Saudi Dental Journal (2022) **34**, 346–354

King Saud University

Saudi Dental Journal

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ORIGINAL ARTICLE

Assessment of ultra-structure, viability and function of lipopolysaccharides-stimulated human dermal fibroblasts treated with chrysin and exosomes (*in vitro study*)

Rabab Hassan [a], **Dina Sabry** [b,c], **Amany A. Rabea** [d,*]

[a] *Associate Professor of Oral Biology, Faculty of Dentistry, Ain Shams University, Cairo, Egypt*
[b] *Professor of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Cairo, Egypt*
[c] *Professor of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Badr University in Cairo, Badr City, Egypt*
[d] *Associate Professor of Oral Biology, Faculty of Oral and Dental Medicine, Future University in Egypt, Cairo, Egypt*

Received 18 February 2022; revised 15 April 2022; accepted 17 April 2022
Available online 25 April 2022

KEYWORDS

BM-MSC exosomes;
Chrysin;
Fibroblast;
MTT;
ELISA

Abstract *Background:* Lipopolysaccharides (LPS) stimulate production of inflammatory cytokines. Chrysin is flavonoid beneficial for treatment of inflammatory conditions. Bone marrow mesenchymal stem cell (BM-MSC) exosomes have regenerative ability in different tissues.

Objective: To assess potential role of chrysin and BM-MSC exosomes on ultra-structure, viability and function of human dermal fibroblasts-adult (HDFa) stimulated by LPS.

Methods: HDFa cells were divided into: **Group I:** Cells received no treatment. **Group II:** Cells were stimulated with LPS. **Group III:** LPS stimulated cells were treated with chrysin. **Group IV:** LPS stimulated cells were treated with exosomes.

Results: After 48 h, ultrastructural examination of HDFa cells in Group I revealed intact plasma membrane and numerous cytoplasmic organelles. Group II displayed destructed plasma membrane and apoptotic bodies. Group III showed intact plasma membrane with loss of its integrity at some areas. Group IV demonstrated intact plasma membrane that showed fusion with exosomes at some areas. Statistical analysis of MTT represented highest mean value of cell viability% in Group IV

* Corresponding author.
E-mail address: Amanyrabea1@gmail.com (A.A. Rabea).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sdentj.2022.04.006

followed by Groups III, I and II respectively. Statistical analysis of enzyme-linked immunosorbent assay (ELISA) showed the highest mean value of interleukin-1β (IL-1β) was in Group II followed by Groups III, IV and I, while highest mean values of interleukin-10 (IL-10), nuclear factor-erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) proteins were in Group I, followed by Groups IV, III and II respectively.

Conclusions: LPS have harmful consequences on ultra-structure, viability and function of HDFa cells. BM-MSC exosomes have better regenerative action on inflamed fibroblasts in comparison to chrysin.

1. Introduction

One of the most important connective tissue cell members are fibroblasts. They are responsible for the synthesis and metabolism of collagen and extracellular matrix, differentiation of the epithelium, regulation of inflammatory response and wound healing, secretion of different growth factors and act as scaffolds for other cell types (Fernandes et al., 2016; Girsang et al., 2019).

Interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine which is important for defense against injury and infection specially in periodontal diseases. IL-1β is produced by fibroblasts when stimulated by lipopolysaccharides (LPS) present in cell wall of periodontal pathogens *"Porphyromonas gingivalis and Escherichia coli*". *In vivo* IL-1β is produced by different cell types such as macrophages, monocytes, Langerhans cells and keratinocytes (Tardif et al., 2004; Lopez-Castejon & Brough, 2011; Steen et al., 2020).

Heme oxygenase (HO)-1 is a cytoprotective enzyme which has anti-inflammatory and antioxidant properties as it enhances heme degradation to produce carbon monoxide "anti-inflammatory" and biliverdin "antioxidant". HO-1 expression is up-regulated in fibroblasts under oxidative stress as one of cellular responses against oxidative damage (Even et al., 2018).

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is the master regulator of antioxidant responses. It modulates the expression of many antioxidants and it is crucial for maintaining the cellular redox status. The Nrf2 regulates the transcription of cytoprotective genes such as those encoding antioxidant proteins, detoxifying enzymes and drug transporters. In fibroblasts, Nrf2 is highly expressed during wound healing (Zhang et al., 2016; Hiebert & Werner, 2019).

