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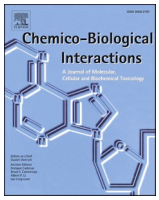

Research paper

Mechanisms of action of mineral fibres in a placental syncytiotrophoblast model: An *in vitro* toxicology study

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ABSTRACT

Asbestos has been widely used due to its unique characteristics. It is known that exposure to asbestos causes serious damage to health but one species, chrysolite, is still used because it is considered less toxic and not biopersistent in some countries. The aim of our study was to investigate if cellular process underlying the proliferation, differentiation and cell death of placental tissues could be modify in presence of asbestos fibres (50 μg/ml final concentration), long chrysolite fibres (CHR-L) and short chrysolite fibres (CHR-S), using BeWo cell line, an *in vitro* model that mimics the syncytiotrophoblast (STB), the outer layer of placental villi. Our data demonstrated that none of the fibres analysed alter syncytiotrophoblast formation but all of them induce ROS formation and reduced cell proliferation. Moreover, we showed that only CHR-L fibre induced was able to induce irreversible DNA alterations that carried cells to apoptosis. In fact, BeWo cells exposed to CHR-L fibre showed a significant increase in cleaved CASP3 protein, a marker of apoptosis. These data suggest that CHR-L may induce death of the placental villi leading to impaired placental development. The impairment of placental development is the basis of many gestational pathologies such as preeclampsia and intrauterine growth retardation. Since these pathologies are very dangerous for foetal and maternal life, we suggest to the gynaecologists to carefully evaluate the area of maternal residence, the working environment, the food used, and the materials used daily to avoid contact with these fibres as much as possible.

1. Introduction

The family of asbestos minerals includes chrysotile (a layer silicate of the serpentine group) and five chain silicates amphibole species: actinolite asbestos, amosite, anthophyllite asbestos, crocidolite and tremolite asbestos [1]. Asbestos has been practically used everywhere because of its unique physical-chemical and technological properties. It is now well established that exposure to asbestos minerals causes adverse health effects leading to ban the use of amphibole asbestos minerals worldwide (with rare exceptions) whereas chrysotile is still mined in many countries like China, India and Russian Federation where its "safe use" is allowed [2]. In these countries, it was assumed that chrysotile fibres are less toxic than amphibole fibres because they are not biopersistent. In fact, chrysotile fibres which enter in alveolar space are internalized by alveolar macrophages where they are dissolved and reduced in shorter fibres easier to be cleared from the lung, however amorphous silica-rich products with probable toxicity are obtained [3].

The 68 % of the extracted asbestos comes from Urals Mountains, in particular commercial Russian chrysotile extracted by Orenburg minerals (Yasny-Russia) was extensively characterized and found to resemble the Italian chrysotile (Balangero, Turin, Italy) [4]. These minerals are classified by the World Health Organization and the

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International Agency for Research on Cancer (IARC) as carcinogens to humans, which can cause lung cancer, malignant mesothelioma of the pleura and peritoneum [5,6]. It is generally accepted that the physical-chemical characteristics of mineral fibres and morphometric parameters (e.g., length, width, aspect ratio) play a key role in carcinogenesis [7]. The toxicity of asbestos is mainly characterized by the production of reactive oxygen and reactive nitrogen species (ROS and RNS) which can cause various types of DNA damages. ROS can be produced through Fenton-type reaction catalysed by iron impurities [8]. Currently, it was affirmed that asbestos deposit generate inflammation [9] and that asbestos exposure can occur not only through inhalation but also with ingestion or dermal contact. It was reported that asbestos fibres could cross the stomach and intestine and circulate in various body districts [10].

Human placenta is an important transitory organ playing a key role in normal fetal development ensuring oxygen and nutrient support to the fetus. Thus, an impairment of placentation process can lead to placental and fetal diseases [11–14].

Some studies carried out in pregnant women have revealed the presence of asbestos fibres in the human placenta, affirming that these fibres could cross the maternal-fetal barrier and reach the fetus [15]. In particular, Haque and colleagues demonstrated the presence of asbestos fibres in some organs including placenta of stillborn infants [16]. Further studies have shown a significant relation between asbestos exposure and gestational trophoblastic disease (GTD) such as hydatidiform moles or choriocarcinoma [17], confirming that these fibres could affect the proper placenta development. The aim of our study was to investigate the toxicity of asbestos fibres in a syncytiotrophoblast *in vitro* model to understand if the contact of these fibres with these cells could modify the cellular process underlying the proliferation, differentiation, and cell death that are key processes of placental development. For this purpose, we used BeWo cell line that mimics the syncytiotrophoblast, the outer layer of placental villi. This layer is in contact with the maternal blood that could carry asbestos fibres altering placental cell homeostasis.

2. Materials and methods

2.1. Mineral fibres

The following mineral fibres were selected for the study:

(i) chrysotile fibres were separated from the commercial produced of Orenburg Minerals mine near Yasniy (in Russian "Ясный" = clear), in the Orenburg region, Ural Mountains (Russia). The chrysotile fibres were identified as clinochrysotile with minor ortho-chrysotile, displaying the mean chemical formula $(Mg_{2.870}Fe^{2+}_{0.027}Al_{0.034}Cr_{0.005}Ni_{0.006})_{2.986}(OH)_4Si_{1.92}O_5$ [18]. The full crystal-chemical characterization of the sample can be found in Ref. [18]. A selection of the fibres under the optical microscope was accomplished to obtain high purity batches for the *in vitro* tests. Cryogenic milling of the chrysotile fibres for the size separation was possible using a Retsch mixer mill MM 400 (Düsseldorf, Germany). A description of the procedure is found in Ref. [19]. Short chrysotile fibres (CHR-S) (95 % L < 5 μm) were obtained by cryogenic milling for 40 min while a milling time of 5 min was sufficient to yield a sample mainly composed of long chrysotile fibres (CHR-L) (90 % L > 5 μm). For the short (L < 5 μm) chrysotile fibres (CHR-S), we measured a mean L of 1.91 (±0.21) μm and a mean W of 0.15 (±0.07) μm while for the fraction of long (>5 μm) chrysotile fibres (CHR-L), we measured a mean L of 29.80 (±3.08) μm and a mean W of 0.4 (±0.1) μm [19] while

(ii) crocidolite has been selected as positive carcinogenic standard fibre as it is classified by the International Agency for Research on Cancer (IARC) in Group 1 "Carcinogen for humans" [1]. The used UICC standard crocidolite (South African, NB #4173-111-3) has mean chemical formula of $(Na_{1.96}Ca_{0.03}K_{0.01})_2(Fe^{2+}_{2.34}Fe^{3+}_{2.05}Mg_{0.52})_{4.91}(Si_{7.84}Al_{0.02})_{7.86}O_{21.4}(OH)_{2.64}$. The sample contains minor (<1 wt%) impurities of hematite, magnetite, and quartz. The measured mean L and W of the fibres are 18 (±1) μm and 0.35(±0.1) μm, respectively.

(iii) fibrous wollastonite has been selected as negative carcinogenic standard. The used NYAD G wollastonite mainly contains wollastonite-1A and minor calcite. It has a mean chemical formula of $Ca_{0.997}Fe^{2+}_{0.0053}Fe^{3+}_{0.0024}Mn_{0.0025}Mg_{0.0009}Si_{0.9786}O_3$, a measured mean L and W of the fibres of 46.6 μm and 3.74 μm, respectively [20].

The length and width of the chrysotile fibres was determined by analysing a series of SEM images by using ImageJ software as previously described [19].

