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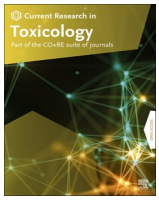

Effects of mineral fibres in an in vitro placental syncytiotrophoblast model

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ABSTRACT

It is known that mineral fibres can be found in placental tissues, but their effect is not known on these tissues. BeWo in vitro model of syncytiotrophoblast, the outer layer of maternal-foetal barrier, is necessary to understand if mineral fibres can alter placental cell turnover and consequently to influence the outcome of pregnancy. We performed in vitro experiments using chrysotile UICC (UICC), chrysotile Valmalenco (VM) and erionite (ERI) to investigate the potential cytotoxic effects of these mineral fibres on BeWo cells. We demonstrated that all fibres are toxic while only UICC fibres caused a DNA damage that the cells were not able to repair through RAD51 activity. In addition, we demonstrated that DNA replication is not altered while cyclin D1 showed a significant decrease in VM and UICC suggesting that the cell cycle is altered in G1 phase. Moreover, UICC increased active form of caspase 3 demonstrating that apoptosis can be induced in BeWo cells. We suggest that although morphological changes are not visible in BeWo cells treated with these mineral fibres, DNA damage can lead to altered placenta physiology that can be seen late when the damage at the foetal tissues has already occurred.

1. Introduction

The placenta is an organ genetically belonging to the *fetus*, disc-shaped with two sides: the fetal one, connected to the cardiovascular system of the *fetus* through the vessels that run in the umbilical cord and the maternal one adhering to the surface of the *uterus* (Fantone et al., 2021; Huppertz, 2008; Tossetta et al., 2016). The maternal blood encounters the maternal-fetal placental barrier but not the fetal blood and it is through this barrier that both oxygen and not only nutrients pass to the *fetus* (Fantone et al., 2021). This barrier is formed by an outer layer of syncytiotrophoblast in contact with maternal blood, an underlying layer of villous cytotrophoblast (which is discontinuous and/or very thin during the second half of gestation), and the endothelium of fetal vessels. Of course, this barrier is not as selective as the blood–brain barrier (Huppertz, 2008). It is a fairly permissive barrier (for some viruses, drugs, alcohol, mineral fibers) even if at the level of the placenta occur metabolic processes that can prevent the passage of certain substances from the mother to the fetus as well as certain drugs (Goasdoue et al., 2017). Asbestos is a term generally used to describe mineral species belonging to the serpentine and amphiboles families, and is known to represent a severe hazard for human health (Gualtieri et al., 2023a; Gualtieri et al., 2023b; Okazaki, 2022; Pugnaloni et al., 2023; Solbes and Harper, 2018). It is known that the asbestos fibres were found in the placental tissue (Haque et al., 1996; Haque et al., 1998), but nothing is known about the possible damage that these fibres can cause to the mother and the unborn child. In this regard, Haque and collaborators have analyzed the presence of asbestos fibres in stillborn demonstrating that in the group of mothers with no previous history of abortions, less than half of the stillborn infants had asbestos fibres in their tissues. In comparison, twice as many stillborn infants of mothers who had a history of previous abortions had fibres in their tissues (Haque et al., 1998). The same research group also demonstrated that, although asbestos

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fibres were also found in control placentas of liveborn infants, the count was lower, and the fibres were longer and thicker than those found in the placentas of stillborn group. The presence of the fibres was also associated to the working mothers compared to the housewives suggesting an exposure of the mothers at work (Haque et al., 1996). However, the placental entry of the fibres is not clear. In addition, it has been demonstrated that erionite or amphibole asbestos increased the frequency of positive anti-nuclear antibodies (ANA) tests in a mouse model. Interestingly, these data suggest that exposures to erionite may induce the production of autoantibodies that can be potentially associated with adverse autoimmune effects not only related to pulmonary cancers and fibrosis (Zebedeo et al., 2014). It is known that autoantibodies can significantly impair pregnancy outcomes causing pregnancy loss and pregnancy complications (Carp et al., 2008; D'Ippolito et al., 2020; di Simone et al., 2006; Iijima et al., 1997). Interestingly, it has been reported that ANA + IgG injection in mice was able to induce foetal resorption (Veglia et al., 2015). Fetotoxicity and teratogenicity were also demonstrated in mice exposed to asbestos (crocidolite, chrysotile and amosite) during pregnancy (Fujitani et al., 2014). We hypothesized that those mineral fibres could cross the placental barrier or simply meet the syncytiotrophoblast, altering the placental environment, behavior and development. The cell cycle and/or cell survival of placental cells could be modified leading, in the worst-case scenario, to the death of the fetus because the placenta is fundamental for fetal survival and development. In the present study, we analyzed if erionite-Na and serpentine asbestos (chrysotile) fibres can cause alterations in syncytiotrophoblast biology using BeWo cells as in vitro model.