Interleukin-10 (IL-10) is a pleomorphic anti-inflammatory cytokine produced by different immune cells such as B cells, granulocytes, dendritic cells, mast cells, macrophages and T cells. It exhibits negative feedback regulating action on inflammation through paracrine and autocrine mechanisms. *In vitro*, it turns out that IL-10 can also be produced by fibroblasts (Hulsmans et al., 2018; Steen et al., 2020).

Chrysin is a cheap and easily extracted flavonoid. It is present in high levels in honey and propolis, but also it exists in mushroom and numerous plant extracts like Passion-flower, Passiflora caerulea and Passiflora incarnata. It has different pharmacological activities such as antioxidant, anti-inflammatory, anti-genotoxic, anti-mutagenic and hypoglycemic effects. Biomedical findings revealed that chrysin is a beneficial treatment for many diseases like neurodegenerative disorders, diabetes and many inflammatory diseases (Hermenean et al., 2017; Farkhondeh et al., 2019; Li et al., 2020).

An exosome is a cell-derived vesicle its size ranges from 30 to 150 nm. Exosomes secreted by bone marrow mesenchymal stem cells (BM-MSCs) can repair damage induced by many diseases including the inflammatory ones. BM-MSC exosomes have a regenerative ability through exerting paracrine effect. They express surface molecules which enable them to bind with the recipient cells and deliver their contents subsequently (Nakamura et al., 2015; Chan et al., 2019; Zhang et al., 2019; Alcaraz et al., 2020).

Based on previous facts, this study aimed to compare the possible effect of chrysin versus that of BM-MSC exosomes on human dermal fibroblasts-adult (HDFa) ultra-structure, viability and function in LPS induced inflammatory condition.

2. Materials and methods

The study was approved by the Research Ethics Committee, Future University in Egypt (FUE.REC (29)/12-2021).

- The purchasing of LPS was from (Sigma-Aldrich Chemical Co., St. Louis, Mo, U.S.A., L3012).
- Chrysin was provided as a 5gm faint yellow powder from (Sichuan Benepure Pharmaceutical Co., Ltd., Chengdu, China).
- Isolation of exosomes, cell culture, MTT assay and ELISA assay were performed at the Biochemistry and Microbiology Unit, Faculty of Medicine, Cairo University.

2.1. Isolation and recognition of exosomes

For isolation of exosomes, BM-MSCs were cultured in Dulbecco's Modified Eagle Medium with 0.5% human serum albumin overnight (Sigma-Aldrich®, USA). The medium was centrifuged for 20 min at 2,000xg, and then ultracentrifuged for 1 h at 100,000*g* and 4 °C in a SW41 swing rotor (Beckman Coulter, Fullerton, CA, USA). The obtained exosomes were washed in serum-free medium M 199 containing 25 mM HEPES buffer solution (pH = 7.4) and submitted to repeated ultracentrifugation. After washing, exosomes were analyzed using a Fluorescence-Activated Cell Sorting, Calibur flow cytometer (Becton Dickinson, FACS Calibur). The analysis revealed that they express CD44, CD29, α4- and α5 inte-

grins as well as CD73, but not α6-integrin (Bruno et al., 2009; Nassar et al., 2016).

2.2. Cell culture

The HDFa cell line was bought from the American Type Culture Collection (ATCC; Minnesota, USAHB-8065). It was cultured in Park Memorial Institute (RPMI -1640) culture media with glutamine 2 mM (bio west, Nampa, cat n L0498-500), 10% fetal bovine serum (PAA, Pasching Austria, cat. no. A11-151) and penicillin with streptomycin 1% (Lonza, Verviers, Belgium, cat. no. DE17-602E). The HDFa cells were grown in 50 cm^2 flask (Greinerbio-one GmbH Maybachstr.272636 Frickenhausen, Germany) and preserved in typical humidified incubator supplied with 5% CO_2, 95% air at 37 °C (New Brunswick Scientific- Innova co-170). Cells were washed with cold phosphate buffered saline, trypsinized, harvested and centrifuged to form cell pellets. The cultured HDFa were divided into four groups as follows:

- Group I (Control): The HDFa cells received no treatment.
- Group II (LPS): The HDFa cells were stimulated with LPS (10 ng/mL, *Escherichia coli* 055:B5, List Biological Laboratories, CA, USA) for 48 h (Skioldebrand et al., 2018).
- Group III (LPS/Chrysin): The HDFa cells were stimulated by LPS and treated with chrysin (25 μM) for 48 h (Zhu et al., 2016).
- Group IV (LPS/Exosomes): The HDFa cells were stimulated by LPS and treated with BM-MSC exosomes (100 μg/mL) for 48 h (Huang et al., 2021).