2.2. Cell culture and treatments

Human BeWo cell line (American Type Culture Collection, Rockville, MD, USA) was used as a model of syncytiotrophoblast, the outer layer of fetal-maternal barrier in contact with the maternal blood in the placenta, to evaluate biological effects of the mineral fibres listed below. These cells were seeded in triplicate at 10^5 cells/well in 6-well plates and cultured in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, MA, USA) supplemented with 10 % foetal bovine serum (FBS; Gibco) and 100 U/ml penicillin and streptomycin (Gibco) in a humidified incubator at 37 °C and 5 % CO_2. The medium was changed every 2 days and cells were split 1:4 every 3 days. To evaluate the biological responses of BeWo cells in contact with mineral fibres, these cells were seed at confluence and the different mineral fibres (50 μg/ml final concentration) were added to each well, and the plates were incubated at 37 °C for the following times: 30 min, 2 h, 4 h, 8 h, 24 h and 48 h. The 50 μg/ml concentration was chosen to compare the cytotoxic action of the different mineral fibres because it produced well-defined cell responses. In addition, this concentration was chosen because of its occupational significance [21,22]. Control cells were grown in culture medium with no treatments.

Syncytialization was induced in BeWo cells using Forskolin 50 μM (sc-3562, Santa Cruz Biotechnology) for 48 h. E-Cadherin was used to validate syncytialization protocol [23].

2.3. MTT assay

To assess the cytotoxic effect of the exposure to the asbestos fibres on BeWo cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was evaluated. For MTT assay these cells were seeded into 96-well microplate and treated as described above. After treatments, the medium was removed and 100 μl MTT solution (Sigma, St. Louis, USA. 5 mg/ml in Phosphate Saline Buffer (PBS)) were added to cells and incubated at 37 °C for 3 h. After discarding supernatants, the formazan crystals were dissolved in 200 μl of solvent (DMSO). The amount of formazan crystals is directly related to the number of viable cells. The optical density was measured at 570 nm (reference filter, 690 nm) using a plate reader (Absorbance microplate reader AMR-100). Data were expressed as relative cell viability using the following equation: relative viability (%) = Absorbance (Treatment)/Absorbance (Vehicle Control) × 100.

2.4. Intracellular ROS levels

Intracellular ROS levels were detected by flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) as probe in BeWo cells in presence of asbestos fibres for 8 h and 24 h. Cells were trypsinized, washed twice with cold PBS, and suspended at a final concentration of 0.5 × 10^6 cell/mL in prewarmed PBS containing 10 μM probe.

After incubation for 30 min in the dark at 37 °C, cells were washed twice in PBS and stained with 10 μg/mL propidium iodide (PI). Fluorescence of labelled cells was measured on Guava easy Cyteflow cytometer (Merck Millipore, Darmstadt, Germany) using an excitation wavelength of 488 nm. Emissions were recorded using the green channel for carboxy-DCF and the red channel for propidium iodide. The cells permeable to PI were excluded from the cell population considered for the ROS production to avoid false negatives. The data acquired were analysed by the FCS Express Program (De Novo Software, CA, USA).

2.5. Protein extracts and western blotting

For Western blotting analysis, BeWo were seeded in 6-well microplates in presence of asbestos fibres for 24 h. Then, the cells were lysed using a lysis buffer consisting of 0.1 M PBS, 0.1 % (w/v) SDS, 1 % (w/w) NONIDET-P40, 1 mM (w/v) Na orthovanadate, 1 mM (w/w) phenyl methane sulfonyl fluoride (PMSF), 12 mM (w/v) Na deoxycholate and 1.7 μg/ml aprotinin, pH 7.5 than centrifuged at 10,000 g for 5 min at 4 °C, aliquoted and stored at −80 °C until use. Protein concentrations were determined by a Bradford protein assay (Bio-Rad Laboratories, Milano, Italy). All protein samples were fractionated on 10 % SDS-polyacrylamide gels (SDS-PAGE), electrophoretically transferred (Trans-Blot® Turbo™ Transfer System; Bio-Rad Laboratories) to nitrocellulose membranes and subjected to Western blot analysis. Non-specific protein binding was blocked with EveryBlot Blocking Buffer (Bio-Rad Laboratories) for 5 min. Blots were incubated with the primary antibodies listed in Table 1. After washing with tris-buffered saline TBS/0.05 % Tween 20 (TBS-T), blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) diluted 1:10,000 in TBS-T for 30 min. Bound antibodies were detected with Clarity Western ECL Substrate (Bio-Rad Laboratories). Images were acquired with Chemidoc (Bio-Rad Laboratories). Bands were densitometrically analysed using ImageJ software (National Institutes of Health; https://imagej.nih.gov/ij/download.html).

2.6. Immunofluorescence analysis

BeWo cells in the presence of asbestos fibres for 24 h were washed in Dulbecco's PBS (Euroclone), fixed in 4 % paraformaldehyde in PBS for 10 min at 4 °C and permeabilized in 0.1 M PBS added with 0.1 % Triton X-100 (Sigma, Milano, Italy) for 5 min. After washing in PBS at RT, cells were blocked with Animal-Free Blocker (Vector Laboratories, Burlingame, CA) 1:5 in PBS and incubated overnight at 4 °C with primary antibody (Table 1). Cells were then washed 3 times in PBS and incubated with Alexa Fluor 488-conjugated donkey anti-rabbit and Alexa Fluor 555-conjugated donkey anti-mouse IgG secondary antibody (both from Thermo Fisher Scientific) for 30 min at RT. The DAPI probe was used for nuclear staining. Finally, the slides were cover-slipped with propyl gallate and evaluated with fluorescence confocal microscopy (Nikon).

Table 1
Primary antibodies used in this study.

Antibody	WB	IF	Company
Rabbit anti-human Phospho-Histone H2A.X (#9718)	1:1000	1:100	Cell Signaling Technology, Danvers, USA
Rabbit anti-human Histone H2A.X (#2595)	1:1000	//	Cell Signaling Technology, Danvers, USA
Mouse anti-human RAD51 (#ab88572)	1:1000	1:100	Abcam, Cambridge, UK
Mouse anti-human E-cadherin (#sc-8426)	1:500		Santa Cruz Biotechnology, Inc. Dallas, US
Rabbit anti-human Caspase-3 (#9662)	1:100		Cell Signaling Technology, Danvers, USA
Rabbit anti-human Cleaved-Caspase-3 (#9662)	1:1000		Cell Signaling Technology, Danvers, USA
Rabbit anti-human Cyclin D1 (#55506)	1:1000		Cell Signaling Technology, Danvers, USA
Mouse anti-human β-ACTIN (#sc-47778)	1:250		Santa Cruz Biotechnology, Inc. Dallas, US
Mouse anti-human PCNA (#sc-56)	1:250		Santa Cruz Biotechnology, Inc. Dallas, US
Mouse anti-human GAPDH (#sc-32233)	1:250		Santa Cruz Biotechnology, Inc. Dallas, US

IF: immunofluorescence; WB: Western blotting.

2.7. Micronuclei assay

Micronuclei assay was performed in BeWo cells in the presence of asbestos fibres for 24 h. Cells were washed in Dulbecco's PBS (Euroclone), fixed in 4 % paraformaldehyde in PBS for 10 min at 4 °C and permeabilized in 0.1 M PBS added with 0.1 % Triton X-100 (Sigma, Milano, Italy) for 5 min. After washing in PBS nuclear staining was carried out by using DAPI probe. Micronuclei were counted in four different microscopic non-overlapped fields from each slide and expressed as mean of micronuclei (MNI) ± standard deviation (SD).

2.8. Statistical analysis

Data were analysed using Student's *t*-test and P values < 0.05 were considered statistically significant. All analyses were carried out using GraphPad Prism (ver 8) statistical software.

3. Results

3.1. Crocidolite, CHR-S and CHR-L have a cytotoxic effect in BeWo cell line

BeWo cells in presence of asbestos fibres demonstrated that not all the fibres are cytotoxic at the same time point as demonstrated by MTT assay. As shown in Fig. 1, only CHR-S and CHR-L fibres showed a cytotoxic effect at 30 min while crocidolite showed a cytotoxic effect later, at 2 h. Moreover, cytotoxicity increased with the increasing of fibre contact time up to 48 h. Cells in presence of wollastonite showed no cytotoxic effect at any time point studied. Thus, we can state that crocidolite, CHR-S and CHR-L are the most cytotoxic fibres among the asbestos fibres analysed, unlike wollastonite (the negative standard) which did not show cytotoxic effects.

