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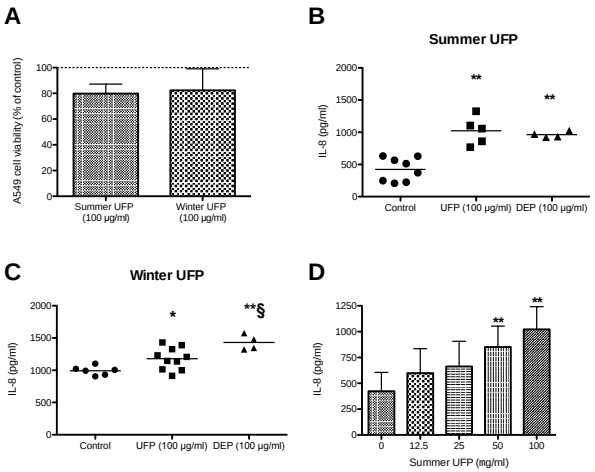
Response to Reviewers:

***Graphical Abstract**



Highlights

- Physical-chemical properties and biological effects of UFPs emitted by wood burning were investigated at an alpine town
- Cell cultures were used as sensors to test the toxicological properties of UFPs.
- UFPs collected in the summer were more active in inducing IL-8 release compared to winter UFPs
- Genotoxic effects induced by UFPs were higher in winter than in summer samples

THE CHEMICAL COMPOSITION OF ULTRAFINE PARTICLES AND ASSOCIATED BIOLOGICAL EFFECTS AT AN ALPINE TOWN IMPACTED BY WOOD BURNING

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ABSTRACT

This work is part of the TOBICUP (TOxicity of BIomass Combustion generated Ultrafine Particles) project which aimed at providing the composition of ultrafine particles (UFPs, i.e. particles with aerodynamic diameter, d_{ae}, lower than 100 nm) emitted by wood combustion and elucidating the related toxicity. Results here reported are from two ambient monitoring campaigns carried out at an alpine town in Northern Italy, where wood burning is largely diffused for domestic heating in winter. Wintertime and summertime UFP samples were analyzed to assess their chemical composition (i.e. elements, ions, total carbon, and polycyclic aromatic hydrocarbons) and biological activity. The induction of the pro-inflammatory cytokine interleukin-8 (IL-8) by UFPs was investigated in two human cells lines (A549 and THP-1) and in human peripheral blood leukocytes. In addition, UFP-induced oxidative stress and genotoxicity were investigated in A549 cells. Ambient UFP-related effects were compared to those induced by traffic-emitted particles (DEP) taken from the NIES reference material "vehicle exhaust particulates". Ambient air UFPs induced a dose-related IL-8 release in both A549 and THP-1 cells; the effect was more relevant on summer samples and in general THP-1 cells were more sensitive than A549 cells. On a weight basis our data did not support a higher biological activity of ambient UFPs compared to DEP. The production of IL-8 in the whole blood assay indicated that UFPs reached systemic circulation and activate blood leukocytes. Comet assay and γ-H2AX evaluation showed a significant DNA damage especially in winter UFPs samples compared to control samples.

Our study showed that ambient UFPs can evoke a pulmonary inflammatory response by inducing a dose-related IL-8 production and DNA damage, with different responses to UFP samples collected in the summer and winter periods.

1. INTRODUCTION

Pollution due to particulate matter (PM) is currently of major concern, and it has been evaluated as the 9th highest risk factor in a study on the global burden of disease (Lim et al., 2012). Air pollution is considered as a major environmental risk to health affecting both developed and developing countries by the World Health Organization, which states that by decreasing air pollution levels, countries can reduce the burden of heart diseases, lung cancer, acute and chronic respiratory diseases (Domenici et al., 2006; Pope and Dockery, 2006; WHO, 2014). These effects are believed to be due to exposure to PM10 and PM2.5 (i.e. particles with aerodynamic diameter lower than 10 and 2.5 μm, respectively), which can penetrate deep into the lungs (Johnson and Vincent, 2003; Pope and Dockery, 2006).

Particulate matter is composed of a complex mixture of inorganic and organic, liquid and solid particles suspended in atmosphere; it is often referred to as atmospheric aerosols (Hinds, 1999). It is one of the most challenging pollutants due to its complex nature having e.g. primary and secondary origin, a variety of size-distributions and chemical compositions, as well as being emitted by both natural and anthropogenic sources. Moreover, it is a priority issue in air quality and climate studies (Colbeck and Lazaridis, 2014). In urban areas major sources are largely ascribed to combustion processes, and among them wood burning for domestic heating is growing more and more also in Europe. Indeed, as promoted by the Biomass Action Plan of the European Commission (EC, 2005), it represents a renewable energy source and contributes to lower green-house-gases emissions. Moreover, considering the increasing trend in the cost of other fuels (e.g. natural gas and oil) residential biomass combustion will become more and more a cheaper alternative.