2.3. Ultrastructural preparation

After the end of the experimental period, HDFa cells from each group were ultra-centrifuged and suspended in 100ul human serum albumin then fixed in 2% glutaraldehyde, 0.1 M sodium cacodylate and 1 mM calcium chloride at 37 °C with pH 7.4 for 30 min; then washed twice with cacodylate buffer (pH 7.4) (0.1 M sodium cacodylate and 0.2 M sucrose) for 10 min at 4 °C to stop fixation. The HDFa cells were post-fixed with 1% osmium tetroxide in cacodylate buffer for 30 min and then dehydrated by ethanol. Then, cells were infiltrated in resin and polymerized. Finally, the specimens were cut into ultrathin sections, collected on copper grids and stained with uranyl acetate and lead citrate (Le et al., 2020). The sections were examined and photographed at 10,000× and 15,000× magnifications using transmission electron microscope (JEOL JEM® 1010, Jeol Ltd, Japan). This procedure was done at Regional Center of Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt.

2.4. MTT assay

The anti-proliferative effect of LPS on HDFa, was investigated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The HDFa cells of the 4 studied groups were fixed with the frequency of ($1x10^3$) cells into 96 well plates for 48 h. The MTT reagent was supplemented to the wells in line with the manufacturer's instructions (Biospes, China, cat. no. BAR1005-1) and left from 4 h to 6 h. When the purple precipitate was clearly visible, detergent reagent was added (100 μl per well) to make the formazan dye soluble. Plates were left with cover in the dim from 2 h to 4 h. The plate cover was detached and the absorbance in each well was assessed at a range from 490 nm to 630 nm using enzyme-linked immunosorbent assay (ELISA) plate reader (Stat Fax 2200, Awareness Technologies, Florida, USA).

2.5. ELISA assay

The concentrations of IL-1β, IL-10, Nrf2 and HO-1 cytokines were measured in cell culture supernatants conditioning medium using ELISA kits according to the manufacture's recommendations. The concentrations of cytokines in fibroblast culture medium were assessed and measured at optical density ranged from 490 nm to 630 nm using microplate reader (Stat Fax 2200, Awareness Technologies, Florida, USA).

2.6. Statistical analysis

Numerical data from MTT and ELISA assays were explored for normality by checking the distribution of data and using tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk tests). All data were presented as mean ± standard deviation (SD). For parametric data, comparison of numerical variables between the studied groups was done using one way analysis of variance (ANOVA) test, after the statistically significant difference was confirmed, it was followed by post hoc test: Least Significant Difference for multiple 2-group comparisons. The high significance level was set at probability value (p-value) < 0.0 01, significance level at p-value ≤ 0.05 and non-significant as P-value > 0.05. Statistical analysis was accomplished with IBM SPSS Statistics for Windows, Version 23.0. (Armonk, NY: IBM Corp).

3. Results

3.1. Ultrastructural results

3.1.1. Group I (Control)

Fibroblast in Group I showed oval nucleus, intact plasma membrane as well as peroxisomes (Fig. 1a). Clusters of condensed chromatin at the nuclear membrane and in the nucleoplasmic space intermingled with areas of loose chromatin were observed. Mitochondria, endosomes, rough endoplasmic reticulum and free ribosomes were seen (Fig. 1b).

3.1.2. Group II (LPS)

In Group II, fibroblast revealed destructed plasma membrane. Cytoplasm appeared scanty with numerous apoptotic bodies (Fig. 1c). Oval nucleus with obviously few sporadic condensed chromatin was noticed. Most of cellular organelles could not be detected (Fig. 1d).