3.2. Asbestos fibres induce oxidative stress but only crocidolite, CHR-S and CHR-L increase BeWo cell death

Another important toxicity parameter is ROS production. Cytoplasmatic ROS level was measured in the presence of all mineral species in BeWo cells. As shown in Fig. 2A, wollastonite, CHR-S and CHR-L fibres increased cytoplasmatic ROS levels at 8 h. However, ROS production was further increased in presence of wollastonite while crocidolite started to induce ROS production at 24 h. Curiously, the presence of CHR-S and CHR-L in culture medium did not show any significant alteration of cytoplasmatic ROS levels at 24 h unlike at 8 h. We have hypothesized that since these fibres caused cell death before 24 h, (as demonstrated by the MTT assay the BeWo cells start to dead at 30 min), either the probe to detect ROS does not enter the cell or the probe leaves the cell due to its death. In any case, the probe is no longer in the cytoplasm, and it can no longer detect ROS. To verify our hypothesis, we performed PI analysis evaluating cell death percentage in the presence of the mineral fibres at 8 h and 24 h. As shown in Fig. 2B, only CHR-L increased cell death at 8 h while crocidolite and CHR-S induced significant increased cell death at 24 h. Although ROS levels were significantly increased in presence of wollastonite, this did not lead to cell death at any time point analysed.

In conclusion, we can state that the probe to detect ROS is not detectable in the cytoplasm of BeWo cells in contact to CHR-L due to cell death and lysis starting at 8 h.

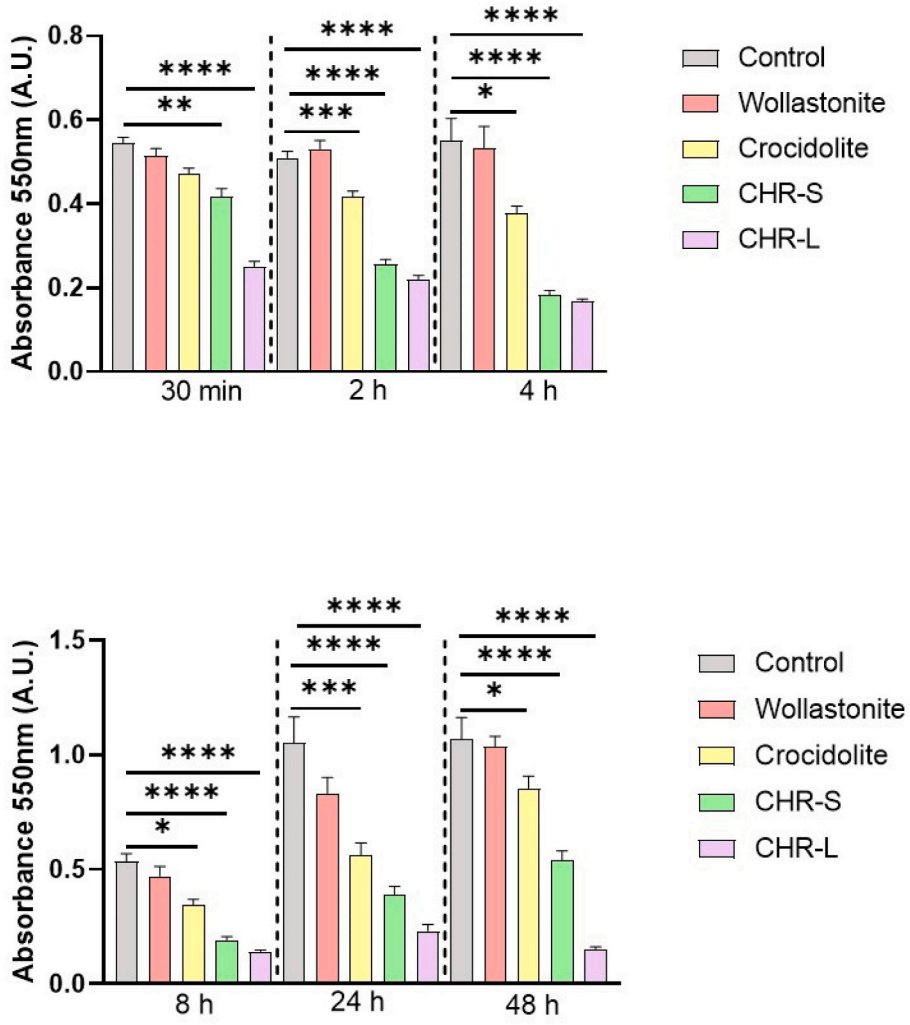


Fig. 1. Histogram representing results of MTT assay (n = 3) of BeWo cells in presence of mineral fibres at increasing times (from 30 min to 48 h). MTT assay shows a significant decrease in BeWo cell viability in presence of CHR-S and CHR-L at 30 min while crocidolite at 2 h. Wollastonite has no cytotoxic effect on BeWo cells. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. CHR-S: russian chrysotile short fibres (<5 μm) CHR-L: russian chrysotile long fibres (>5 μm).

3.3. BeWo cells morphology was not altered in presence of wollastonite, crocidolite, CHR-S and CHR-L fibres

BeWo cells morphology was evaluated after presence of wollastonite, Crocidolite, CHR-S and CHR-L for 24 h. As shown in Fig. 3, all fibres did not modify cell morphology and were detectable in culture medium.

3.4. BeWo cells syncytialization was not altered in presence of wollastonite, crocidolite, CHR-S and CHR-L fibres

BeWo cell line can be used as a model of syncytiotrophoblast if the syncytialization is induced by Forskolin [24]. E-cadherin decrease expression proved that syncytialization has occurred (E-cadherin was used as a marker of syncytialization). As shown in Fig. 4, BeWo cells in the presence of all the asbestos fibres, after syncytialization induced by foskolin (FK), did not alter the syncytializzation process in these cells.

3.5. Micronuclei formation was detected in presence of CHR-L fibres in BeWo cell line

Micronuclei are irregular and smaller nuclei due to acentric chromosomes or inappropriately segregate entire chromosomes generation resulting in genetic damage caused by chemicals or physical agents [25]. We counted the micronuclei formation to evaluate the genotoxic damage generated by the presence of wollastonite, crocidolite, CHR-S and CHR-L in BeWo cells using DAPI probe which binds to nucleic acids. Only the presence of CHR-L showed a statistically significant increase of micronuclei in BeWo cells at 24 h (Fig. 5).

3.6. The presence of asbestos fibres altered cell cycle, damage and repair mechanisms in BeWo cells

DNA damage was investigate in presence of wollastonite, crocidolite, CHR-S and CHR-L in BeWo cells for 24 h by evaluating phosphorylated H2A.X (γH2A.X) expression, a known marker of DNA damage [26]. Moreover, we studied RAD51 protein expression as repair marker of double-stranded DNA breaks (DSBs) under the same conditions [27]. Wollastonite and CHR-S caused a significant decrease of γH2A.X protein expression, while crocidolite did not show any significant variation. In the same condition, wollastonite, CHR-S and crocidolite showed no variation of RAD51 protein. On the contrary, CHR-L fibres showed a significant increase of γH2A.X protein levels and a significant decrease of RAD51 protein (Fig. 6A). These data suggest that CHR-L fibres caused a DNA damage that the cells were not able to repair by RAD51 activity. Moreover, we analysed Cyclin D1 protein, an important regulator of cell cycle [28]. As shown in Fig. 6B, all the fibres lead to a significant decrease of Cyclin D1 protein that could be due to a block on cell cycle at G0/G1 phase. We can note that no Cyclin D1 expression was detected

