


Low-Dose Pesticide Mixture Induces Accelerated Mesenchymal Stem Cell Aging In Vitro

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ABSTRACT

The general population is chronically exposed to multiple environmental contaminants such as pesticides. We have previously demonstrated that human mesenchymal stem cells (MSCs) exposed in vitro to low doses of a mixture of seven common pesticides showed a permanent phenotype modification with a specific induction of an oxidative stress-related senescence. Pesticide mixture also induced a shift in MSC differentiation toward adipogenesis. Thus, we hypothesized that common combination of pesticides may induce a premature cellular aging of adult MSCs. Our goal was to evaluate if the prolonged exposure to pesticide mixture could accelerate aging-related markers and in particular deteriorate the immunosuppressive properties of MSCs. MSCs exposed to pesticide mixture, under long-term culture and obtained from aging donor, were compared by bulk RNA sequencing analysis. Aging, senescence, and immunomodulatory markers were compared. The protein expression of cellular aging-associated metabolic markers and immune function of MSCs were analyzed. Functional analysis of the secretome impacts on immunomodulatory properties of MSCs was realized after 21 days' exposure to pesticide mixture. The RNA sequencing analysis of MSCs exposed to pesticide showed some similarities with cells from prolonged culture, but also with the MSCs of an aged donor. Changes in the metabolic markers MDH1, GOT and SIRT3, as well as an alteration in the modulation of active T cells and modifications in cytokine production are all associated with cellular aging. A modified functional profile was found with similarities to aging process. *STEM CELLS* 2019;37:1083–1094

SIGNIFICANCE STATEMENT

Although the general population is chronically exposed to multiple environmental contaminants such as pesticides, the in vitro results of this study show that a mixture of seven pesticides, frequently detected in food samples, induces on adult mesenchymal stem cells (MSCs) an ensemble of characteristics found in aged tissue. MSCs are important components of hematopoietic niche, and their accelerated cellular aging, induced by noncytotoxic doses of combined pesticides, may participate in the acceleration of different degenerative pathologies.

INTRODUCTION

Exposure to pesticides is a problematic public health issue, due to their intensive use in agriculture, which leads to occupational contamination, but also to a wide exposure of the general population through consumption of treated food. Even if pesticide quantities are limited to a maximal theoretical safety threshold, called the acceptable daily intake (ADI), the exposure of the general population is constant, chronic, and consists mainly of a polyexposure. A number of studies have shown that this polyexposure results from both the polycontamination of food and the sum

of daily exposures of contaminated sources by a single component [1, 2]. Various studies regularly show the presence of multiple pesticide residues in the blood or metabolites in the urine [3–5]. In a previous work [6], we studied the risks of multiple exposures by the evaluation, on a long-term basis, of mixtures of pesticides of different chemical families.

Hematopoietic tissue is a main site of exposure to pesticides [7]. Mesenchymal stem cells (MSCs) are important components of the hematopoietic niche and contribute to its homeostasis [8]. They can also be found in other compartments. MSCs have the capacity to differentiate

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into many cell types, including osteoblasts, chondrocytes, and adipocytes [9], which enlarges their functional contribution. Moreover, these cells also show an immunosuppressive potential for the innate and adaptive immune system [10] and were first demonstrated to modulate T-cell proliferation [11].

We have previously shown that the exposure of MSCs for 21 days *in vitro* to low doses of a mixture of seven pesticides, frequently detected in food samples, induced permanent phenotypic modifications with alteration of MSCs proliferation, a specific induction of an oxidative stress-related senescence, and a shift in MSCs differentiation toward adipogenesis [6]. The adipogenesis shift is also an indicator of a senescent reprogramming of the MSCs by pesticides.

Cellular senescence has been implicated in the loss of regenerative potential in aging tissues [12] and there is evidence linking cellular senescence and aging process with a correlation between *in vitro* senescence and *in vivo* aging [13]. Of note, the effect of age versus multiple passages on MSCs property differences is small [14]. Thus, to strengthen our hypothesis on our pesticide cocktail-induced cellular aging, we compared MSCs exposed *in vitro* to pesticides obtained from young adults with those of untreated older donors. We used both RNA sequencing analysis of MSCs with or without exposure to pesticides and the determination of cellular aging associated functional alterations of exposed MSCs.

MATERIALS AND METHODS

Materials

All pesticides (chlorpyrifos ethyl, dimethoate, diazinon, iprodione, imazalil, maneb, and mancozeb) were obtained from Sigma-Aldrich (St. Louis, MO). Human peripheral blood mononuclear cells (PBMCs) of three different donors came from "Etablissement Français du Sang" (convention number: CPDL PLER 2018 021). MSCs from different young and healthy donors (aged of 23–35 years) were provided by the "Tissus Bank of IRCNA/CHU Nantes-Angers and ICO." The written consent was obtained from informed patients in accordance with French law (Art. L. 1245-2 of the French public health code, law number 2004-800 of August 6, 2004, official journal of August 7, 2004). Some MSCs were obtained from bone marrow aspirates from the iliac crest of healthy human donors (21–26 years old) after receiving informed consent according to the Declaration of Helsinki. The project was approved by the Ethical Committee of Ulm University (Dr. P. Layrolle). MSCs were characterized as previously described [15]. Briefly, the surface markers, including CD34, CD45, CD73, CD90, and CD105, and the differentiation capacity toward four lineages (osteoblast, adipocyte, chondroblast, or myoblast) were assessed and confirmed as recommended [16]. Cell media: Minimum Essential Medium - Alpha Modification (α -MEM), Dulbecco's modified Eagle's medium, Roswell Park Memorial Institute (RPMI-1640), and fetal calf serum (FCS; #10270) were from Thermo Fisher Scientific (Villebon-sur-Yvette, France). MesenCult and the corresponding serum were from Stem Cell Technology (Grenoble, France). Medium and supplements for human microvascular endothelial cells endothelial cell growth medium-2 were from Lonza (Viviers, Belgium).

Pesticide Mixture Doses and Preparation

The doses of pesticides used were extrapolated from three values (Supporting Information Table S2). The first is the high

nutritional daily intake (hNDI), calculated for the French population of all ages based on the method of the European Food Safety Authority [11]. hNDI are the low doses of pesticides used in our study. The second one is the international ADI representing the threshold of safety in humans for lifetime exposure. The last one is a value of three times the ADI (3ADI) for positive control. An average value (in %ADI) of the highest estimated exposure for each pesticide was converted to mg/kg body weight per day considering the ADI value for each pesticide. For the three values of hNDI, ADI, and 3ADI, a further conversion has been made in order to extrapolate the aforementioned doses (estimated in mg/kg body weight) to an *in vitro* model. We considered a total absorption of this ingested amount and then its dilution in 5 l of blood in a subject of 60 kg, in order to obtain the blood concentration (mg/l and μ mol/l) to which the various organs could be theoretically exposed.