3.1.3. Group III (LPS/Chrysin)

Fibroblast in Group III displayed small rounded nucleus with condensed chromatin. Most of plasma membrane appeared intact, however some areas showed loss of its integrity (Fig. 2a). Cytoplasmic organelles such as mitochondria, rough

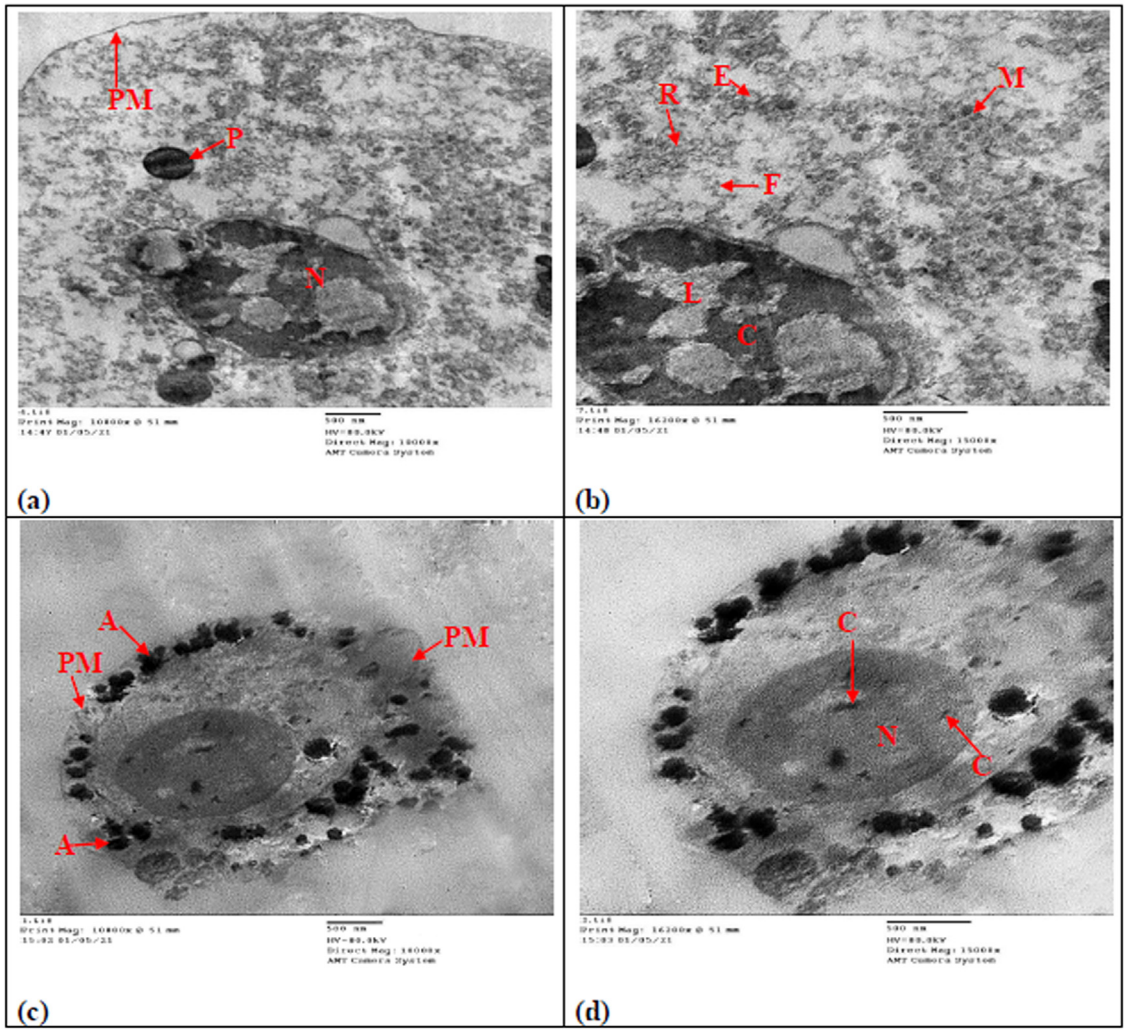

Fig. 1 Transmission electron micrographs of fibroblast showing: **(a)-** Group I with oval nucleus (N), intact plasma membrane (PM) and peroxisomes (P), **(X10000). (b)-** At a higher magnification, clusters of condensed chromatin (C) intermingled with areas of loose chromatin (L). Mitochondria (M), endosomes (E), rough endoplasmic reticulum (R) and free ribosomes (F), **(X15000). (c)-** Group II with destructed plasma membrane (PM) and apoptotic bodies (A), **(X10000). (d)-** At a higher magnification, oval nucleus (N) with sporadic condensed chromatin (C), **(X15000).**

endoplasmic reticulum, free ribosomes and cytoplasmic vacuoles were spotted (Fig. 2b).

3.1.4. Group IV (LPS/Exosomes)

Examination of fibroblast in Group IV revealed markedly large rounded nucleus with dense nucleolus. Plasma membrane appeared intact and showed fusion with exosomes in some areas (Fig. 2c). Peroxisomes, exosomes, mitochondria, rough endoplasmic reticulum and free ribosomes were detected (Fig. 2d).