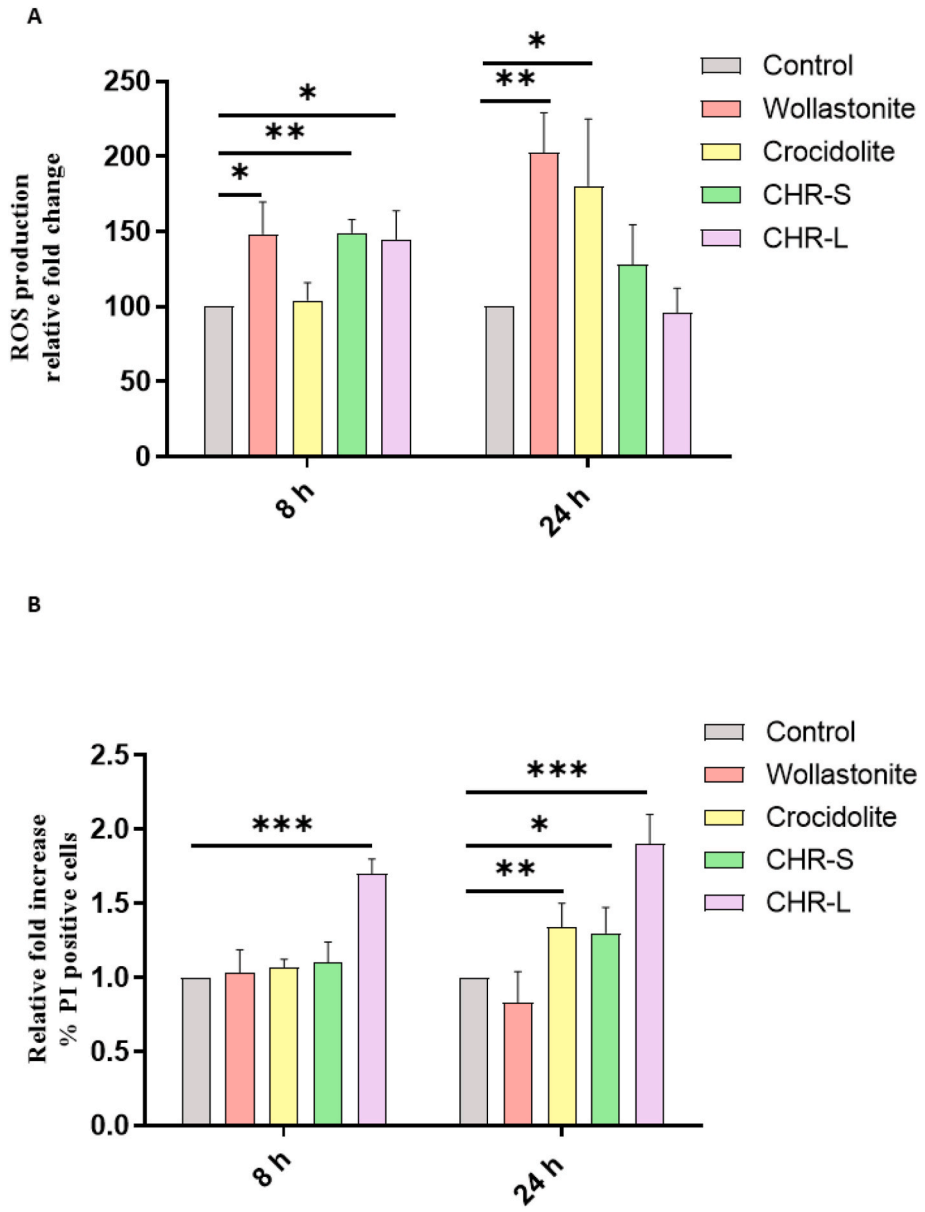


Fig. 2. A) Histogram representing the intracellular ROS levels detected by H2DCF-DA (n = 3) of BeWo cells in presence of asbestos fibres at 8 h and 24 h. ROS levels significantly increase after wollastonite, CHR-S and CHR-L exposure at 8 h while crocidolite induces increase of ROS levels at 24 h. CHR-S and CHR-L shows no significant alteration of ROS levels at 24 h. B) Propidium iodide (PI) nuclear staining of BeWo cells (n = 3) in presence of mineral fibres. CHR-L shows a significant increase of percentage of nuclear positivity at 8 h revealing a significant increase in dead cells. Crocidolite and CHR-S show significant increase of nuclear staining at 24 h *p < 0.05, **p < 0.01, ***p < 0.001. CHR-S: Russian chrysotile short fibres (<5 μm) CHR-L: Russian chrysotile long fibres (>5 μm).

with CHR-L suggesting that this fibre could cause cellular death differently to the other fibres that could resume the cell cycle (Fig. 6B). In addition, we evaluated if γH2A.X protein expression alterations were due to a modulation of total H2A.X expression or to a modulation of its phosphorylation (γH2A.X). Wollastonite, crocidolite, and CHR-S showed a significant decrease of total H2A.X while only CHR-L did not show any effect on total H2A.X. In conclusion, we showed that wollastonite, crocidolite, and CHR-S showed a decrease of γH2A.X due to a decrease of total H2A.X while, only CHR-L caused an increase of γH2A.X that was not due to total H2A.X increase (Fig. 6B). Thus, we can confirm that only CHR-L caused a DNA damage.

3.7. γH2A.X and RAD51 localization in BeWo cells in the presence of the asbestos fibres

As shown in Fig. 7, γH2A.X and RAD51 are present in the nuclei of BeWo cells in all conditions. Thus, the mechanism of DNA damage/repair is active in the nuclei of these cells. The previously reported western blotting data (Fig. 6A) demonstrated a higher expression of γH2A.X and a low expression of RAD51 in CHR-L compared to the other fibres.

3.8. Proliferation and apoptosis in BeWo cells in presence of asbestos fibres

To evaluate if wollastonite, crocidolite, CHR-S and CHR-L fibres could alter cell proliferation and apoptosis in BeWo cell line, we performed western blotting analysis to investigated PCNA, caspase-3 and its cleaved form, respectively. As reported in Fig. 8A, PCNA expression was significantly lower in all conditions compared to the control. However, only CHR-L fibre (Fig. 8B) led to a significant increase in cleaved caspase-3, suggesting that this fibre can stimulate programmed cell