Cells Culture and Treatment

MSCs (used between passages 4 and 7) were cultured at 2,000 cells per cm^2 in α -MEM modified with ribonucleosides and deoxyribonucleosides and supplemented with FCS (10%) and 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.2 ng/ml of FGF2 in an atmosphere of 5% CO_2 and 95% humidity at 37°C. Pesticide mixture at doses of hNDI, ADI, or 3ADI was dissolved in Dimethyl sulfoxide (DMSO) and added to the cell media over the 21-day exposure. DMSO doses versus media did not exceed 1/1,000 (vol/vol) for control and pesticides-treated cells. Media were replaced every 2–3 days.

Measurement of NAD/NADH Report

Total intracellular nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide diaphorase (NADH) levels were measured using the NAD/NADH Quantification Colorimetric Kit (BioVision, Inc., Milpitas, CA) as described by manufacturer.

RNAseq Analysis

Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was quantified using a NanodropND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, United States). Quality and integrity of RNA samples were assessed using the 2100 Bioanalyzer and RNA 6000 Nano LabChip Kit series II (Agilent Technologies, Santa Clara, California, United States). Library construction was performed from 500 ng of total RNA with SureSelect Strand-Specific RNA Library Prep for Illumina Multiplexed Kit (reference 5190-6410, Agilent Technologies) according to Agilent_PreLib_G9691-90010_juillet2015_vD protocol. Purifications were carried out with NucleoMag NGS Clean-up and Size Select (reference 744970.50, Macherey-Nagel). Fragment size of libraries was controlled on D1000 ScreenTape with 2200 TapeStation system (Agilent Technologies). Libraries with P5–P7 adaptors were specifically quantified on LightCycler 480 Instrument II (Roche Life Science, Penzberg, Germany) and normalized with DNA Standards (1–6; reference KK4903, KAPABIOSYSTEMS—CliniSciences, Nanterre, France). Then, 12 pM of each library was pooled and prepared according to denaturing and diluting libraries protocol for the HiSeq and GAIIx, part#15050107 v02 (Illumina) for cluster generation on cBot system. Paired-end sequencing (2 \times 100 cycles) was carried out in four lanes on HiSeq 2500 system (Illumina, San Diego, California, United States) in TruSeq v3 chemistry according to the instructions of HiSeq 2500 System Guide, part#15035786 v01 (Illumina).

Details for library construction (Agilent_PrepLib_G9691-90010_juillet2015_vD protocol): poly(A) RNA was purified from total RNA using two serial rounds of binding with oligo(dT) magnetic particles. Purified poly(A) RNA is chemically fragmented to the appropriate size for RNA sequencing library preparation (fragments between 200 and 600 bp). Then, cDNA is synthesized and processed for 5' ends-repair, phosphorylation, and A-tailing in 3' ends. After ligation of adaptors, cDNA samples were indexed and amplified for 14 Polymerase Chain Reaction (PCR) cycles.

After demultiplexing and quality control with fastQC_0.11.2 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), illumina adapter was trimmed with cutadapt-1.2.1 [17] and reads with Phred quality score below 30 were filtered with prinseq-lite-0.20.3 [18]. Reads were aligned against human hg19 reference genome with tophat2.0.10; reads count was realized with htseq-count from HTSeq-0.5.4p5 [19] and differential analysis with [20].

Validation of RNA-Seq Results by Quantitative Real-Time PCR

For further analysis of the expression of MSCs transcripts, total RNA was extracted using NucleoSpin RNA II (Macherey-Nagel, Düren, Germany) at days 7, 14, and 21. Reverse transcription was performed using 1 µg d'ARN with the Maxima First strand cDNA synthesis kit (Thermo Scientific). Quantitative PCR analysis was then performed with the qTower system (Analytik Jena, DE) and the sybr green technology using the qScript XLT cDNA superMix kit (Quanta Biosciences). Triplicate using 10 ng of cDNA was done for each sample. For each PCR, the specificity of the PCR product was verified using the melting curve. Gene names and primer sequences are in Supporting Information Table S3. Fold-change was obtained using the $2^{-\Delta\Delta Ct}$ method.

Interleukin Enzyme-Linked Immunosorbent Assay (ELISA) Quantification

Cytokine levels were measured in cell culture supernatants using the ELISA technique, according to the manufacturer's instructions for interleukin (IL), IL-10 and IL-6, monocyte chemoattractant protein (MCP)-1, leukemia inhibitor factor (LIF), Transforming Growth Factor β (TGF- β) and Interferon γ (IFN γ) (R&D Systems Europe, Ltd., U.K.) for LIF (Life Technology).

Test PBMCs

Fifty microliters per weight of OKT3 antibodies (10 µg/ml final solution in phosphate-buffered saline [PBS]) were used in a 96-well plates and incubated overnight at 4°C. The wells were washed three times with PBS, then 5×10^4 PBMCs, diluted into 60 µl culture medium, were placed in each well. In addition, 60 µl RPMI medium/10% FCS or MSC conditioned medium (exposed or not) were added. After 24 hours/37°C incubation, supernatants (100 µl) were evaluated for IFN γ levels using the ELISA technique, according to the manufacturer's instructions (R&D Systems Europe, Ltd., U.K.).

Statistical Analysis

Results are expressed as mean \pm SEM. Statistical analysis was performed when appropriate using one-way or two-way analysis of variance (ANOVA) followed by Tukey or Dunnett's multiple comparison tests. For in vivo comparison, "Gehan-Breslow-Wilcoxon test" was used. Statistics were run with GraphPad Prism. Differences with $p < .05$ were considered statistically significant.

Miscellaneous

For SA- β -galactosidase activity and immunoblot, see Supporting Information Methods.

Data Availability

The RNAseq data have been deposited in the SRA database under accession number PRJNA510912. To view the SRA accession PRJNA510912, go to <https://www.ncbi.nlm.nih.gov/sra/PRJNA510912>.

RESULTS

Transcriptome Profiling of MSCs Exposed to Pesticide Mixture Showed Multiple Similarities with MSCs from Long-Term Culture and MSCs from a 72-Year-Old Donor in Terms of Aging and Senescence Markers

To demonstrate that pesticide exposure may lead to a premature aging of adult MSCs, we used RNA-sequencing approach and compared the transcriptomic profiles of MSCs under different conditions: normal MSCs at low passage (P4, control cells), MSCs exposed (for 21 days; at P4) to intermediate doses of pesticide mixture (ADI: in vitro extrapolation of the ADI as defined by Hochane et al.), MSCs maintained in long-term culture to high passages (P14, called MSC-P14), and MSCs from a 72-year-old donor. In vitro, MSC-P14, MSCs from aging donor, and ADI-treated MSCs show similar β -galactosidase staining indicative of the similar induction of senescence (Fig. 1A).