3.2. Statistical results

3.2.1. Cell viability

Statistical analysis of fibroblast cells viability% showed that, there was no statistically significant difference between Groups I, III & IV, however they showed statistically significant difference with Group II. Group IV showed the highest mean value of cell viability%, followed by Groups III, I and II respectively (Table 1 & Fig. 3).

3.2.2. ELISA results

Statistical analysis of ELISA results regarding IL-1β protein showed that, there was statistically significant difference between different studied groups. Group II showed the highest mean value of IL-1β, followed by Group III then Group IV. Group I showed the least mean value of IL-1β (Table 2 & Fig. 4a). The statistical analysis of IL-10, Nrf2 and HO-1 proteins showed that, there was statistically significant difference between different studied groups. Group I showed the highest mean value, followed by Group IV then Group III. Group II showed the least mean value (Table 2 & Fig. 4b, c & d).

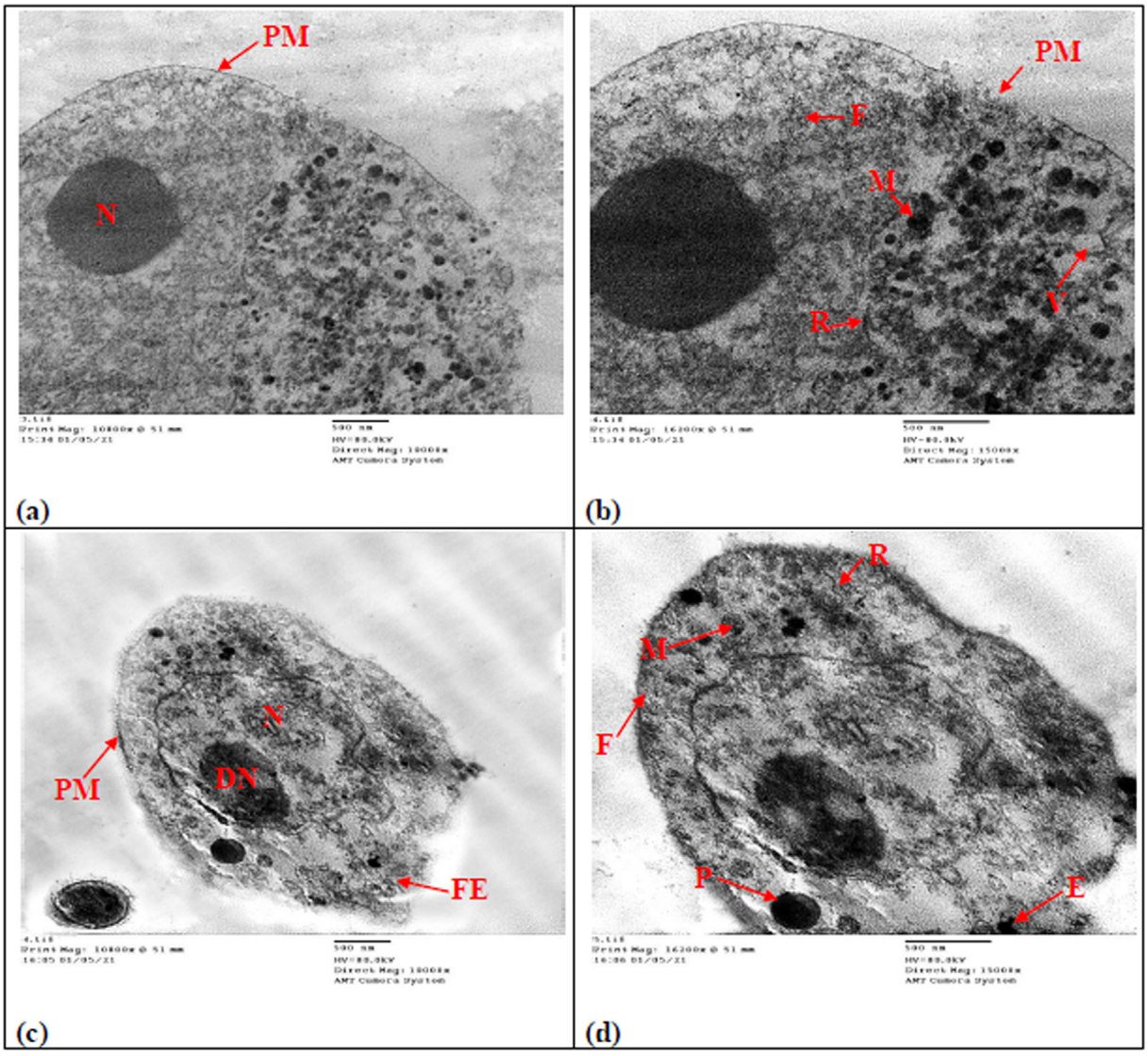

Fig. 2 Transmission electron micrographs of fibroblast showing: **(a)-** Group III with small rounded nucleus showing condensed chromatin (N), intact plasma membrane (PM), **(X10000). (b)-** At a higher magnification, destructed plasma membrane (PM) in some areas. Mitochondria (M), rough endoplasmic reticulum (R), free ribosomes (F) and cytoplasmic vacuoles (V), **(X15000). (c)-** Group IV with markedly large nucleus (N) and dense nucleolus (DN). Intact plasma membrane (PM) and areas of fusion with exosomes (FE), **(X10000). (d)-** At a higher magnification, peroxisomes (P), exosomes (E), mitochondria (M), rough endoplasmic reticulum (R) and free ribosomes (F), **(X15000).**

Table 1 Showing comparison between studied groups regarding cell viability%.

Cell Viability%	Group I	Group II	Group III	Group IV	ANOVA	p-value
Mean ± SD	100.64 ± 29.17	32.70 ± 10.11[a]	101.62 ± 36.22[b]	120.38 ± 29.61[b]	9.442	0.002

According to Least Significant Difference post hoc test, superscript letters indicate; a: significant difference with Group I; b: significant difference with Group II.