2. Materials and methods

2.1. Mineral fibres

This study was performed using three different asbestos fibres: Chrysotile (serpentine asbestos) from Valmalenco (Central Alps, Sondrio, Italy) (VM), Chrysotile (serpentine asbestos) standard of the Union for International Cancer Control (UICC) and Fibrous erionite-Na from Jersey, Nevada (USA) (ERI). More in detail, VM with measured chemical formula, calculated on the basis of 5 oxygen atoms, of $Mg_{2.93}Fe^{2+}_{0.03}Al_{0.01}Fe^{3+}_{0.03}Ni_{0.01}(OH)_{3.93}Si_{2.02}O_5$ (see the full characterization in Pollastri et al., 2016). Instead UICC is a mixture of fibres from the firms Bells, Carey, Cassair, Flintkote, Johns-Manville, Lake, Normandie and National, proportioned roughly to represent Canadian production of asbestos products at that time. The measured chemical formula on the basis of 5 oxygen atoms is $Mg_{2.97}Fe^{2+}_{0.02}Al_{0.01}Fe^{3+}_{0.04}(OH)_{3.83}Si_{2.01}O_5$ (see the full characterization in (Pollastri et al., 2016). At last, ERI with chemical formula $Na_{5.35}K_{2.19}Ca_{0.15}Mg_{0.11}Ti_{0.05}(Si_{28.01}Al_{7.90})\ O_{72}{\cdot}28.13H_2O$ with traces of clinoptilolite (<1 wt%) (Gualtieri et al., 2016; Gualtieri et al., 2018).

2.2. Cell culture and treatments

Human BeWo cell line (kindly provided by S. Alberti, Laboratory of Cancer Pathology, CeSI-MeT, University "G. d'Annunzio", Chieti, Italy) were used as an in vitro model of syncytiotrophoblast (Butler et al., 2017; Orendi et al., 2010). Cells were maintained in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, MA, USA) supplemented with 10 % fetal 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 study the biological effect of mineral fibres to the materno/fetal barrier, BeWo cells were seeded in 6-well plates and after reach the confluence they were put in contact with the different mineral fibres resuspended in the complete culture medium (50 μg/ml final concentration). The plates were incubated at 37 °C for the following times: 10 min, 30 min, 2 h, 4 h, 8 h, 24 h and 48 h. The 50 μg/ml fibres concentration was chosen because of its occupational significance and because this concentration produced well-defined cell responses (Giantomassi et al., 2010; Pugnaloni et al., 2015). Control cells were grown in culture medium with no fibres.

To study the syncytialization capacity of BeWo, cells was treated with Forskolin 50 μM (sc-3562, Santa Cruz Biotechnology) for 48 h. The syncytia formation was evaluated studying E-Cadherin protein expression (Fantone et al., 2023; Tossetta et al., 2023).

2.3. MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess the cytotoxic effect of asbestos fibres on BeWo cells. BeWo cells were seeded into 96-well microplate and grown in the presence of the different mineral fibres. After the different time points mentioned above, 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 the medium, the formazan crystals were dissolved in 200 μl of dimethyl sulfoxide (DMSO). The homogenized MTT-formazan solutions were measured by a microplate reader (Absorbance microplate reader AMR-100) at 550 nm (reference filter, 690 nm). The amount of formazan crystals is directly related to the number of viable cells. Data were expressed as arbitrary units (A.U.).

2.4. Protein extracts and Western blotting

BeWo cells were seeded in 6-well plates in presence/absence 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 15,000*g* for 5 min at 4 °C, the obtained supernatant was stored at −80 °C until use. Bradford assay was used to evaluate the protein concentration in each sample (Bio-Rad Laboratories, Milano, Italy). All protein samples were analyzed by 10 % SDS-polyacrylamide gels (SDS-PAGE), then transferred (Trans-Blot® Turbo™ Transfer System; Bio-Rad Laboratories) to nitrocellulose membranes. 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 horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology) diluted 1:10,000 in TBS-T for 30 min.