The analysis of RNA sequencing data showed that the exposure to pesticide mixture modified 612 gene expressions, 2,150 with MSC-P14, and 3,648 with MSCs from aging donor, compared with control MSCs (P4). Only the genes whose modulation was greater than two times ($\log_2 > \text{onefold change}$) were taken into account. Principal component analysis showed an important similarity between pesticide-treated MSCs and MSC-P14. However, fewer similarity with MSCs from older donor was also observed (Fig. 1B). The cells exposed to pesticides are closer to the MSC-P14 cells in the main component PC1 (77.3%) but closer to the controls with the main component PC2 (17.8%). As shown in Figure 1C, higher similarities in the upregulated and downregulated genes were found between the pesticide mixture-exposed cells and MSC-P14 rather than with the older donor. Figure 1D shows 116 genes upregulated and 106 genes downregulated compared with control MSCs (Supporting Information Table S1).

To further evaluate the functional network modification by pesticide mixture, the STRING database was used, and protein-protein interaction networks for upregulated (Fig. 2A) and downregulated genes (Fig. 2B) were produced. In the group of upregulated genes, two nodes are observed corresponding in large part to common gene identified. We observed an upregulation of genes implicated in cell survival after stress induction or during aging process, and downregulation of genes involved in mitosis, kinetochore elements required for chromosomal segregation, or signaling pathway components governing cellular proliferation.

We searched for senescence markers and those associated with aging in MSCs. Figure 3 (upper panel) shows a similar profile of different aging markers (intercellular adhesion molecule-1 [ICAM1], CXCL5, Leptin, IL1 β) under three conditions. In addition, senescent cells specifically secrete a number of factors that generally constitute the senescence-associated secretory phenotype (SASP) [21]. Some markers associated with physiological aging

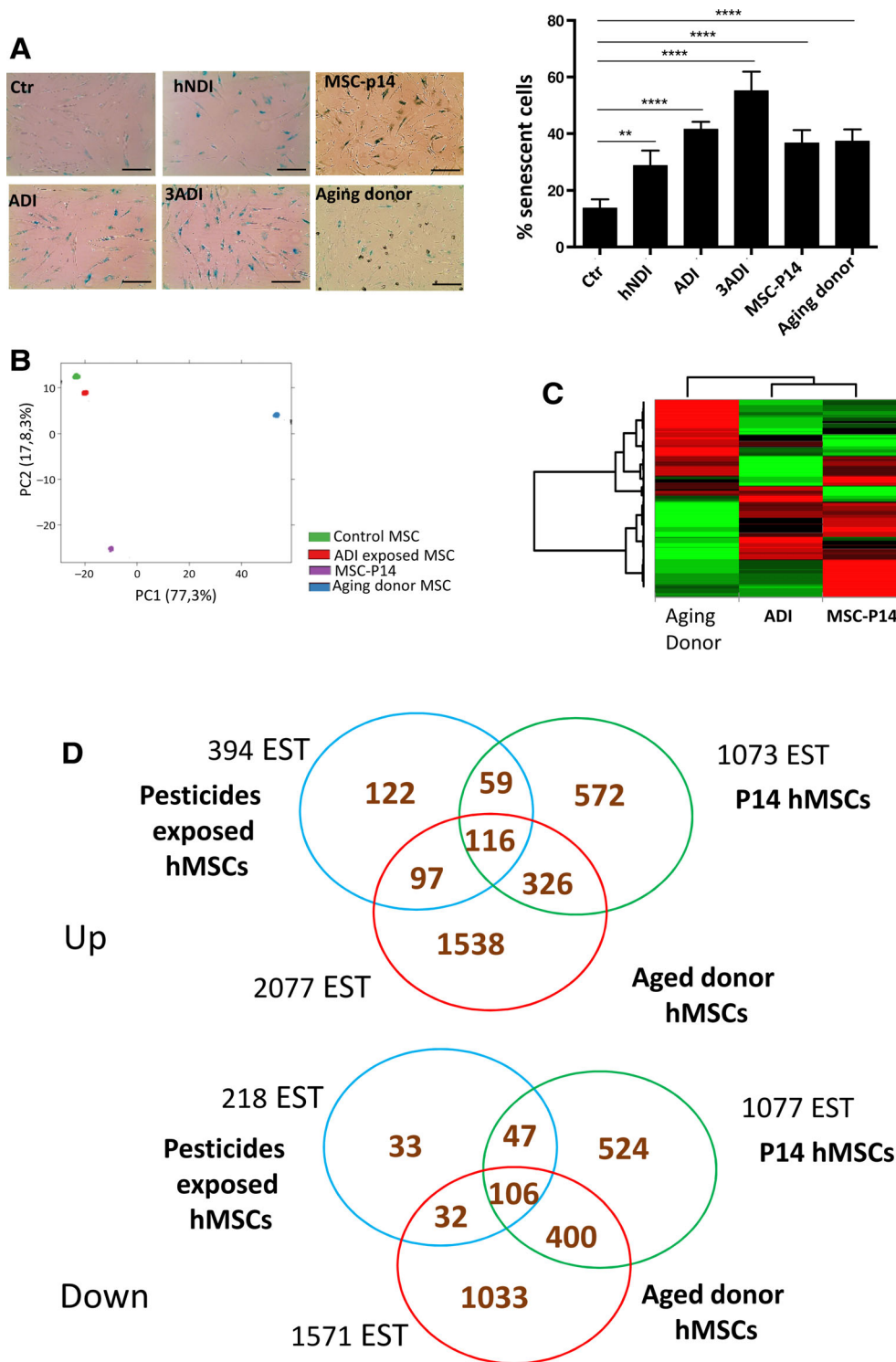


Figure 1. RNAseq analysis reveals similarity between mesenchymal stem cells (MSCs) exposed 21 days to pesticide mixture and aging cells. **(A):** β-Gal staining of MSCs after 21-day exposure to pesticide mixture, MSC-P14, and MSCs from aging donor. The percentages of senescent cells were then determined ($n = 3$; *, $p < .05$; **, $p < .01$; ***, $p < .001$; scale bars: 200 μm). **(B):** Principal component analysis of negative control MSCs (P3; green), 21 days pesticide mixture (acceptable daily intake) exposed MSCs (red), long-term culture MSC-P14 (purple), and MSCs from aging donor (72 years; blue). **(C):** Pesticide-induced gene expression in MSCs is similar to long-term cultured MSCs. Global gene expression profiles of four MSC samples were analyzed by Affymetrix technology (control MSCs [P3], 21 days exposed pesticide mixture MSCs [P4], long-term cultivated MSCs cells [P14], and MSCs from aging donor [72 years old]). **(D):** Analyses revealed, respectively, that 394, 1,073, and 2,077 ESTs were significantly upregulated (red) from pesticide exposed, MSC-P14, and MSCs from aged donor and 218, 1,077, and 1,571 ESTs were downregulated (green). Color-coding in the heat map demonstrates that gene expression changes upon aging are also reflected by replicative senescence of MSCs in vitro. **(C):** Differential gene expression of the three datasets (MSCs-donor age, exposed-MSCs, and long-term culture MSC-P14) was further compared. Venn diagrams demonstrate the overlap of differentially expressed genes that are upregulated (A) or downregulated (B). Differentially expressed genes are indicated as shortcut (Hugo gene nomenclature). There was a relatively high overlap with the three conditions.