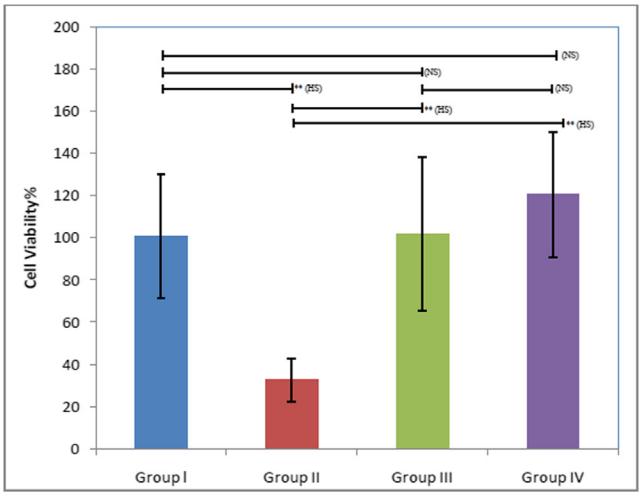

Fig. 3 Bar chart representing comparison of mean and SD values between different studied groups regarding cell viability%. **P < 0.001, ^{NS}P > 0.05.

4. Discussion

The LPS are considered as inflammatory agents as they upregulate IL-1β and other inflammatory cytokines in the inflamed tissue as well as in cultured cells (Ren & Torres, 2009).

Chrysin has potent antioxidant and anti-inflammatory effects as it reduces the reactive oxygen species levels and suppresses the expression of many inflammatory biomarkers "accompanied by interfering with the inflammatory pathways" also; it increases the expression of various anti-inflammatory cytokines (Farkhondeh et al., 2019).

The BM-MSC exosomes have antioxidant effect, beside their ability of down-regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory ones (Lv et al., 2018; Alcaraz et al., 2020).

The ultra-structure features of fibroblasts described in Group I of current study are in agreement with those of (Zhang et al., 2013; Le et al., 2020).

The apoptotic changes of ultra-structure findings observed in Group II of herein research could be implicated to the effect of oxidative stress created by IL-1β that leads to reduction of resting mitochondrial membrane potential which is an initial and irreversible step towards apoptosis. In addition to the mitochondrial dysfunction, the oxidative stress leads also to alterations in plasma membranes permeability as well as changes in structure of various macromolecules such as DNA, lipids and proteins which eventually end up by destruction of different cell organelles and secretory granules (Frisard & Ravussin, 2006; Wang et al., 2017; Nogueira et al., 2019).

The nearly normal ultra-structure results explored in Group III of the present research might be attributed to the antioxidant and anti-inflammatory effects of chrysin which provide protection against free radicals, preservation of organelles' membranes and cytoplasmic structures as well as reduction of mitochondrial degeneration along with inhibition of the mitochondrial apoptosis pathway (Aishwarya & Sumathi, 2015; Hermenean et al., 2017).