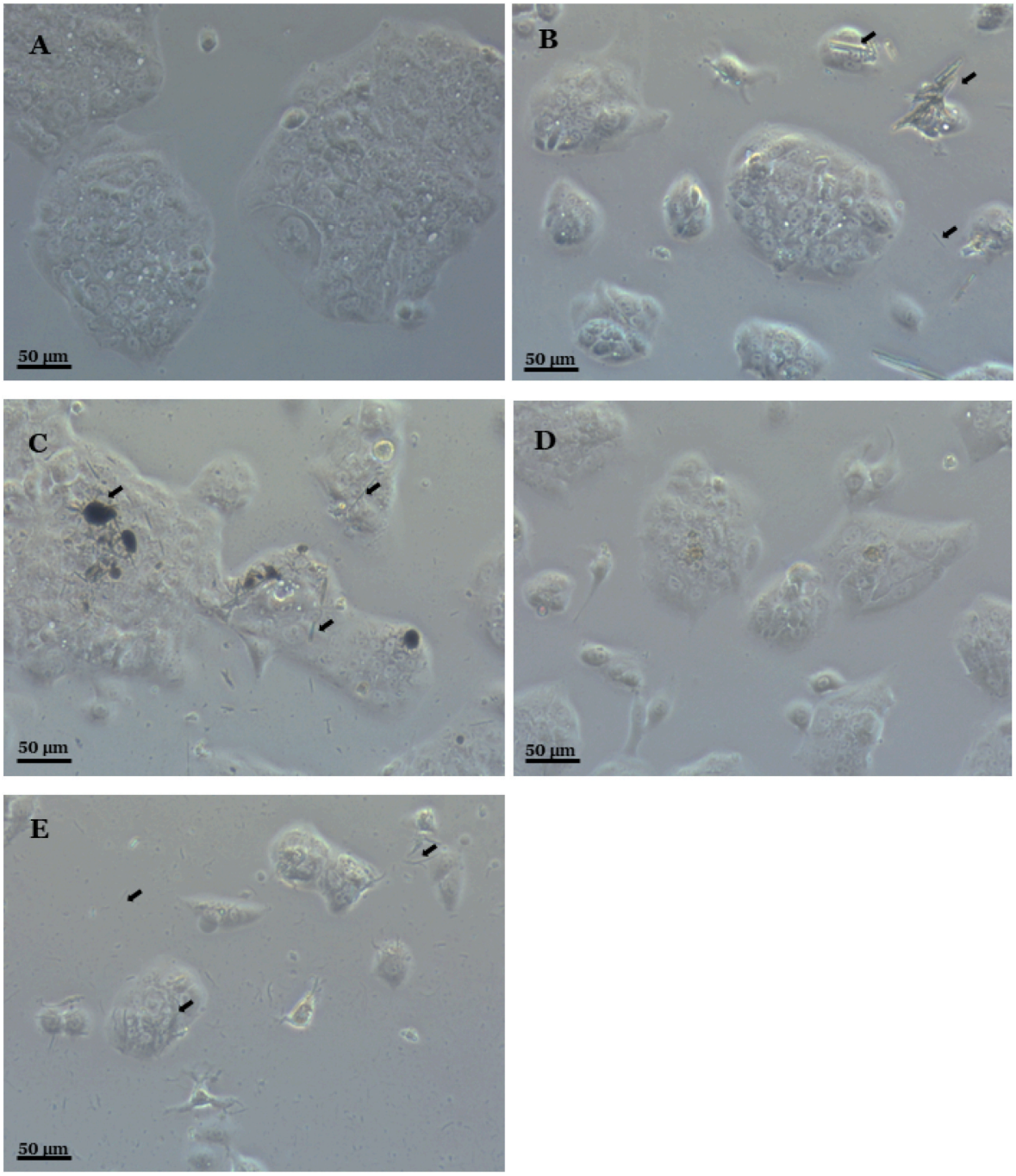


Fig. 3. Morphology of BeWo cells in presence of mineral fibres. A) no fibres, B) wollastonite, C) crocidolite, D) CHR-S and E) CHR-L. No evident differences in cell morphology are present in Bewo cells in presence of mineral fibres compared to control (untreated cells) (n = 3). CHR-S: russian chrysotile short fibres (<5 μm) CHR-L: russian chrysotile long fibres (>5 μm).

death in BeWo cells.

4. Discussion

It is suggested that the risk of chronic non-communicable diseases in adulthood is influenced by altered environment during the phase of embryonic development [29,30]. Some environmental agents enter the umbilical cord blood and amniotic fluid through the placenta and can affect normal foetal development and growth [29,31]. The placenta represents the only route of communication between the fetus and the outside environment, and it is known that asbestos fibres can cross the placenta [15,16]. Generally, it is reported that asbestos increases the risks of systemic autoimmune and cardiovascular diseases; causes not only pulmonary cancer but it is also involved in larynx, ovary, colorectum, pharynx and stomach cancer onset. Moreover, it is associated with increased inflammatory response, oxidative stress, fibrosis and histological alterations [32–35]. Particularly, it has been reported that asbestos fibres can increase ROS production [36,37]. In pregnancy, oxidative stress has been associated to an impaired trophoblast development and it is involved in many pregnancy complications such as preeclampsia [38], preterm delivery [39] and gestational diabetes mellitus [40]. We showed that wollastonite, CHR-S and CHR-L fibres significantly increased ROS formation in our cell model proving an important role of these fibres in inducing oxidative stress in BeWo

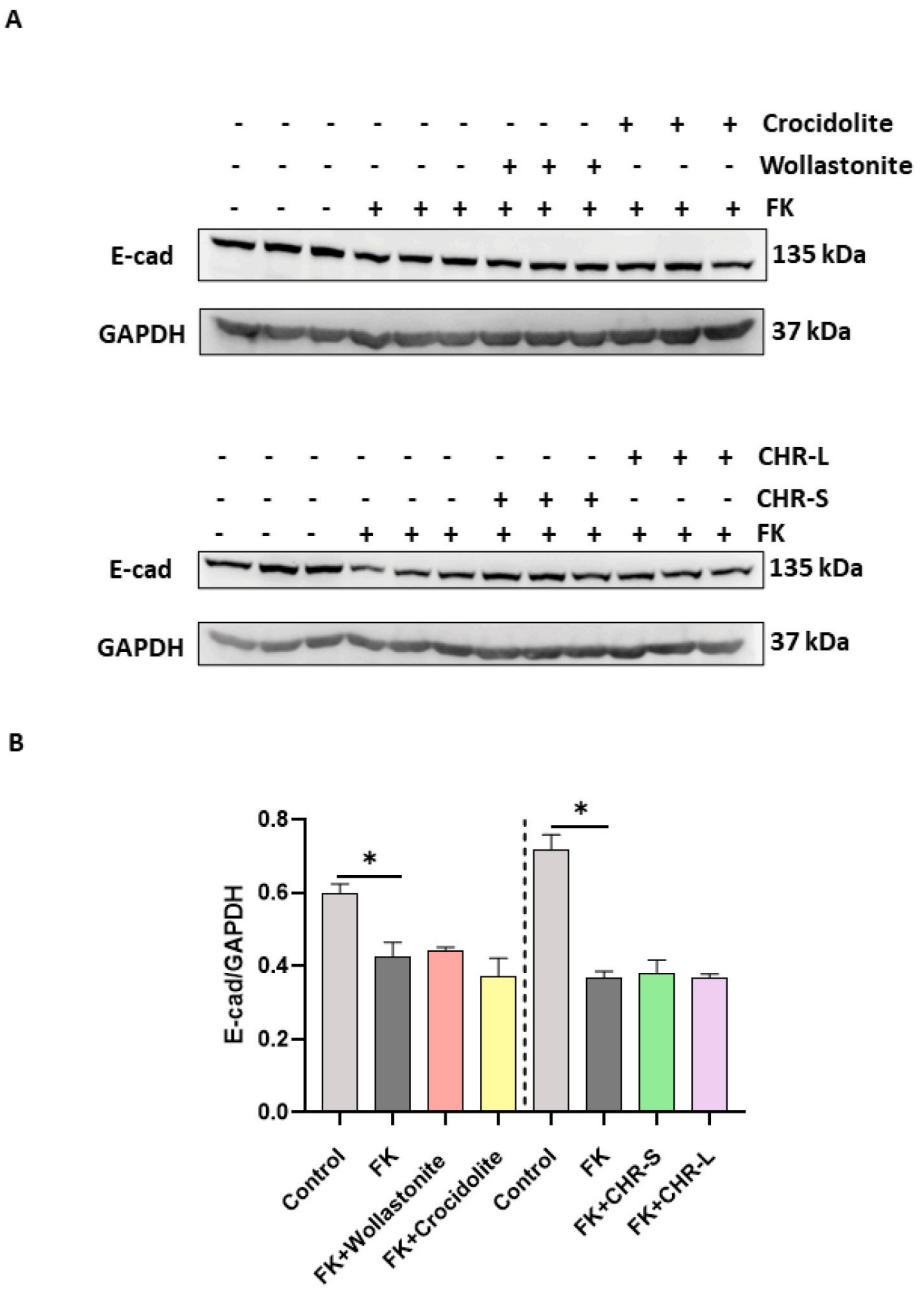


Fig. 4. E-cadherin expression in BeWo cells after treatment with foskolin (FK). A) Western blotting analysis (n = 3). B) Histograms representing densitometric analysis of western blotting. The presence of all the mineral fibres did not alter the syncytializzation. *p < 0.05.

placental cell line suggesting that these fibres can induce oxidative stress altering normal placental development. In addition, we demonstrated that only CHR-L showed a fast significant increase in the percentage of dead cells under oxidative stress conditions while crocidolite and CHR-S increase cell death later. Moreover, we showed a decrease of Cyclin D1 expression in BeWo cells in presence of all asbestos fibres but highly significant in presence of CHR-L fibres suggesting that these fibres can influence placenta development leading to abnormal placentation causing pregnancy complications. In fact, cyclin D1 is a key protein that regulates the G1/S transition [41]. Interestingly, during normal pregnancy, trophoblast cells express cyclin D1 protein suggesting that this protein plays a key role in normal development and function of the human placenta. It was demonstrated that in pregnancy complicated by preeclampsia or in foetal growth restriction, there is a decrease of cyclin D1 in placenta tissues indicating a reduction of proliferation and migration of trophoblast cells [42,43]. Interestingly, all fibres showed a significant decrease in cell proliferation (by PCNA expression), but only CHR-L fibre induced DNA alterations that carried cells to apoptosis. In fact, our data showed that BeWo cells only in presence of CHR-L fibre showed a significant increase in cleaved CASP3 protein, a marker of apoptosis. It is known that CHR-L induces ROS production due to its physical-chemical characteristics [44]. Thus, our data are in agreement with the data reported in literature showing that CHR length (>5 μm) plays a key role in asbestos-related diseases onset [45]. The only difference between CHR-S and CHR-L is the length since the width and impurities are the same. The toxicity associated to the CHR length may be probably due to a greater surface area in contact with the cell membrane causing a greater exposure of the cell to the ROS produced by the fibre. So, we can conclude that fibre length is a critical parameter for cell survival. In fact, the damage caused by the short fibres can be repaired while that one caused by the long fibres is too high to be repaired leading the cell to apoptosis.