Chemidoc (Bio-Rad Laboratories) was used to visualize the protein bands by using the Clarity Western ECL Substrate (Bio-Rad

Table 1
Primary antibodies used in this study.

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

Laboratories). Bands were densitometrically quantified using ImageJ software (National Institutes of Health; https://imagej.nih.gov/ij/download.html).

2.5. Statistical analysis

All analyses were carried out using GraphPad Prism (ver 8) statistical software. When two groups were compared, Student's *t*-test was used. When comparing three or more groups, one-way ANOVA followed by a Dunnett post-test was used. Data were considered statistically significant with P values < 0.05.

3. Results

3.1. Cytotoxic effects of ERI, VM and UICC on BeWo cells

In order to evaluate the cytotoxic effects of ERI, VM and UICC in BeWo cell line, we treated cells with 50 μg/mL of fibres, which is the concentration at which population is commonly exposed (Giantomassi et al., 2010). As shown in Fig. 1, only ERI (Fig. 1A) showed a cytotoxic effect at 30 min while UICC (Fig. 1B) and VM (Fig. 1C) showed a cytotoxic effect later, at 2 h. Moreover, cytotoxicity increased with the increasing fibre contact time up to 48 h.

3.2. Effects of ERI, VM and UICC on BeWo cell syncytialization

Since syncytiotrophoblast is the first barrier of plancental villous, we evaluated if ERI, VM and UICC fibres could alter syncytialization process in BeWo cells. We used E-cadherin expression as marker of syncytialization since its expression decreases when syncytialization occurred (Coutifaris et al., 1991). As shown in Fig. 2 all fibres did not modify BeWo cell morphology (Fig. 2A–D, see the enlargement of the framed area in A′–D′) and did not alter the syncytializzation process (Fig. 2E and F).

3.3. Effects of ERI, VM and UICC on DNA damage and repair

Since obvious morphological damage may be late compared to genetic damage, we wanted to investigate if there were imbalances in the mechanisms of damage and repair of DNA. DNA damage was investigated in presence of ERI, VM and UICC in BeWo cells for 24 h by evaluating phosphorylated H2AX (γ-H2AX) expression, a known marker of DNA damage (Sharma et al., 2012). Moreover, we studied RAD51 protein expression as repair marker of double-stranded DNA breaks (DSBs) under the same conditions (Wassing and Esashi, 2021). All the fibres caused a significant increase of γ-H2AX protein expression, particularly with UICC treatment ($p < 0.001$) (Fig. 3A–D). However, RAD51 expression (Fig. 3A and E) was significantly decreased in cells treated with UICC fibre demonstrating that cells were not able to repair DNA damage induced by this fibre. In addition, we detected that the alteration of γ-H2AX protein expression in presence of ERI was due to a modulation of total H2AX expression (Fig. 3C and D).

3.4. Effects of ERI, VM and UICC on BeWo cell apoptosis

Since an impairment of DNA damage/repair mechanism can lead to apoptosis and alter cell proliferation, we investigated the expression of PCNA, cyclin D1 and caspase 3 proteins. As reported in Fig. 4A and B, PCNA expression was not altered in all conditions compared to the control suggesting that DNA replication is not altered while cyclin D1 (Fig. 4A and C) showed a significant decrease in VM ($p = 0.005$) and UICC ($p = 0.023$) suggesting that the cell cycle could be altered in G1 phase. In addition, UICC led to a significant increase in cleaved caspase 3 (Fig. 4A and E).