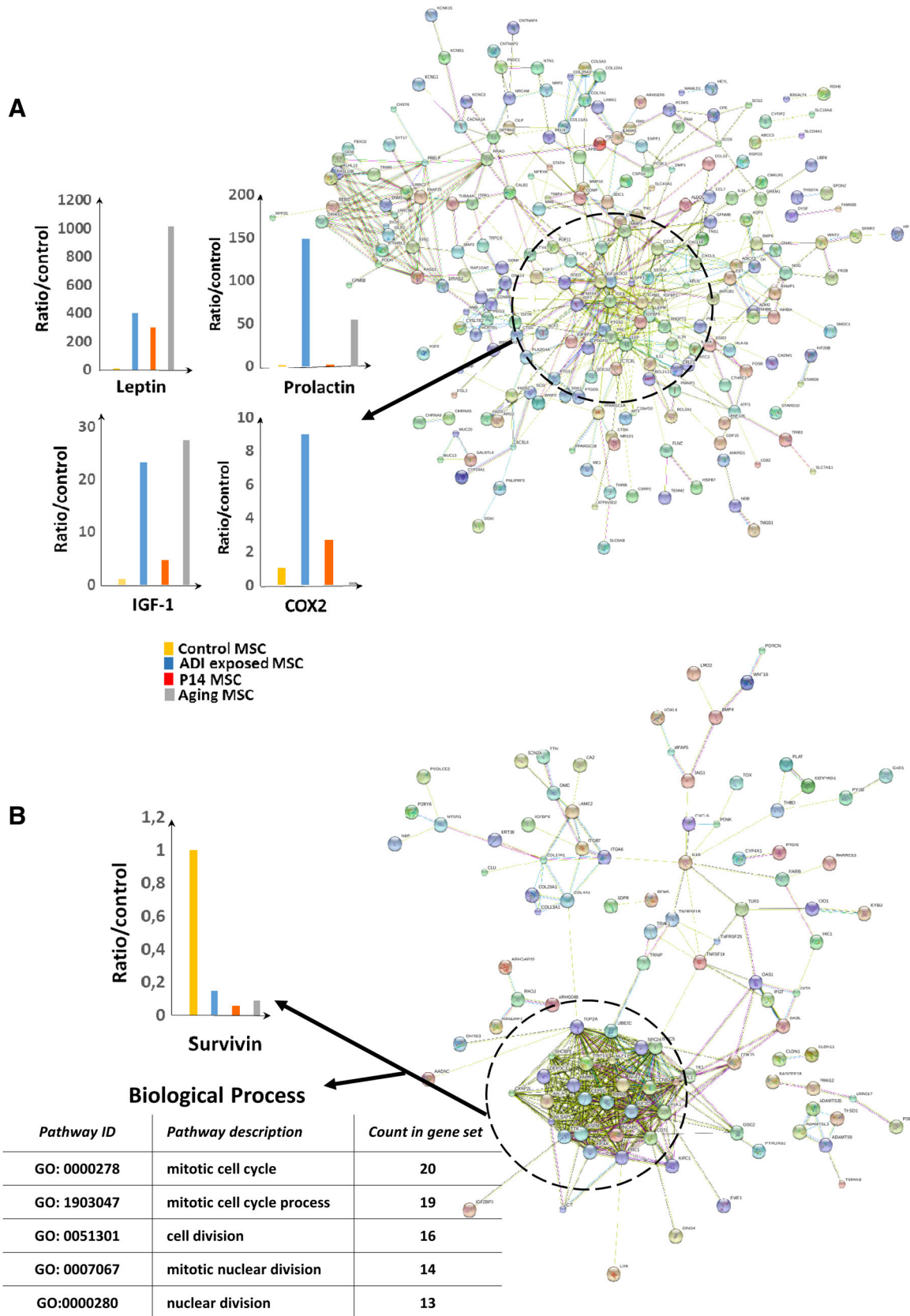


Figure 2. Protein–protein interaction network for dysregulated mesenchymal stem cell genes after 21 days exposure with pesticide mixture and analyzed by STRING software. **(A):** The string diagram shows the predicted protein–protein interactions of the 186 upregulated differentially expressed genes using medium confidence (0.4), key genes (*igf-1*, *prolactin*, *leptin*, and *cox-2*) from main group of genes (Figure legend continues on next page.)