The almost normal ultra-structure features observed in Group IV of this study could be implicated to the antioxidant properties, anti-inflammatory and paracrine effects "release of multiple growth factors, cytokines and chemokines" of BM-MSC exosomes which lead to restoration of mitochondrial membrane potential and function, inhibition of mitochondrial apoptosis pathway, reconstruction of cell organelles and recovery of cellular homeostasis (Wang et al., 2017; Xian et al., 2019).

The MTT and ELISA results of fibroblasts recorded in Group I of this study are in accordance with those of (Oyewole et al., 2014; Lee et al., 2017; Shochet et al., 2017; Girsang et al., 2019).

The fibroblasts in Group II of current research recorded the least mean of viability%. Moreover, statistical analysis of ELISA in Group II showed the highest mean value of IL-1β and the least mean values of HO-1, Nrf2 and IL-10. These findings could be attributed to the effect of LPS that up-regulate the expression of IL-1β in fibroblasts which leads to initiation of inflammation and generation of reactive oxygen species responsible for creation of oxidative stress that consequently induces suppression of anti-inflammatory cytokines and enzymes (Tardif et al., 2004; Lavieri et al., 2016; Saha et al., 2020).

Statistical analysis of MTT and ELISA results reported in Group III of herein study could be ascribed to the antioxidant and anti-inflammatory roles played by chrysin in down-regulation of IL-1β expression along with attenuation of the oxidative stress, activation of HO-1 as well as up-regulation of Nrf2 and IL-10 (Farkhondeh et al., 2019).

The viability% and ELISA results explored in Group IV of present research might be attributed to the inhibitory effect of exosomes on inflammatory pathways mediated by IL-1β. Also, BM-MSC exosomes exert paracrine effects and deliver different bioactive molecules like DNA, messenger-RNA, proteins, microRNA and other nuclear materials to target cells after fusion with them. Consequently, this is accompanied by HO-1 activation and significant up-regulation of both Nrf2 and IL-10 (Zhang et al., 2016; Cosenza et al., 2018).

Table 2 Showing comparison between studied groups regarding IL-1β, IL-10, Nrf2 & HO-1 proteins.

ELISA	Group I	Group II	Group III	Group IV	ANOVA	p-value
IL-1β (pg/ml)Mean ± SD	21.90 ± 2.95	66.50 ± 10.32^{a}	33.10 ± 3.06^{ab}	31.13 ± 2.05^{b}	35.473	<0.001
IL-10 (ng/ml) Mean ± SD	4.83 ± 0.35	1.33 ± 0.15^{a}	3.27 ± 0.31^{ab}	3.70 ± 0.10^{ab}	102.062	<0.001
Nrf2 (ng/ml)Mean ± SD	4.13 ± 0.32	1.20 ± 0.20^{a}	2.80 ± 0.20^{ab}	3.10 ± 0.36^{ab}	56.521	<0.001
HO-1 (ng/ml) Mean ± SD	3.53 ± 0.15	0.97 ± 0.15^{a}	2.53 ± 0.21^{ab}	2.80 ± 0.20^{ab}	107.786	<0.001

According to Least Significant Difference post hoc test, superscript letters indicate; a: significant difference with Group I; b: significant difference with Group II.