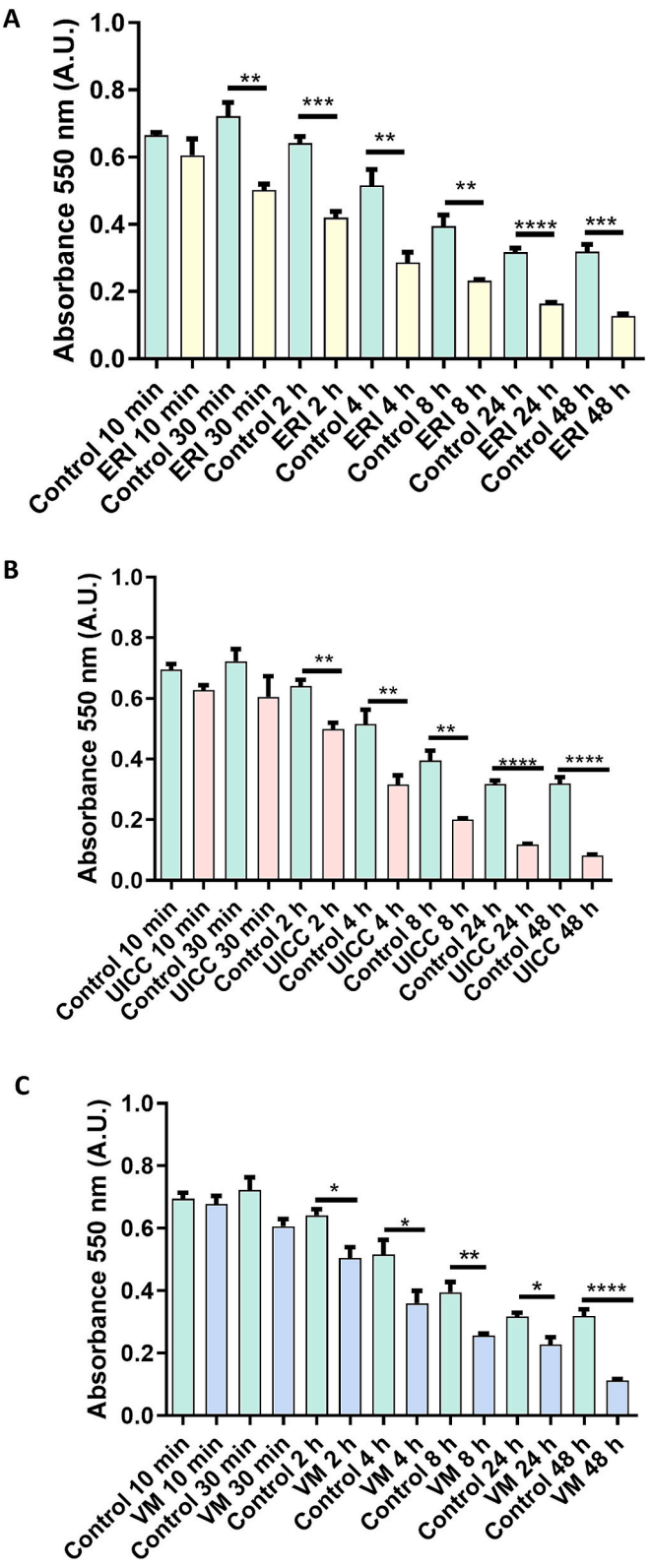

Fig. 1. MTT assay of BeWo cells exposed to ERI, UICC and VM. Cells were cultured in presence of mineral fibres at increasing times (from 10 min to 48 h). MTT assay shows a significant decrease in BeWo cell viability in presence of ERI at 30 min (A) while UICC and VM at 2 h (B, C) ($N = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