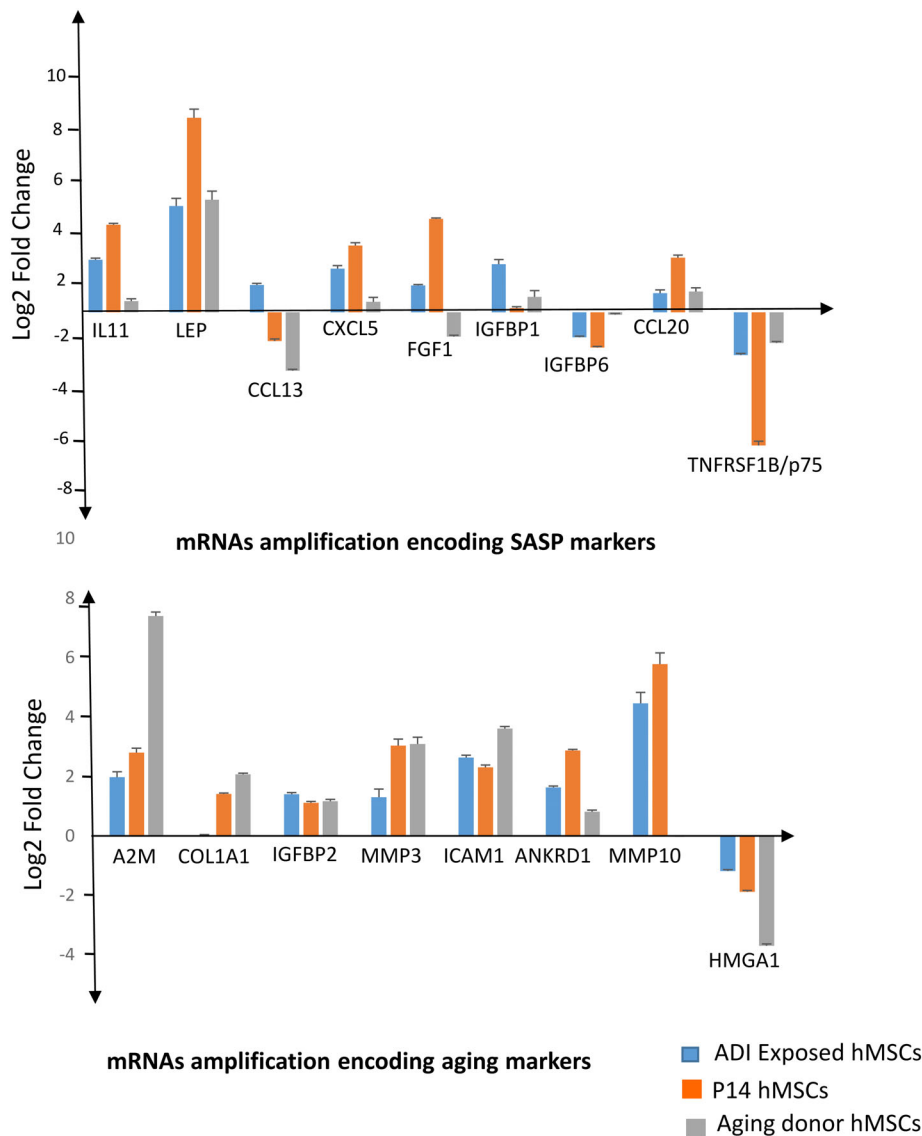


Figure 3. Pesticide mixture induces senescent and aging profile on exposed mesenchymal stem cells (MSCs). Analysis of the differentially expressed genes between the exposed MSCs (blue bars), long-term culture MSC-P14 (orange bars), or MSCs from aging donor (gray bars) versus control MSCs. Upper panel for genes encoding markers established in senescence-associated secretory phenotype, and lower panel for genes encoding markers established in cell aging profile.

whose genes implicated in extracellular matrix organization altered with aging process (α -2-macroglobulin, collagen type I, matrix metalloproteinase 3, ICAM1) were also found to be dysregulated in all our conditions (Fig. 3, lower panel).

Pesticide Exposure Induced Metabolic Modifications Similar to those Encountered During Aging Process

Donor age and long-term passage of MSCs seem to modify their proliferation and differentiation in a similar manner [22];

however, the aspects of correlation of ADI and MSC-P14 profiles on the heat map have influenced us to use MSC-P14. Thus, we decided to use the MSC-P14 for further comparisons with MSCs exposed to pesticide for the functional analysis of aging.

Wiley and Campisi have established links between the cellular metabolism and phenotypes commonly associated with senescent cells and physiological aging [23]. Some characteristic changes involve the inhibition of aspartate aminotransferase (GOT1), which decreases the cytosolic NAD^+/NADH ratio and

(Figure legend continued from previous page.)

highlighted, were evaluated by qPCR. (B): The string diagram shows the predicted protein–protein interactions of the 186 downregulated differentially expressed genes using medium confidence (0.4). Disconnected nodes in the network were hidden. Colored nodes represent query proteins and first shell of interactors; white nodes represent second shell of interactors. Edges represent protein–protein associations and different colors of the edges represent different associations. The known interactions were found from curated databases (blue) or experimentally determined (purple); the predicted interactions were suggested by gene neighborhood (green), gene fusions (red), or gene co-occurrence (dark blue); and other protein–protein associations were suggested by text-mining (yellow–green), coexpression (black), or protein homology (gray); key gene (*survivin*) from main group of under expressed genes was evaluated by qPCR.

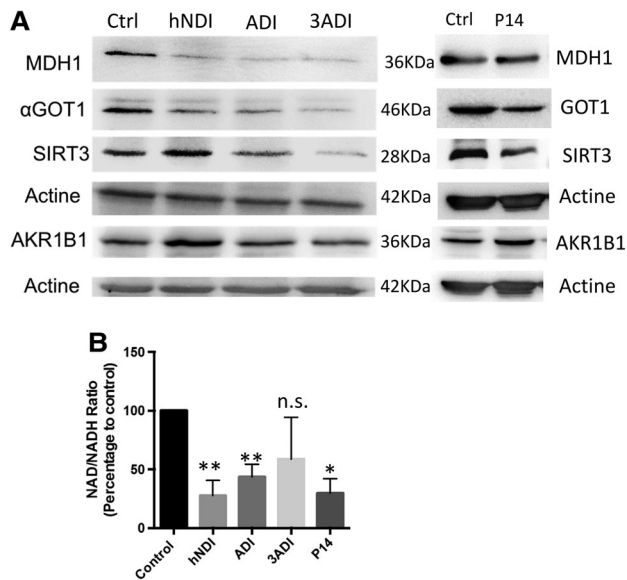


Figure 4. Prolonged pesticides exposure of mesenchymal stem cells (MSCs) induce metabolic profile associated with aging. **(A):** Expression of metabolic enzyme implicated in senescence process and aging was evaluated after exposure to pesticide mixture for 21 days by immunoblot in control MSCs (Ctrl; left panel) and long culture MSC-P14, or MSCs from aged donor (right panel; $n = 3$); **(B):** MSCs were treated with pesticide mixture for 21 days and the NAD^+ and NADH levels in cell extracts were quantified. The optical density at 450 nm was recorded and used to calculate the NAD^+ /NADH ratio compared with control MSCs and MSC-P14. Values (mean \pm SE) were obtained from at least three independent experiments ($n = 3$; *, $p < .05$; **, $p < .01$; ***, $p < .001$).

induces senescence. Similarly, malate dehydrogenase-1 (MDH1) was identified as increasingly modified during aging [24]. We compared the metabolic profiles of MSCs after pesticide exposure to that of high-passage MSC-P14 (Fig. 4A).

MDH1 and GOT1 decreased in a dose-dependent manner with pesticide exposure. The same modification was observed for GOT1, but not with MDH1, in MSC-P14.

Sirtuin 3 (SIRT3) is the major mitochondrial protein deacetylase [25]. Its expression is decreased during aging [26]. We observed a decrease of SIRT3 protein expression in a dose-dependent manner as compared to MSC-P14.

In addition, we controlled Aldo-keto reductase family 1, member B1 (AKR1B1) (aldose reductase) protein expression, a member of the AKR superfamily. The induction of AKR1B1 expression in response to oxidative stress plays a role in the antioxidant response [27]. Pesticide treatment modestly increased AKR1B1 expression with hNDI doses; no modification was observed for MSC-P14 cells.

NAD^+ is a critical cofactor of the previous enzymes and decreased with aging [28]. The ratio of the oxidized and reduced forms (NAD^+ and NADH, respectively) after pesticide mixture exposure and in MSC-P14 was assayed (Fig. 4B). Our results show a significant decrease in the NAD^+ /NADH ratio with pesticide treatment only for hNDI and ADI doses. This was also observed with the MSC-P14.

Prolonged Exposure to Pesticide Mixture Induce Alteration of Immunomodulatory Properties of MSCs

MSCs exert anti-inflammatory and immunomodulatory effects on cells of the innate and adaptive immune systems via cytokine

and chemokine secretion or direct cell-to-cell contacts [29]. To determine if pesticides had any effect on the immune properties of MSCs, we first investigated whether the expression of genes associated with immune and inflammatory function have been altered through the analysis of the differential expression of genes identified under the gene ontology. Panther pathway for activated genes implicated in immune pathways and/or inflammation is depicted in Figure 5.

