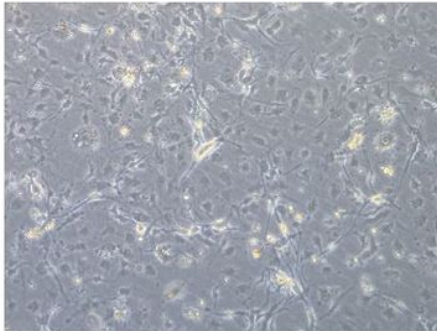
(a) Low density



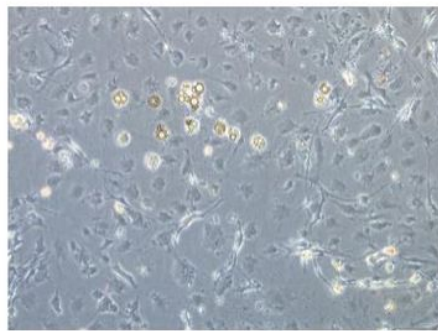
(b) High density



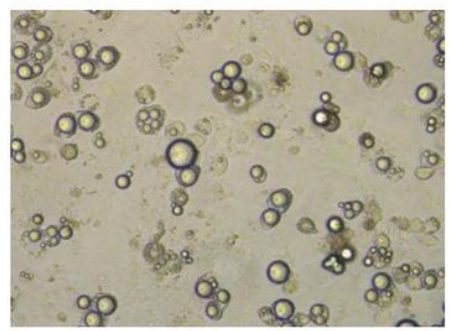
(c) Contact inhibition



(d) After 24 h of culturing in
MDI induction medium



(e) Differentiation
in progress



(f) Mature
preadipocytes

Figure 2

Culturing and differentiating 3T3-L1 cells (200× magnification)