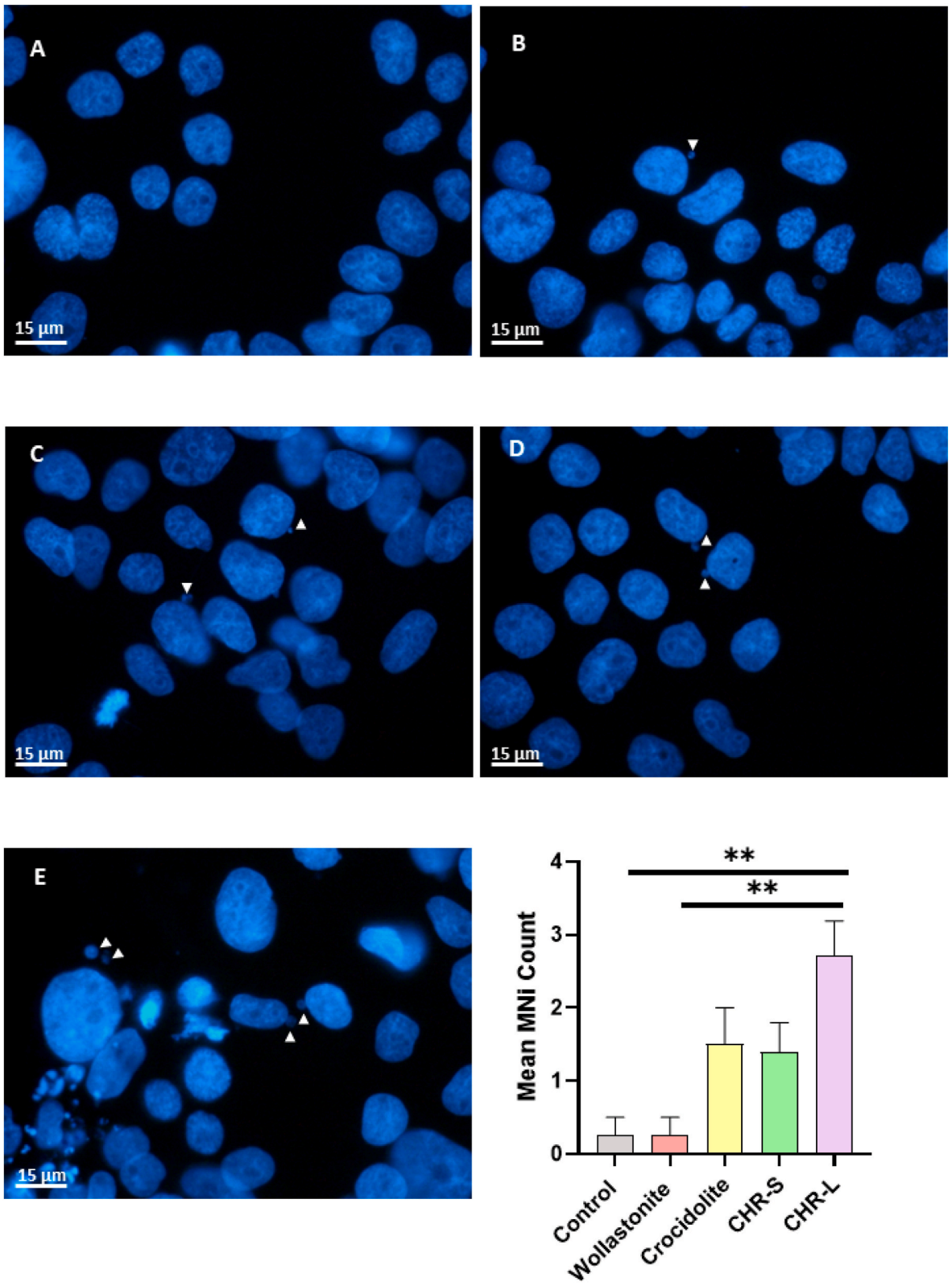


Fig. 5. Micronuclei count. A) no fibres, B) wollastonite, C) crocidolite, D) CHR-S and E) CHR-L. A significant increase of micronuclei (arrowheads) is present in BeWo cells in presence of CHR-L compared both to control and wollastonite (n = 3). **p < 0.01. CHR-S: Russian chrysotile short fibres (<5 μm) CHR-L: Russian chrysotile long fibres (>5 μm).