Regulation of the immune response by MSCs declined with age. Number of factors identified through RNA sequencing (ICAM-1, LIF, MCP-1/CCL2, TGF- β , IL6, IL7R) and others implicated in immunomodulation properties of MSCs (IL10, Human Leukocyte Antigen – DR isotype [HLA-DR]) have been assayed either by immunoblotting (Fig. 6A) or by ELISA (Fig. 6B) both in pesticides-exposed MSCs and in MSC-P14. The expression of IL-7R, which plays a role in the differentiation and survival of T cells, was increased dose-dependently in pesticide-exposed MSCs and in MSC-P14.

MSCs are in fact a heterogeneous population of cells that express CD73, CD90, and CD105 and lack the hematopoietic lineage surface markers CD45, CD34, CD11c, CD14, CD19, CD79A, and HLA-DR [16]. However, class II HLA has been found to be expressed in the intracellular compartment of MSCs [30]. An increase of HLA-DR was observed in hNDI-treated MSCs exposed and in MSC-P14. ICAM-1, which is important for T-cell activation and leukocyte recruitment [31], was increased in a dose-dependent manner in MSCs exposed to pesticides but not in MSC-P14.

Senescence is associated with functional changes and secretion of several proteases, inflammatory cytokines, and growth factors [32]. For MSCs, the findings are less clear. We have compared elements from MSC secretome in our different pesticide exposure conditions and compared with MSC-P14 cells secretome. Cytokines were selected on the basis of SASP compounds and cytokines implicated in immunomodulation properties of MSCs (Fig. 6B). Pesticide exposure reduces, in a dose-dependent manner, IL-10 production in correspondence with MSC-P14. We observed an increase of IL-6 production, which was significant only for ADI and 3ADI doses as for MSC-P14. TGF- β , anti-inflammatory factor, was decreased significantly in a pesticide dose-dependent manner after long culture condition. The protein expression of MCP-1 (MCP-1/CCL2), important for immune cell migration, was increased after both pesticide exposure and prolonged culture.

Finally, to compare chronic pesticides exposure and long culture MSCs for immunomodulatory properties, human PBMCs were preactivated with OKT3 and incubated with supernatants of MSCs (P4) cultivated 21 days with or without pesticide mixture. As expected, MSC supernatants repress $\text{INF}\gamma$ secretion by PBMCs, and pesticides inhibit this capacity except for hNDI doses without inducing a decrease in their proliferation (Supporting Information Fig. S1). This effect is similar to MSC-P14 supernatant (Fig. 7).

DISCUSSION

The self-renewal ability of stem cells is known to decline with advancing age [33]. Increasing evidence suggests that dysregulated formation of reactive oxygen species may conduct stem cells into premature senescence.

We have previously demonstrated that pesticide mixture of chemicals commonly found in food induce selective modifications on MSCs, decreasing proliferation and inducing partial

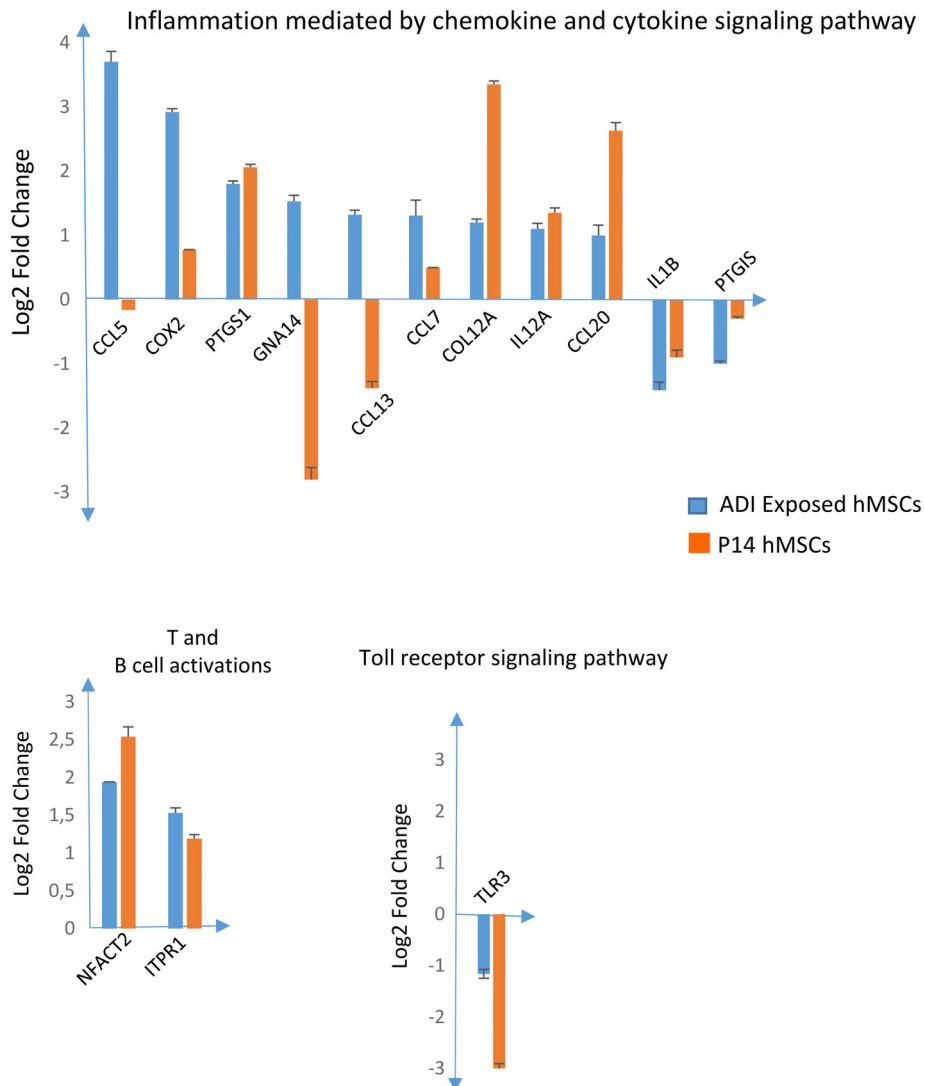


Figure 5. Panther pathway analysis revealed similar modulations for pesticide exposed mesenchymal stem cells (MSCs) and MSC aging. Panther pathway analysis of the differentially expressed genes, among the exposed MSCs, long-term culture MSC-P14, and the MSCs control P4 (Ctrl), reveals similar modulations in both conditions, showing immune activation and a proinflammatory profile. Blue bars represent log₂ fold-change gene expression for pesticides exposed MSCs versus control and orange dots MSC-P14 log₂ fold-change gene expression versus control MSCs P4 (Ctrl).