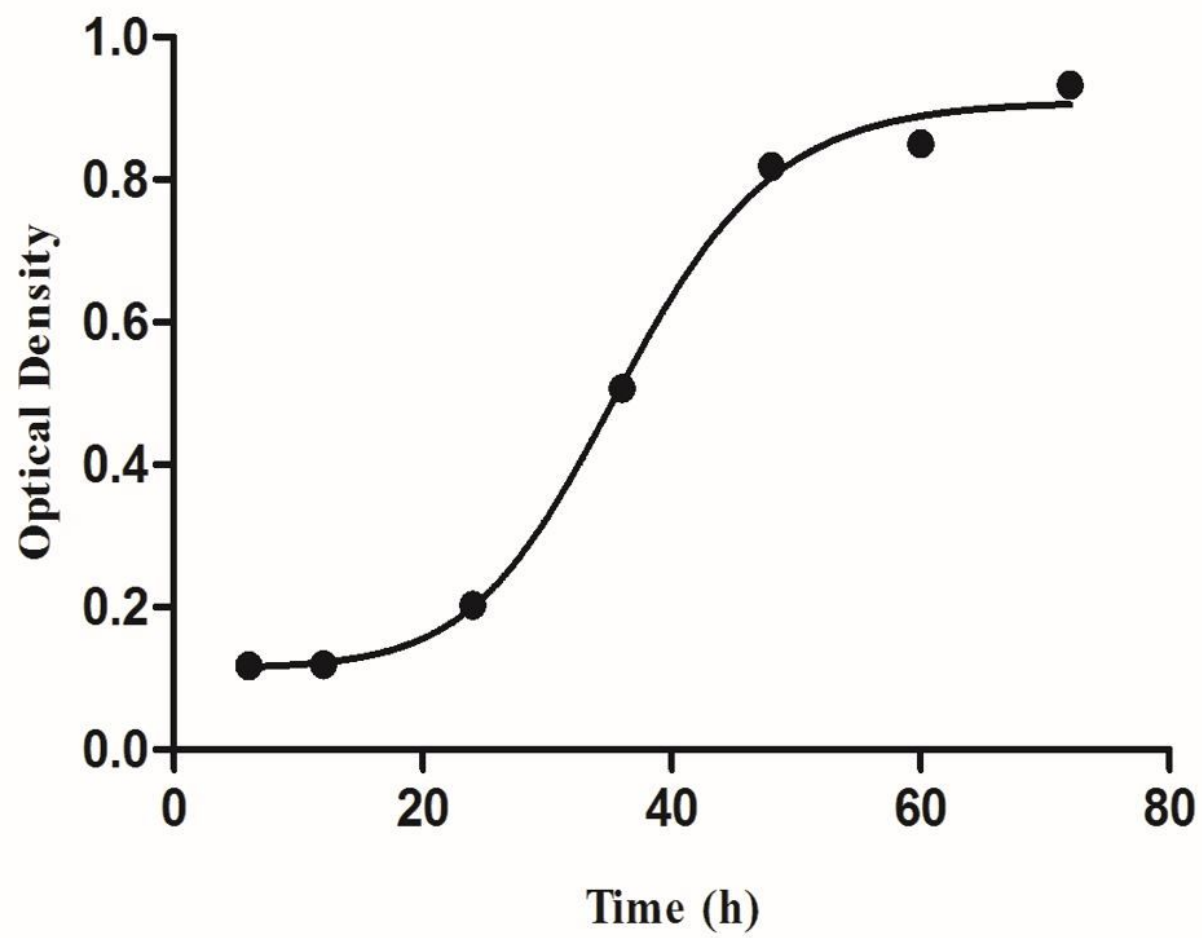


Figure 3

Growth curve of 3T3-L1 cel

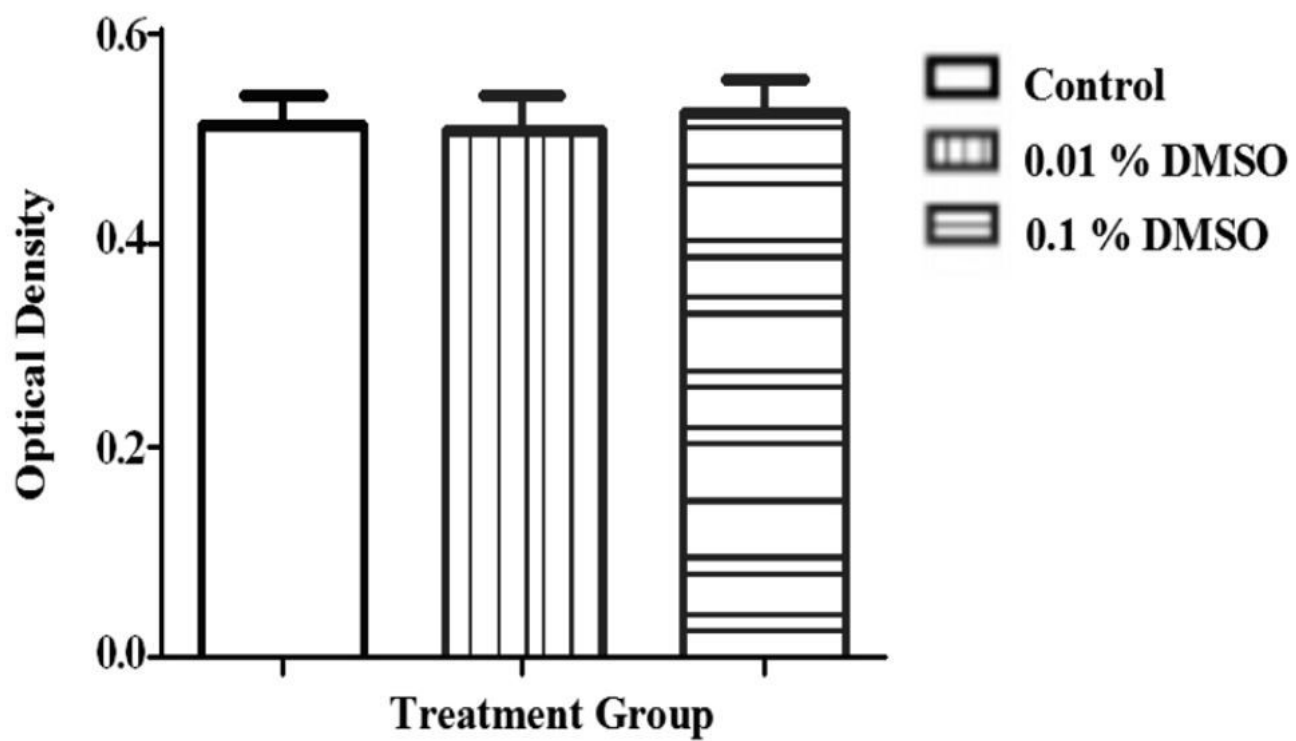


Figure 4

Influence of DMSO on the viability of 3T3-L1 cells

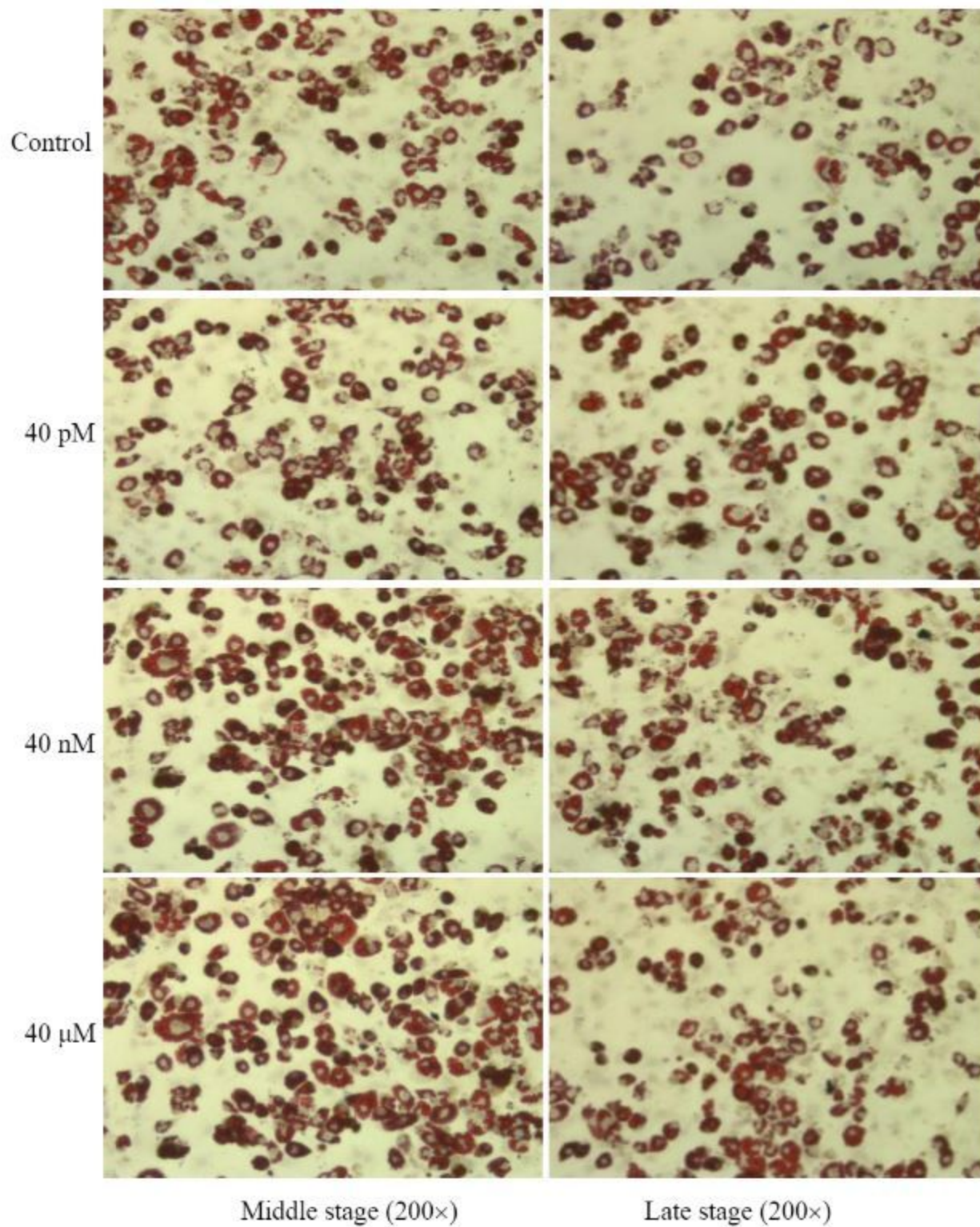


Figure 5

Influence of NP exposure on 3T3-L1 preadipocytes proliferation during the middle and late stages of the induced differentiation.