Wood combustion is a relevant source for organic particulate matter (Daellenbach et al. 2016). In several areas - including the Po Valley in Northern Italy, which is one of the continental hot-spots for air pollution - wood combustion gives a significant contribution to

the high PM concentration observed (Bernardoni et al., 2011; Piazzalunga et al., 2011; Bernardoni et al., 2013; Amato et al., 2016). Wood burning impact on air quality is mainly due to the use of low efficiency combustion appliances, especially in small villages where open fireplaces are largely in use. A survey carried out by Pastorello et al. (2011) reported that in Lombardy region (which is located in the Po valley) about 16% of households have generally old and inefficient wood burning appliances, and 10% of the houses are equipped with new generation combustion systems (e.g. pellet or chips automatic stoves, log wood innovative stoves).

In PM10 and PM2.5 samples collected in the Po Valley atmospheric organic pollutants with a strong toxicological impact, such as Polycyclic Aromatic Hydrocarbons (PAHs) and dioxins were detected and related to wood combustion (Belis et al., 2011; Piazzalunga et al., 2012). Indeed, PAHs are a class of substances of great interest because of their adverse effects on human health (IARC, 2010) due to their mutagenic and carcinogenic properties (Agudelo-Castañeda and Teixeira, 2014). PAHs are known to cause DNA adducts (Godschalk et al., 2000; Squadrito et al., 2001), and transition metals may induce DNA strand breakage by inducing ROS (Chapman et al., 1997).

Recent studies indicate that inhalation of wood smoke emissions can affect pulmonary immune defence mechanisms. Moreover, macrophages (i.e. the main defence of the lung providing the link between the non-specific and specific defence mechanisms of the respiratory tract) together with lung epithelial cells are the principal targets for wood smoke-induced immunotoxicity (Zelikoff et al., 2002). Several epidemiological studies (reviewed in Naeher et al., 2007) concerning exposure to biomass smoke both indoor and outdoor indicate an association with increased risk of respiratory illness and decreased lung functions. Susceptible subpopulations include asthmatics and children, this being consistent with results observed in studies on ambient air pollution impact on human health.

There is still a gap of knowledge on UFPs (i.e. airborne particles with aerodynamic diameter, d_{ae}, lower than 100 nm) health impact as they are usually not monitored on a routine basis and data on UFPs physical-chemical properties are also very scarce in the literature (Ntziachristos et al., 2007; Terzano et al., 2010). UFPs are typically emitted by combustion processes, so that in urban areas traffic and domestic heating are often the most relevant ones. As UFPs have a large specific surface area and longer residence times in lung compared to larger size particles, they are believed to induce strong and prolonged lung inflammation. There are few epidemiological studies on UFPs and (cause-specific) mortality with inconsistent results presented. Cardiovascular effects due to UFPs were reported in few epidemiological studies (Wichmann et al., 2000; Oberdorster et al., 2005). These can be explained by induction of pulmonary inflammatory response, translocation of UFPs from the lung into circulation with subsequent toxicity to vascular epithelium, alteration of blood coagulation, interference with autonomic nervous system activity, all of which constitute the causal link between particle inhalation and risk of cardiovascular diseases (Terzano et al., 2010; Saber et al., 2014; Chen et al., 2016). Despite the documented respiratory health effects of PM2.5, in contrast, the literature remains inconclusive and the respiratory health effects appear independent from particle mass exposures although evidence for a relationship between UFPs and children's respiratory is increasing (Heinzerling et al., 2016; Lanzinger et al., 2016).

Animal studies have shown that exposure to DEP and other carbonaceous nanoparticles induces carcinogenic effects (Heinrich et al., 1995) but - due to the issue of rat lung overload - the relevance for humans is still a matter of debate (ILSI, 2000).

This work is part the TOBICUP (TOxicity of BIomass Combustion generated Ultrafine Particles) project which aimed at providing the composition of ultrafine particles emitted by wood combustion and elucidating the related toxicity. The first part of the project focused on

laboratory tests in which room heaters were fed with wood and pellets of two different types and their emissions were investigated in terms of UFPs physical-chemical properties and biological effects (details in Corsini et al., 2017; Ozgen et al., 2017). The second part of the project investigated the composition and toxicity of UFPs emitted in outdoor air at a site where wood burning is a relevant source for domestic heating during wintertime.