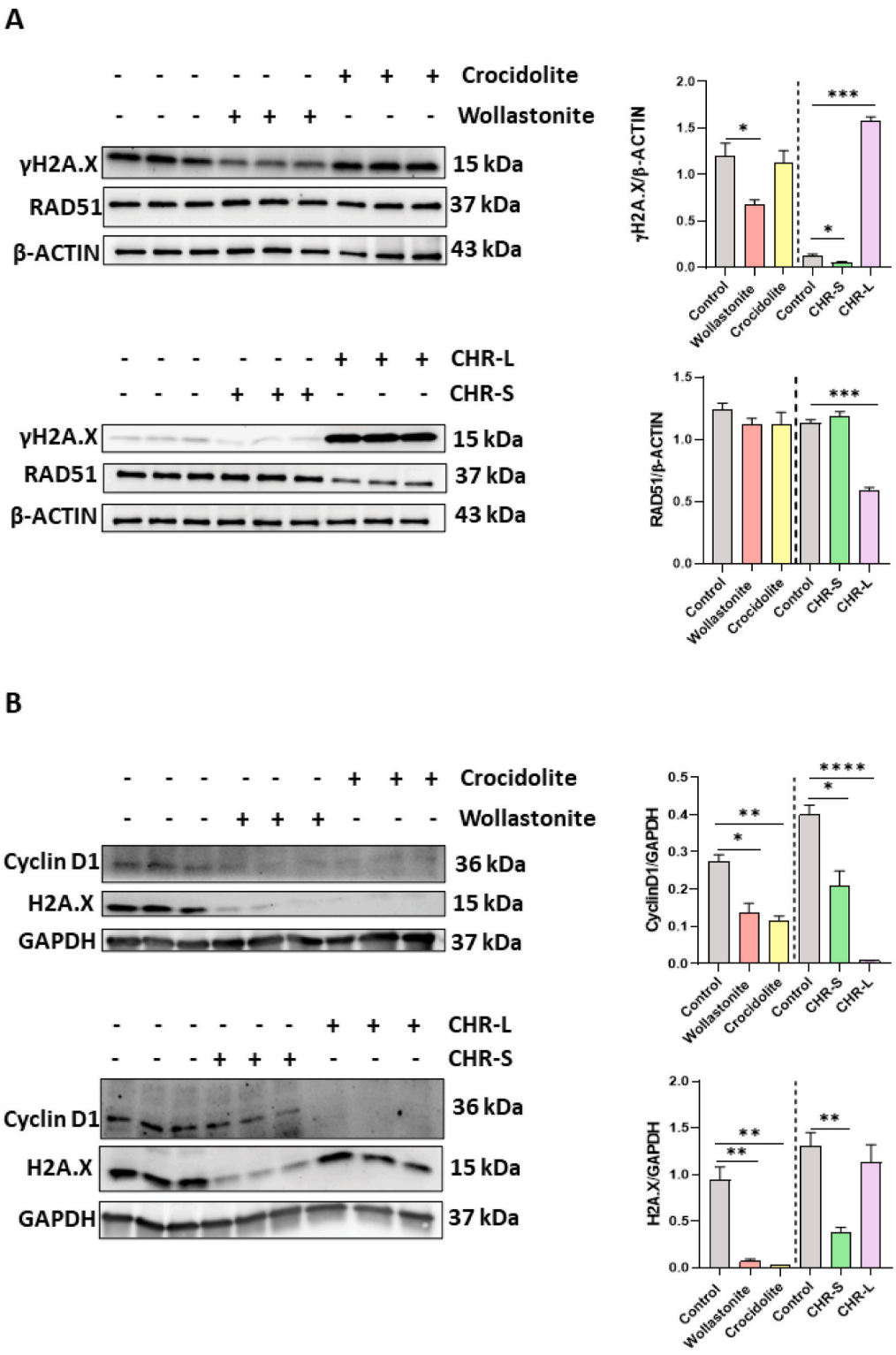


Fig. 6. A) Western blotting of γH2A.X and RAD51 in presence of mineral fibres (n = 3). γH2A.X expression is decreased in BeWo cells in presence of wollastonite and CHR-S. No differences are present with crocidolite. CHR-L significantly increased γH2A.X expression. RAD51 expression is significantly decreased only in presence of CHR-L. B) Western blotting of Cyclin D1 and H2A.X in presence of mineral fibres (n = 3). Cyclin D1 is significantly decreased in presence of all mineral fibres. H2A.X is significantly decreased in presence of wollastonite, crocidolite and CHR-S while is not altered in presence of CHR-L. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. CHR-S: Russian chrysotile short fibres (<5 μm) CHR-L: Russian chrysotile long fibres (>5 μm).

These data are supported by studies reported in literature showing that environmental pollution can be characterized by the presence of genotoxic agents that can cause cell death or DNA damage [46]. Moreover, it has been reported that DNA damage after asbestos exposure can lead to DNA double-strand break and abnormal mitosis causing micronucleus formation which can inhibit trophoblast proliferation [47, 48]. Impairing the normal development of the syncytiotrophoblast (STB) could have foetal repercussions because STB is the outer layer of

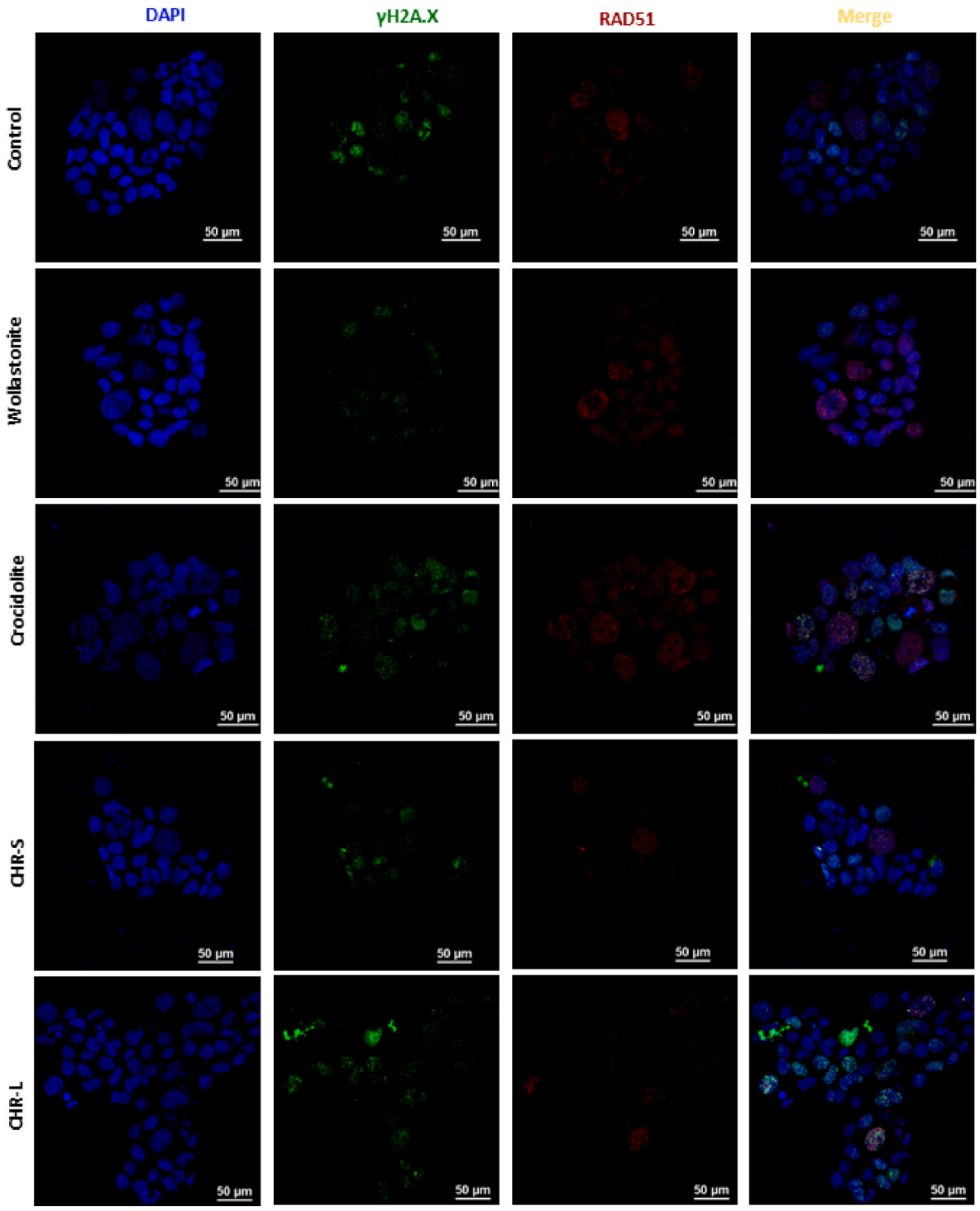
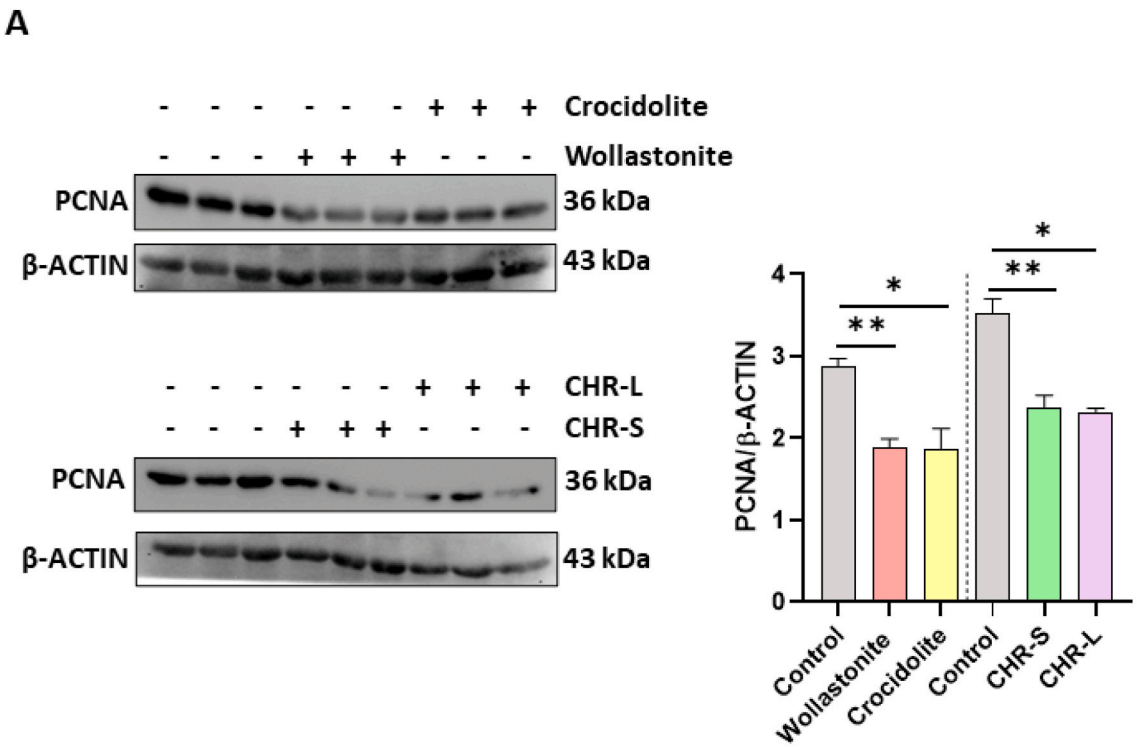