Image not available with this version

Figure 6

Influence of NP exposure on the lipid-metabolism-related proteins C/EBP α , FAS, PPAR γ , and SREBP1 in 3T3-L1 preadipocytes during the middle and late stages of differentiation. vs Control group, $P \leq 0.05$. vs 40 pM group, $P \leq 0.05$. vs 40 nM group, $P \leq 0.05$.

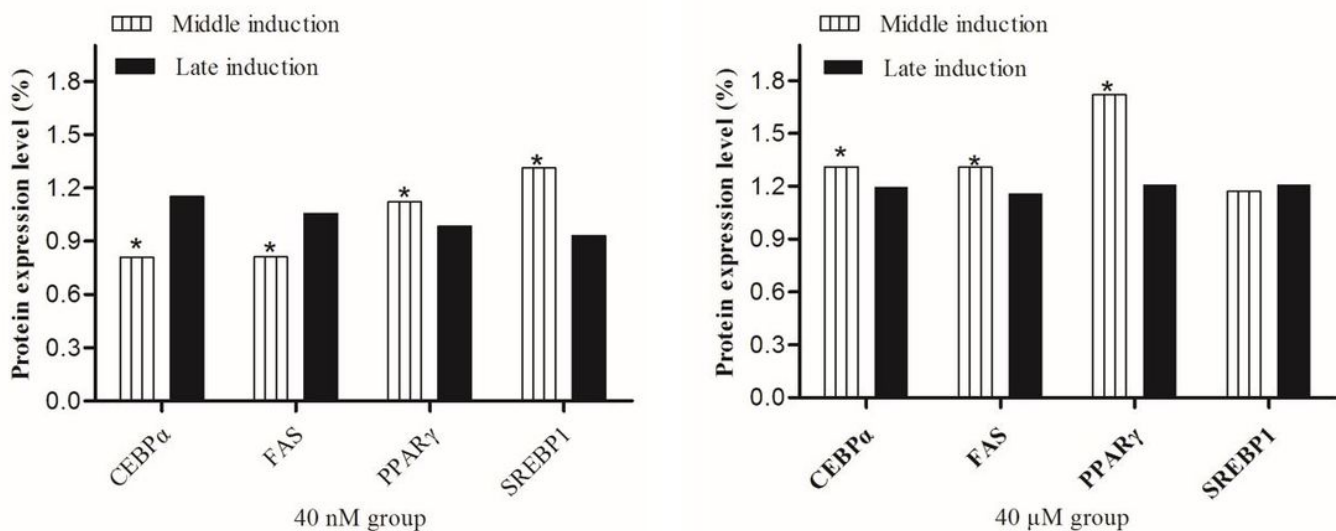


Figure 7

The levels of lipid-metabolism-related proteins in 3T3-L1 preadipocytes During the middle and late stages of induced differentiation. *vs late stage, $P < .05$.

Supplementary Files

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- [GraphicalAbstract.docx](#)