In both cases cell cultures were used as sensors to test the toxicological properties of UFPs.

Results here reported are from two ambient monitoring campaigns performed in Morbegno, a little town in Valtellina, an alpine valley in Northern Italy. Wintertime and summertime UFPs mass concentration was determined and the chemical composition analyzed in terms of elements, ions, total carbon, and PAHs. Differences in biological effects were studied in relation to variations in UFPs composition. As marker of inflammatory effects, the release of interleukin-8 (IL-8) was evaluated in two human cells lines, namely the THP-1 and A549 cells used as surrogates of alveolar macrophages and lung epithelial cell, respectively, and in human peripheral blood leukocytes. In parallel to the inflammatory potential and its possible link with genotoxicity, UFP-induced DNA damage was investigated in A549 cells.

2. MATERIALS AND METHODS

As reported in the previous section, key aspects of UFPs are the large specific surface area and their chemical composition characterized by heavy metals and organics, including PAHs, which can produce oxidative stress, pro-inflammatory gene expression and exert genotoxic effects (Donaldson et al., 2005). Particulate matter emitted by wood burning is highly enriched in carbonaceous components (Daellenbach et al. 2016) and in alpine regions wood is largely used as fuel; therefore, research on UFPs emitted by wood burning is needed to enhance current understanding. Taking into account the research needs above mentioned, the TOBICUP research outputs included both a comprehensive chemical characterization of UFP

samples and biological tests to estimate IL-8 production, genotoxic damage in A549 cells, and ROS/RNS formation due to wood burning emissions of UFP.

All the methods are summarized in Table 1 and described in detail in the following.

It is noteworthy that in the investigated town major sources other than wood burning (e.g. traffic and industries) were expected not to show large seasonal differences in their emissions so that our sampling strategy (see below) allowed the comparison of the overall biological effects due to atmospheric particles with (in wintertime) and without (in summertime) the additional contribution due to the source of interest, i.e. wood burning.

2.1 Site description

Ambient UFPs sampling was carried out in 2015 during a summer and a winter period at the alpine town of Morbegno (Sondrio), Northern Italy. It is a town of approximately 12,000 inhabitants located at 242 meters a.s.l. The monitoring sites were kindly provided by the Municipal Administration. During wintertime the monitoring site was located in the city center inside the courtyard of the town Council, and during summertime – due to logistic problems – it was moved in the Council open-air stock, a place with very similar characteristics to the wintertime one as for air pollution and major emission sources (i.e. low traffic area).

2.2 Sampling methodology

UFPs were collected in a winter period when wood burning was expected to be a relevant source (Jan-Feb) and in a summer period when conversely wood burning was considered to be very limited and almost negligible (Jun-Jul). Indeed, a previous study (Piazzalunga et al., 2011) carried out in Sondrio (a town about 30 km far from Morbegno) showed that PM10 levels were significantly impacted by wood burning only during wintertime (up to 16-23%).

UFPs were collected by three Multistage Cascade Impactors: 1 Small Deposit Impactor (SDI, Dekati), and 2 Micro-Orifice Uniform-Deposit Impactors (MOUDI by MSP Corporation) which operated in parallel during the winter (from 20 Jan 2015 to 27 Feb 2015) and the summer (from 8 Jun 2015 to 16 Jul 2015) campaign. Due to expected differences in average particulate matter concentrations during the two periods, with typically higher PM levels in winter as a consequence of reduced mixing layer heights and the existence of additional sources like domestic heating (Vecchi et al., 2004), sampling times were integrated over three/four days in the winter and seven days in the summer campaign. The rationale for this choice was related to the possibility of collecting a sufficient quantity (i.e. no less than 100 µg per sample) of UFPs on each sample in order to be able to perform all the chemical and biological analyses listed in Table 1. In the TOBICUP project a 5-weeks measurement campaign in different seasons was planned so that differences in the sampling times produced a different number of samples per campaign (i.e. 11 in winter and 5 in summer).

For all multistage impactors, only UFPs collected on the two lower impaction stages and the back-up filter were analyzed in order to select particles with d_{ae}<100 nm in each sampling. The multistage impactors operated on different substrates (see Table 1), depending on the subsequent analysis to be performed: SDI collected UFPs on polycarbonate impaction foils for elemental analysis; one MOUDI operated with pre-fired quartz fiber filters for chemical analyses, and on the other MOUDI aluminum foils were used as impaction substrates for collecting UFPs devoted to toxicological tests. A PTFE (on SDI and MOUDI with Al) and a quartz-fiber filter (on MOUDI with quartz-fiber filters) were mounted as back-up filters in order to collect also those particles not retained on the upper impaction stages.