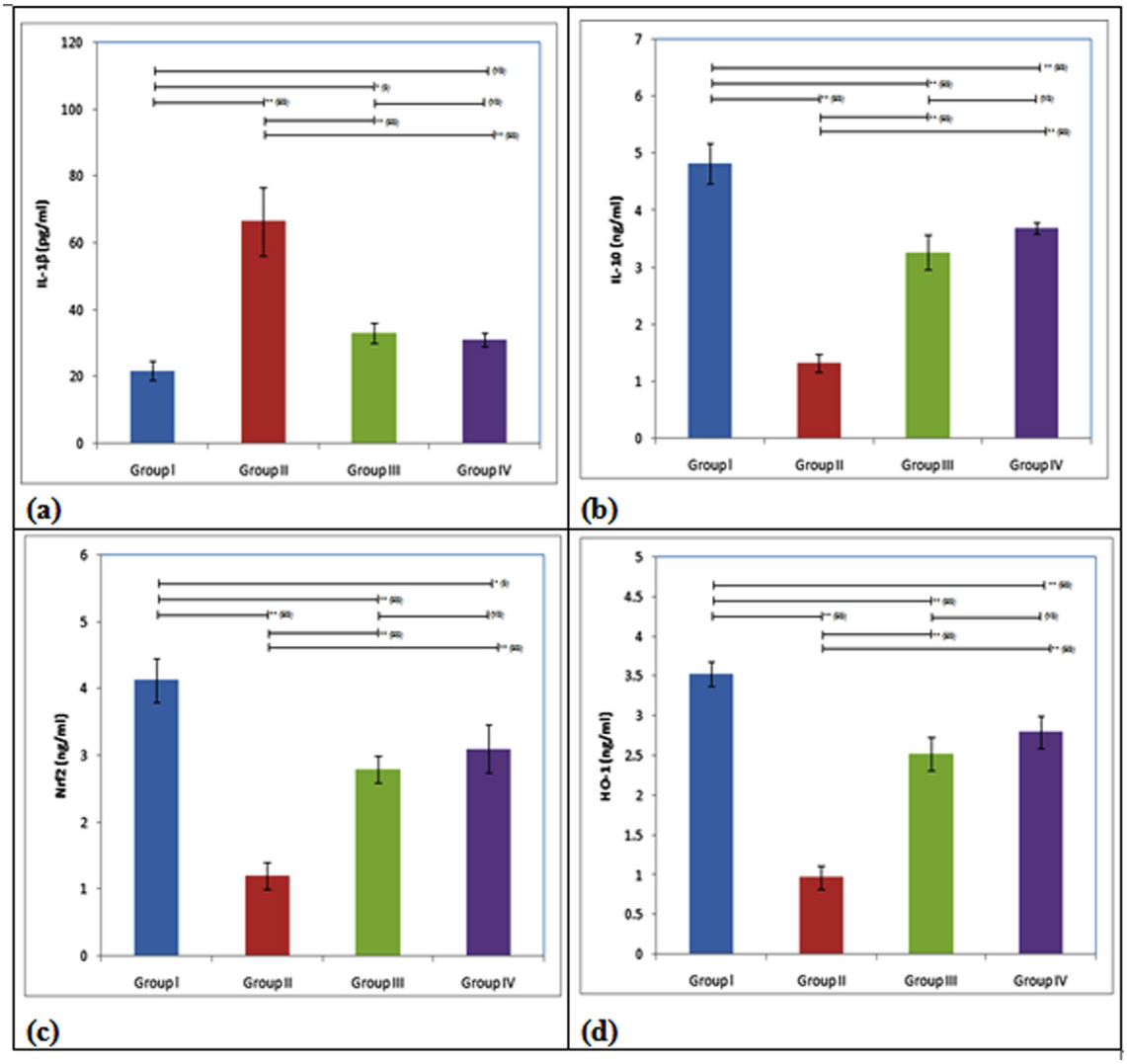

Fig. 4 Bar charts representing comparison of mean and SD values between different studied groups regarding ELISA assay of proteins (a)- IL-1β. (b)- IL-10. (c)- Nrf2. (d)- HO-1. **P < 0.001, *P < 0.05, ^{NS}P > 0.05.

In addition, the higher mean value of Group IV fibroblast viability% compared to Group I in the current study could be attributed, as reported in our ultrastructural results, to the regenerative power of exosomes on mitochondria and plasma membrane which are essential for the chemical reduction of MTT (Berridge et al., 2005). Moreover, Huang et al. (2021) found that, human dental pulp stem cells in inflammatory microenvironment induced by LPS produce more exosomes compared to those present in normal condition.

In vivo studies are needed to compare the potential effect of BM-MSC exosomes versus chrysin on structure and ultrastructure of different oral tissues affected by oxidative stress-related diseases.

5. Conclusions

The LPS have deleterious effect on the ultra-structure, viability and function of HDFa cells. Chrysin and BM-MSC exosomes have regenerative effect against the destructive outcomes of LPS. Ultra-structural and statistical results confirmed that BM-MSC exosomes have intensive regenerative effect on inflamed fibroblast in comparison to chrysin.

Administration of BM-MSC exosomes in patients with oral manifestations of systemic diseases associated with inflammatory conditions may be beneficial.

Ethical statement

All steps of this experiment were reviewed and approved by Research Ethics Committee at Future University in Egypt, (approval number: (FUE.REC (29)/12-2021).

Funding

Self-funded research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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