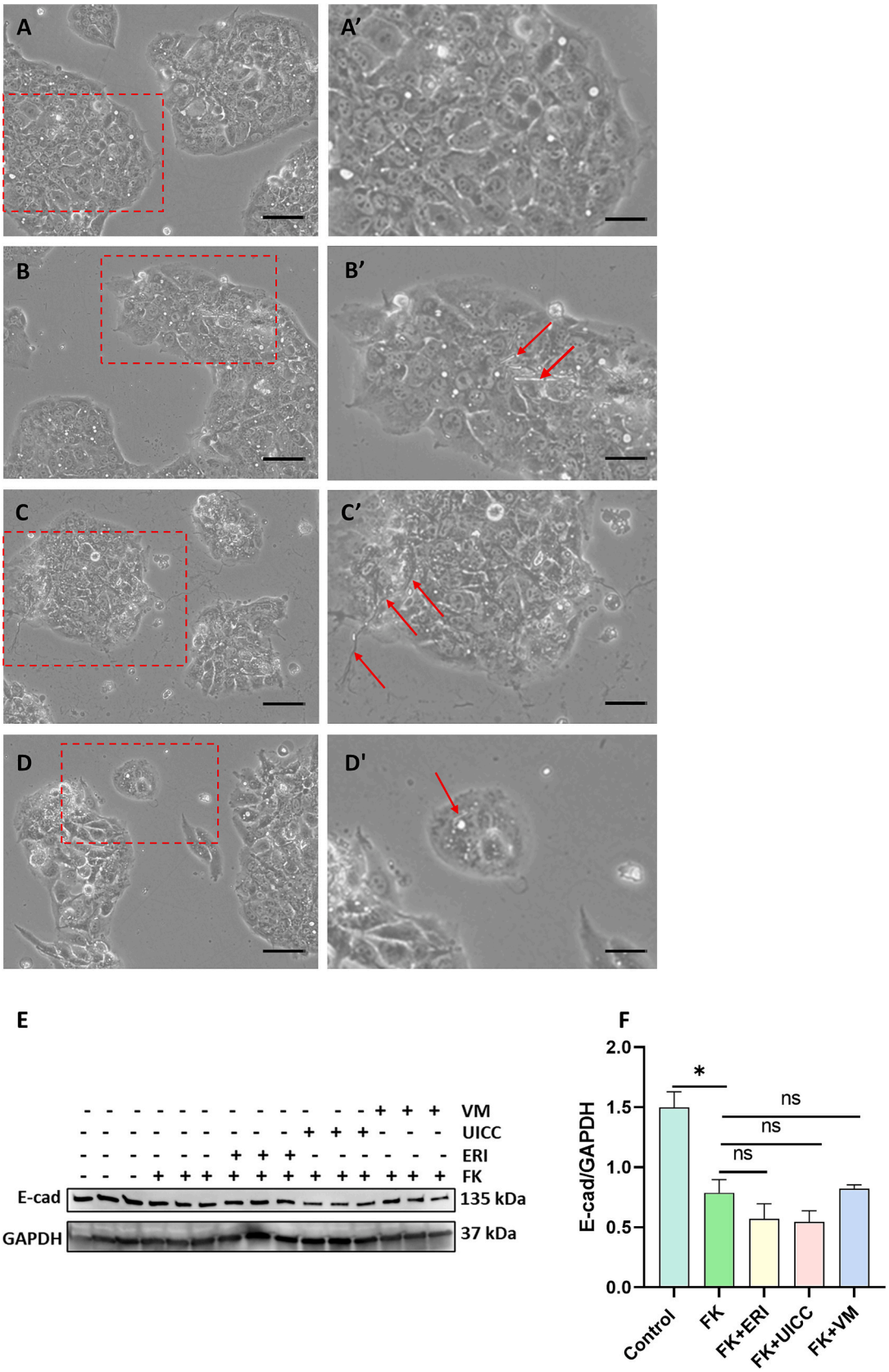

Fig. 2. Morphology of BeWo cells in presence of mineral fibres after 24 h of culture. (A-A′) no fibres (control), (B-B′) ERI, (C-C′) UICC, (D-D′) VM. A′–D′ show the enlargement of the framed area in A–D: the arrows point to fibres in contact with or within BeWo cells. No evident differences in cell morphology are observable. (E) Western blotting analysis of E-cadherin expression in BeWo cells after treatment with forskolin (FK); (F) Histograms representing densitometric analysis of western blotting of E-cadherin. The presence of all the mineral fibres do not alter the syncytializzation (N = 3). *$p < 0.05$. Bars: A, B, C, D = 50 μm; A′, B′, C′, D′ = 25 μm.