senescence without apoptosis or autophagy, metabolic dysfunctions, and adipogenic switch when differentiated [6]. These elements suggested a premature cellular aging profile. In order to explore pesticides-induced aging profile hypothesis, we realized RNA sequencing analysis comparing 21-days pesticide mixture-exposed MSCs, long culture MSCs (MSC-P14), and MSCs from aged donor (72 years old). Similarities were found with more than 200 modified common genes. The STRING database was used, and protein–protein interaction networks for upregulated and downregulated genes were visualized for common genes and selectively with pesticide mixture exposition. At the same time, cluster of upregulated genes implicated for stress resistance and survey was found. Most of them connected to the insulin/IGF-1 signaling pathway, which plays a key role in the coordination of growth, differentiation, and metabolism in response to modification of environmental conditions and nutrient availability [34]. Moreover, a link was established between IGF signaling and resistance for the oxidative stress [35]. An upregulation of

genes connected to pathways IGF-1/IGF-1R can stimulate the process of physiological aging. Associated Leptin gene upregulation could promote MSCs senescence through activation of the phosphatidylinositol 3-kinase/Akt pathway [36] and take part in cell cycle arrest and senescence by activating the p53/p21 pathway and inhibiting the Sirt pathway [37]. In contrast, an important cluster of downregulated gene was observed under three conditions, corresponding to a decrease in proliferation in agreement with our previous observation [6] and associated with cellular aging process. Cellular senescence contributes to aging and similar mRNA amplification encoding SASP or cellular and physiological aging markers profile was observed between MSCs from aging donor, MSC-P14, and exposed pesticides cells.

Because links between metabolism and phenotypes associated with senescent cells and aging were established [23], we investigated whether pesticide exposure induces a metabolic profile similar to that after long-term culture of MSCs (MSC-P14). MDH1 encodes an enzyme that catalyzes the NAD⁺/NADH-dependent

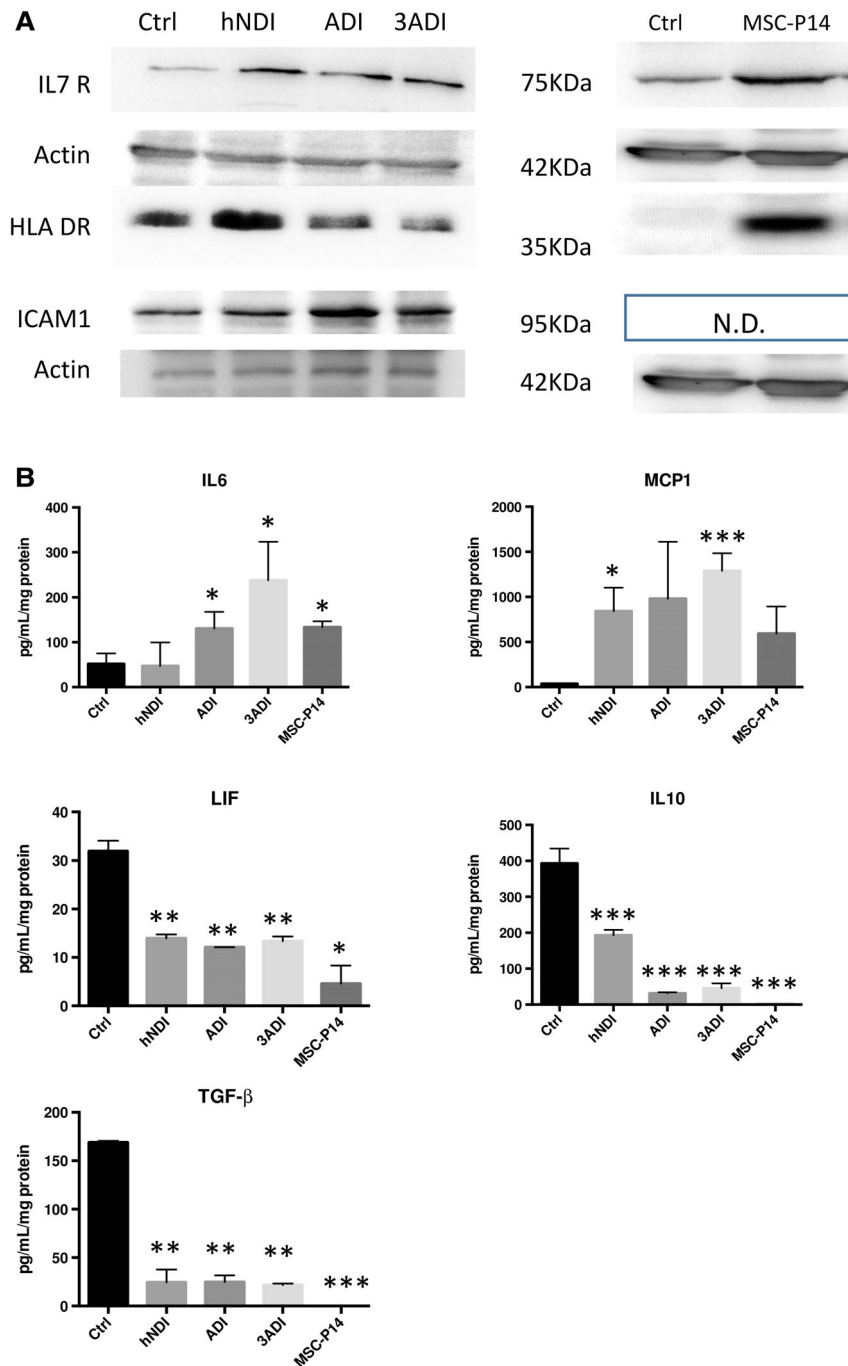


Figure 6. Pesticide mixture altered immunological properties of mesenchymal stem cells (MSCs) and produced proinflammatory secretome. **(A):** Twenty-one days’ exposed control MSC-P4 (Ctrl) cells were evaluated for interleukin IL-7R, HLA-DR, and intercellular adhesion molecule-1 protein expression compared with long culture MSC-P14. **(B):** After 21 days’ pesticide mixture exposure, cell culture medium with serum was changed for 48 hours and supernatants obtained were evaluated by ELISA for IL-6, IL-10, monocyte chemoattractant protein-1, leukemia inhibitor factor, and transforming growth factor-β expression ($n = 3$; *, $p < .05$; **, $p < .01$; ***, $p < .001$).

reversible oxidation of malate to oxaloacetate in many metabolic pathways. MDH1, overexpression, extends life span and its levels and activity decline in senescent cells [38]. Pesticide mixture decreases MDH1 expression in a dose-dependent manner as in MSC-P14 cells. We further confirmed these metabolic elements for a premature metabolic cellular aging induced by pesticide exposure with a SIRT3 modulation. Indeed, SIRT3, a NAD⁺-dependent protein deacetylase, is reported to affect human life

span [39]. SIRT3-knockout mice are prone to age-related disorders [40] and SIRT3 partially abrogates p53 activity with growth arrest and senescence [41]. Low NAD⁺/NADH ratios promote cellular senescence. A significant decrease in NAD⁺/NADH ratio occurs with pesticide treatment only for hNDI and ADI doses. In our previous paper, we showed that MSCs treated with 3ADI had a higher mitochondrial ATP production and basal respiration due to aerobic OXPHOS. These cells upregulated their ATP synthase