Fig. 7. Immunofluorescence of γH2A.X and RAD51 in presence of mineral fibres. The nuclei of BeWo cells express γH2A.X and RAD51 in presence of all mineral fibres (n = 3).

the trophoblast in direct contact with maternal blood. It derives from villous cytotrophoblastic cells (CTB), a monolayer underlying STB that proliferate and then fuse and differentiate in STB. Altered cell fusion may lead to an abnormal STB layer and subsequent functional deficits [49]. Hence, our data suggest that CHR-L fibre could alter these processes in key zones of placenta miming a series of gestational pathologies in women without known risk factors.

A final remark regards wollastonite. Despite being used as negative standard, wollastonite displays activity *in vitro* and prompts production of ROS. This finding is not surprising. It has been reported that, compared to asbestos, wollastonite produces higher levels of ROS in PMN suspensions, likely because the larger size of its fibres may prevent complete ingestion by phagocytes [50]. These authors agree with previous studies [51,52] showing that, in any case, fibrous wollastonite is less toxic than asbestos. The cytotoxic action of wollastonite does not make it carcinogenic [20]. Cytotoxic action *in vitro* is a predictive tool for carcinogenicity but does not automatically make an "agent" carcinogenic. "Strong evidence that the agent exhibits key characteristics of carcinogens" is required to make an "agent" a *probable* or *possible* carcinogen. For the specific case of wollastonite, stated that there is



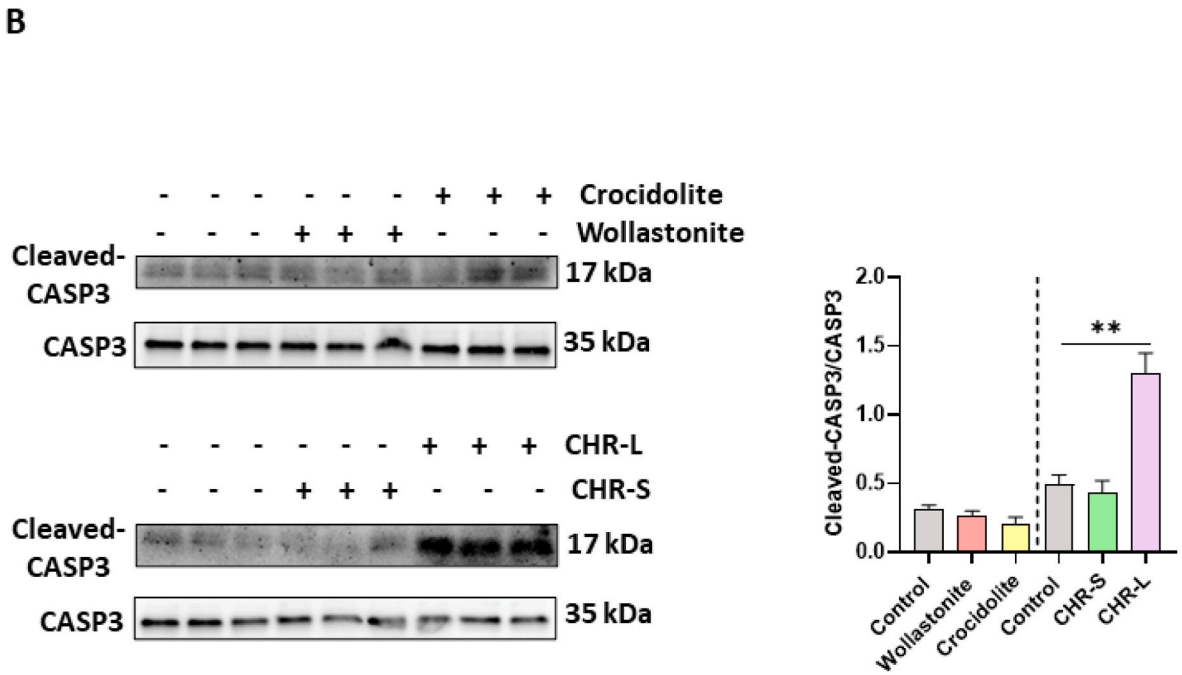


Fig. 8. Western blotting and densitometric analysis (histograms) of PCNA (A) and, Caspase 3 (CASP3) and cleaved form (cleaved CASP3) (B) in presence of mineral fibres (n = 3). PCNA expression is significantly decreased in BeWo cells in presence of all mineral fibres. Cleaved-CASP3 (active form) is significantly increased only in BeWo cells in presence of CHR-L. *p < 0.05, **p < 0.01. CHR-S: Russian chrysotile short fibres (<5 μm) CHR-L: Russian chrysotile long fibres (>5 μm).

insufficient evidence in both humans and animals for its carcinogenicity [53].

In general, for non-animal *in vitro* toxicity tests, a bias that must be taken into account is the so-called "background interference due to inclusion of particles" [54]. Any cell culture in contact with "foreign" bodies such as dust particles invariably undergoes a detrimental physical effect compared to the control cell culture alone. This effect is regarded as a background interference and must be considered to avoid or reduce false positives and false negatives. The reduction in cell viability usually occurs due to physical-mechanical damage of cell membranes in contact with foreign particles [54]. For these reasons, the use of a negative standard is of paramount importance to verify the accuracy of the system and avoid classifying any safe powder as toxic. The effect of reduced cell viability over time is also likely not due to the

chemical-physical nature of the fibres only but also physiological, due to an increase in the number of cell membranes that come into physical contact with the dust and become damaged.

5. Conclusions

This is the first study to explore the possible role of mineral fibres on syncytiotrophoblast development and maintenance. We demonstrated that the analysed fibres could cause damage during syncytiotrophoblast development and, we showed that not all fibres caused damage that cannot be repaired, in our hands only CHR-L fibres caused irreparable damages leading the cells in apoptosis. Since the syncytiotrophoblast is a fundamental structure of the maternal-foetal barrier, together with the endothelium of the foetal vessels, cell apoptosis causes damage that could be fatal to the placental villus. The death of the placental villi leads to impaired placental development which may be the co-cause of gestational pathologies such as, preeclampsia. This pathology is characterized by the presence of infarcted placental zones, therefore with dead and non-functioning villi. Furthermore, if the damage to the placental villi were extensive it would result in a reduced supply of oxygen and nutrients to the fetus resulting in reduced foetal growth and lead to pathologies such as Intrauterine Growth Retardation (IUGR). In conclusion, although our data have been obtained using an *in vitro* model, they suggest serious implications for the health of the mother and child to suggest an assessment by gynaecologists of the maternal environment intended not only as residence geographical area but also as a working environment, maternal feeding and materials used in daily, to avoid contact with asbestos fibres as much as possible.

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CRediT authorship contribution statement

Sonia Fantone: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation, Conceptualization. **Giovanni Tossetta:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Laura Cianfruglia:** Validation, Investigation. **Andrea Frontini:** Methodology, Investigation, Data curation. **Tatiana Armeni:** Methodology, Investigation, Data curation. **Antonio D. Procopio:** Writing – review & editing, Funding acquisition. **Armanda Pugnaloni:** Writing – review & editing, Methodology. **Alessandro F. Gualtieri:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Daniela Marzioni:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors did not use AI.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alessandro F. Gualtieri reports financial support and equipment, drugs, or supplies were provided by Italian Research and University Ministry. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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