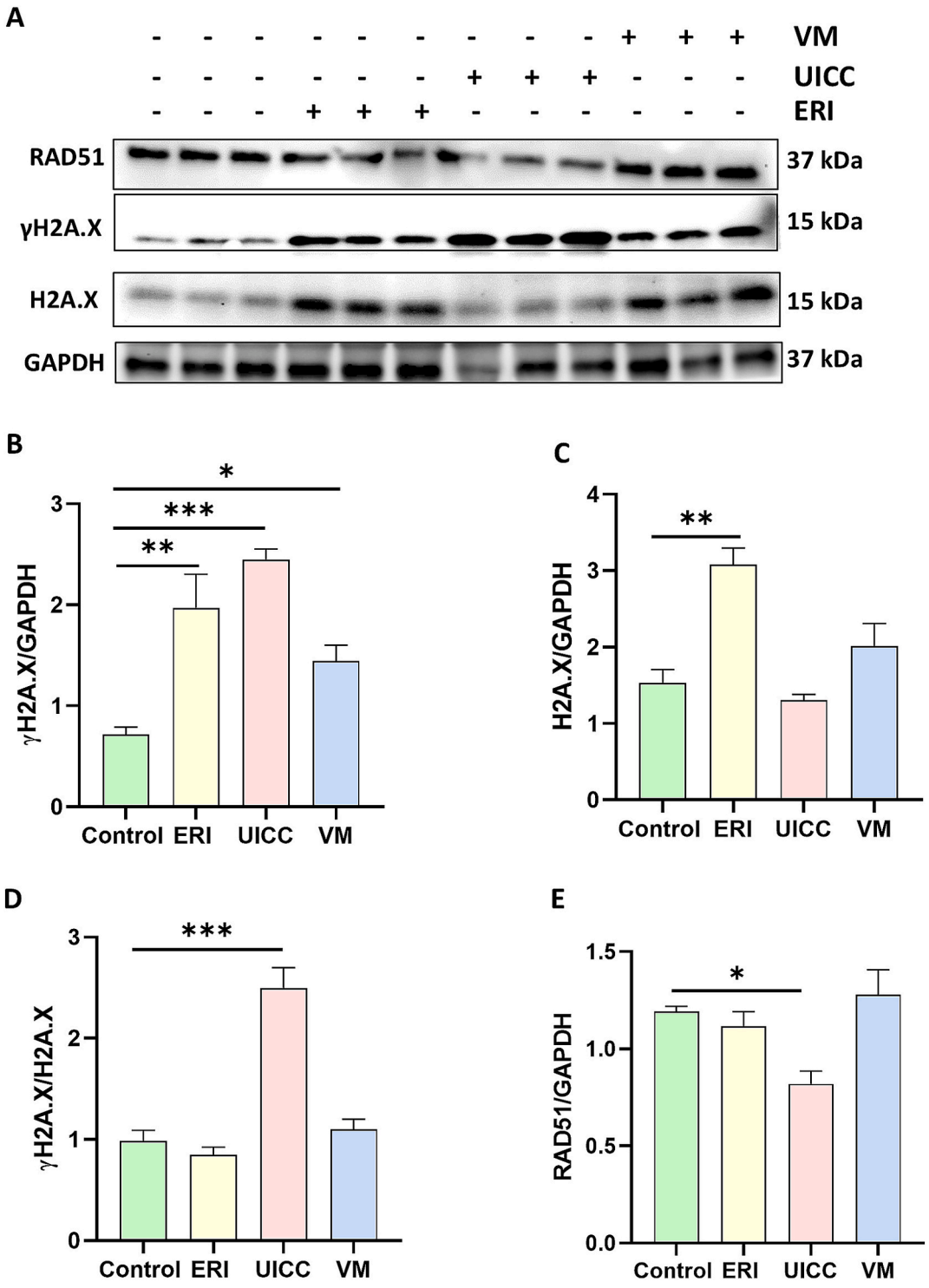

Fig. 3. (A) Western blotting of γH2A.X, H2A.X and RAD51 in presence of mineral fibers. γH2A.X expression is increased in BeWo cells in presence of all mineral fibers (B) while H2A.X is increased only in ERI (C). (D) γH2A.X/H2A.X ratio. (E) RAD51 expression is significantly decreased only in presence of UICC (N = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4. Discussion

The effects of ERI, VM and UICC on syncytiotrophoblast have never been demonstrated to date. In our study, we used BeWo cells to mimic placental syncytiotrophoblast layer, which is in contact with maternal blood present in the intervillous space, and ensure the proper exchanges between the mother and the fetus (Renaud and Jeyarajah, 2022). Probably the best technology to study the interaction of mineral fibers with the placental barrier could be the placenta-on-chip technology. To date, however, there are very few manuscripts that use this technology and with non-standardized methods. Therefore, it requires more comprehensive research into placenta-on-a-chip technology that could lead to a standardized protocol (Elzinga et al., 2023). For this reason, we opted for an in vitro model, i.e. BeWo cell line, that has been widely used for many years. To this end, Pastuschek and co-workers analyzed the seven most widely used in vitro model lines (BeWo, JEG-3, HTR-8/