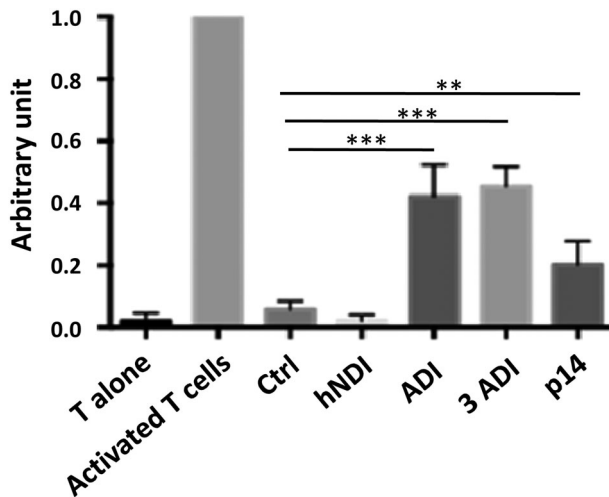


Figure 7. Prolonged exposure to pesticide mixture induces alteration of immunomodulatory mesenchymal stem cell (MSC) properties. Modification of immunomodulatory properties of hMCS: CD3-stimulated peripheral blood mononuclear cells (PBMCs) were preincubated with MSCs supernatants of control MSCs, MSC-P14, and pesticides exposed (hNDI, ADI, 3ADI) MSCs. Concentrations of the stimulatory/proinflammatory cytokines IFN γ -induced production were quantified by ELISA. Results are expressed in arbitrary units considering activated T cells IFN γ production as the maximal response, other concentrations are expressed as a fraction of this maximum response. Results represented means of several experiments ($n = 3$; *, $p < .05$; **, $p < .01$; ***, $p < .001$) with three different PBMCs donors.

and showed a clear exhaustion of their mitochondrial spare metabolic capacity. This may suggest that perhaps, with such high pesticide doses, the NAD/NADH ratio seems to uncouple from SIRT3 and GOT1 downregulation and either associates with changes in other NAD-consuming mediators or show a more stochastic fluctuations with a nonlinear profile compared with lower doses [42, 43]. Sensibly, NAD⁺/NADH ratio was also decreased after pesticide exposure as for aging cells MSC-P14. PGs are derived from arachidonic acid through the activity of two PGH synthases and cyclooxygenases [44]. PGF_{2 α} synthesis can be catalyzed by enzymes of the aldo-keto reductase (AKRs) family [45] and AKR1B1 expression is increased by a proinflammatory stimuli [46] explaining observed modifications with pesticides and MSC-P14.

Other hallmarks of MSCs aging profile, with alteration of immunomodulatory properties, were associated with SASP. The presence of an inflammatory environment has the ability to drastically alter the immunosuppressive potential of MSCs [47].

ICAM-1 and HLA-DR, major histocompatibility complex class II protein levels [48] were upregulated by inflammatory cytokines in MSCs and rendered them more adhesive to T cells. Cell–cell adhesion mediated by ICAM-1 is important for T-cell activation and leukocyte recruitment [31]. ICAM-1 expression was induced in a dose-dependent manner by pesticides exposure. However, it was not possible to evaluate ICAM-1 MSC-P14 cells expression with several antibody experiences. However, RNAseq revealed an increased ICAM-1 mRNA expression compared with control MSCs (Fig. 3B, lower panel).

IL7R is important for the body's innate and adaptive immune responses and plays a role in regulating development, differentiation, and survival of T cells [49] and constitutes element for

proinflammatory IL7 signaling. IL7R expression was induced by pesticide mixture exposure in the same way as prolonged culture MSC-P14.

Immunomodulation by MSCs is mediated by cell–cell contact and the release of soluble factors. We studied pesticide mixture impact on MSCs secretome. Some modifications (IL10, IL-6) were similar to cytokine plasma level observed by Li et al. in senescence-accelerated mice that show aging-related diseases [50]. Despite the dual nature of IL-6, it is commonly described as a proinflammatory cytokine that stimulates T cells [51].

As for MSC-P14, pesticide mixture induces MCP-1 production which is a key chemokine regulating the recruitment and migration of cells of the monocyte–macrophage system [52]. The MCP-1 expression level in aged MSCs is increased [53]. In the same way, a decrease in IL-10 and TGF- β in MSCs exposed to pesticides demonstrates the loss of repressing the immune system because of these pollutants. IL-10 suppresses macrophage and neutrophil functions [54] and inhibits the Th1 immune response [55]. TGF- β regulates multiple fundamental cellular functions and biological processes including the immune response [56]. LIF, decreased by mixture, can act as a mediator of immune modulation and a positive correlation between LIF and human leukocyte antigen (HLA-G) gene expression by MSCs was found [57].

To demonstrate the alteration of immunomodulatory properties of MSCs, we performed a functional *in vitro* test. MSCs can modulate several T-cell functions and exert a profound immunosuppressive effect [58]. MSCs appeared capable of inhibiting antigen-specific T-cell activation by impairing the production of IFN γ [59]. Furthermore, the exposure of PBMCs to our MSCs supernatants functionally confirmed, in a dose-dependent manner, a modification in PBMCs capacity to produce interferon.

Pesticide mixture induces common activation pathways with aging process although differences between aging donor and pesticide-exposed MSCs exist in terms of gene expression as shown by RNAseq. In part, this is possibly due to the accelerated induction of MSCs senescence and aging through pesticide exposure within 21-days in culture. It is also probable that the microenvironment of MSCs during physiological aging also affects the gene expression profile in a way that is different from any *in vitro* conditions.

CONCLUSION

Our results show common characteristics of an aged tissue induced by exposure to a mixture of pesticides, long culture and to some extent MSCs from aging donor, suggesting that accelerated cellular aging can be induced by noncytotoxic doses of combined pesticides. Our results suggest that, through their MSCs premature aging effect, might participate in the acceleration of pathologies such as obesity, degenerative diseases, and cancers. In the latter case, these modifications can directly influence the fate of numerous dysplastic tissues by affecting the tumoral niche, in particular through the modifications of MSCs immunomodulatory properties.

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AUTHOR CONTRIBUTIONS

X.L., F.G., C.G., C.G.: collection and/or assembly of data; M.H.: collection and/or assembly of data, manuscript writing, data analysis and interpretation; S.D.: data analysis and interpretation; L.O.: collection and/or assembly of data, manuscript writing;

V.T.: conception and design, provision of study material or patients; P.L., O.H.: provision of study material or patients; D.H.: conception and design, final approval of manuscript; F.M.V., C.O.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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