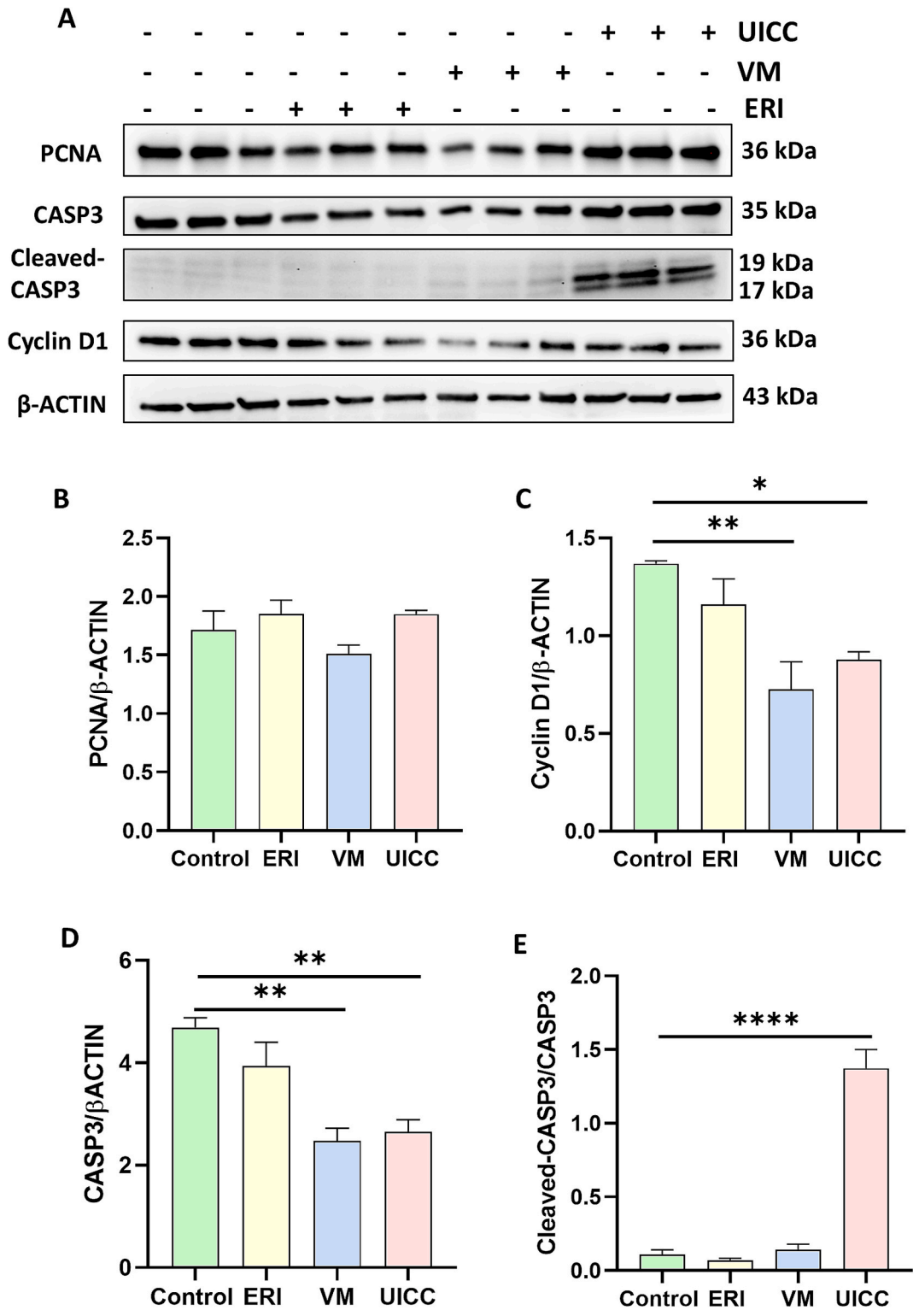

Fig. 4. Western blotting and densitometric analysis (histograms) of PCNA, Cyclin D1 and CASP3 (A). (B) PCNA expression is not affected by the presence of all mineral fibers in BeWo cells while Cyclin D1 (C) is decreased in UICC and VM. (D) Total CASP3 is significantly decreased in presence of UICC and VM while cleaved form of Caspase 3 (active form) is significantly increased only in UICC (E) (N = 3). *p < 0.05, **p < 0.01, p < 0.001, ****p < 0.0001.

SVneo, AC1-M59, AC1-M32, ACH-3P, Swan71) and concluded that BeWo cells seem to be a model for studying syncytiotrophoblast, while the other choriocarcinoma-derived cell lines may be good candidates for studying features of cytotrophoblast and extravillous trophoblast (Pastuschek et al., 2021). Obviously, the best model would be using primary trophoblast cells differentiated into syncytiotrophabst or in vivo experiments on pregnant women, but both have greater limitations than the BeWo cell model. That is, the former has objective limits, i.e., fetal variables, undiagnosed fetal pathologies early, and the latter is untraceable. Our results showed that ERI, VM and UICC fibres have a cytotoxic effect that could modify the physiology of placental tissue. However, not all the fibres are cytotoxic at the same time-point as

demonstrated by MTT assay. Moreover, none of the fibres tested altered syncytiotrophoblast formation. We also found that all fibres significantly induced DNA damage, as demonstrated by the increased expression of γ-H2AX, a known marker double-stranded DNA breaks (DSBs) (Kinner et al., 2008), but UICC-induced damage was more evident. Furthermore, UICC-induced damage could not be repaired by RAD51 since its expression was significantly decreased in UICC treated cells. Contrarily, the damage induced by ERI and VM fibres could be repaired by RAD51 since its expression did not change in cells treated with ERI and VM fibres. The importance of this damage/repair balance has been demonstrated by the fact that only UICC fibre significantly increased apoptosis.

Moreover, erionite and chrysotile mineral fibres in contact with BeWo cells cause genetic alterations that, in the short term, do not appear to bring morphological changes that could be highlighted late due to early cell death due to a decreased expression of Cyclin D and an increase of cleaved caspase 3. These data agree with our previous study reporting cytotoxic effects of short (length < 5 μm) and long (length > 5 μm) Russian chrysotile mineral fibres in BeWo cell line (Fantone et al., 2024). So, UICC exposure reduced Capase-3 protein levels and increased Cleaved-capase-3 protein levels, two typical indicators for cell apoptosis. We suggest that UICC induces not only DNA damage, demonstrated by γ-H2AX increase, like other mineral fibers but also syncytiotrophoblast apoptosis generally mediated by reactive oxygen species (ROS) productions. The syncytiotrophoblast plays an essential role in the first phases of the placenta implantation and reproduction (Gauster et al., 2022). Excessive apoptosis of the syncytiotrophoblast is associated with various adverse pregnancy outcomes and, among these, miscarriages (Zhang et al., 2024). In addition, it has been demonstrated that trophoblast cells are very sensitive to environmental toxicants inducing trophoblast cell apoptosis via ROS or UPR signaling pathway, which alters balance critical to the normal development and function of the placenta (Du et al., 2022). Although the presence of asbestos fibres has been found in placental tissue (Haque et al., 1996, 1998), the way of entry is still not clear. However, it is reasonable to think that a possible way of entry could be the maternal blood stream, but biochemical tests are not available to detect asbestos fibres in the blood. The availability of these tests may detect the presence of asbestos fibres in the mother's blood during early pregnancy, allowing you to identify high-risk pregnancies.

Specific studies aimed to evaluate the frequency of abortions in places contaminated with asbestos are missing but they may clearly demonstrate a correlation between asbestos exposure and pregnancy complications.

5. Conclusions

In conclusion, our study clearly demonstrated that asbestos fibres can significantly impair trophoblast cell development by inducing DNA damage. Moreover, we found that UICC fibre was also able to induce apoptosis. Only UICC chrysotile induced apoptosis while VM fails to do so. This is apparently due to the different sizes of the fibres. In fact, chrysotile UICC displays a mean length of 52.0 μm and a mean width of 0.27 μm (Pollastri et al., 2016) while chrysotile VM displays a mean length of 8.1 μm and a mean width of 0.07 μm (Cattaneo et al., 2012). This in vitro study requires further specific studies to evaluate the relationship between the presence of asbestos fibres and the frequency of pregnancy complications in women who were surely exposed to asbestos.

CRediT authorship contribution statement

Giovanni Tossetta: Conceptualization, Methodology, Software, Investigation, Writing – original draft, Writing – review & editing. **Sonia Fantone:** Conceptualization, Methodology, Software, Investigation, Writing – original draft, Writing – review & editing. **Antonio Domenico Procopio:** Supervision, Visualization. **Armanda Pugnaloni:** Supervision, Visualization. **Alessandro Francesco Gualtieri:** Project administration, Funding acquisition, Visualization, Writing – review & editing. **Daniela Marzioni:** Project administration, Funding acquisition, Visualization, Writing – review & editing.

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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Data availability

Data will be made available on request.

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