Both polycarbonate and Al substrates were weighed (following the procedure described in the Supplementary Material) in order to obtain the ambient UFPs mass concentration and the UFPs mass collected for biological analyses, respectively.

2.3 UFPs chemical characterization

Elemental concentrations (Al, P, Ti, V, Mn, Fe, Co, Ni, Cu, Zn, As, Sr, Mo, Cd, Ba, Pb) were determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES, analytical procedure described in Perrone et al., 2013) on UFPs samples collected on polycarbonate substrates.

One-half of each quartz-fiber filter was used for the determination of ions, total carbon, and anhydrosugars (levoglucosan, mannosan, and galactosan, which are considered markers for wood burning). Water soluble cations (NH_4^+, K^+, Ca^{2+}, Na^+, Mg^{2+}) and anions (NO_3^-, SO_4^{2-}, Cl^-, NO_2^-,) were analyzed by Ion Chromatography (IC). High Performance Anion-Exchange Chromatography (HPAEC-PAD) was exploited for measuring levoglucosan and its isomers. Total carbon (TC) concentration was assessed by Thermal Optical Transmittance (TOT). Details on the above-mentioned analytical methodologies can be found in Piazzalunga et al. (2010 and 2013).

The other half of the quartz-fiber filter was used to quantify 8 PAHs: benzo(a)anthracene, chrysene, benzofluoranthene, benzo(a)pyrene, phenanthrene, anthracene, fluoranthene, pyrene by GC-MS (see Supplementary Material for a short description).

2.4 Experimental methods for biological studies

The experimental methods for biological studies used in this work were already described in previous publications (e.g. Corsini et a., 2013; Corsini et al., 2017) and some more details are given in the Supplementary material.

UFPs devoted to toxicological tests were detached from Al filters by sonication, evaporated for 24 and finally re-suspended in phosphate-buffered saline. Blanks contribution was assessed extracting unexposed filters under the same conditions as the sampled filters.

The induction of the pro-inflammatory cytokine interleukin-8 (IL-8) by UFPs was investigated in two human cells lines (A549 and THP-1) and in human peripheral blood leukocytes. IL-8 is also known as neutrophil chemotactic factor and is a member of the CXC chemokine family. It induces chemotax primarily of neutrophils but also of other leukocytes, including T lymphocytes, and stimulates phagocytic activity. IL-8 has been involved in the pathogenesis of several inflammatory lung diseases (Pease and Sabroe, 2002; Mukaida, 2003).

In addition, UFP-induced oxidative stress and genotoxicity were investigated in A549 cells by comet assay and γ-H2AX. Nucleoids were classified in five categories (A undamaged, B-D damaged, E ghost) on the basis of the area and intensity of the tail staining, and DNA damage quantified as the percentage DNA in the tail (% DNA-Tail), Tail length (TL, μm), and Tail Moment (TM= % DNA-Tail x TL, μm).

ROS formation was assessed as described by Wang and Joseph (1999) with slight modifications that are reported in the Supplementary Material.

As reference particulate matter, samples of vehicle exhaust particles (DEP) obtained from NIES certified reference material n°8 (NIES, Ibaraki, Japan) were used. Similarly to UFP ambient samples, DEP was suspended in phosphate-buffered saline PBS.

A total of sixteen UFPs samples were tested: 5 collected during summertime and 11 during wintertime in the year 2015. Statistical analysis was performed using InStat software version 3.0a (GraphPad Software, La Jolla, CA, USA). Statistical analysis was performed with One-Way ANOVA test, followed by Tukey's multiple comparison test. Effects were designated significant if $p < 0.05$.

3. RESULTS

3.1 Ambient air UFPs composition

A summary of the results on chemical characterization are reported in Table 2, where UFPs ambient air concentrations in terms of mass and chemical composition are given as averages over the summer and winter campaigns. The Welch t-test (i.e. a modified t-test which is more appropriate when the datasets to be tested have not the same number of samples and have unequal variances) was carried out on the concentration means reported in Table 2; means were considered statistically different if $p<0.05$ (indicated with an asterisk in the table). Average ambient air UFPs concentrations did not show significant seasonal differences (2.2 $\mu g/m^3$, range:1.6-3.2 $\mu g/m^3$ in the winter period and 2.0 $\mu g/m^3$, range 1.0-3.1 $\mu g/m^3$ in the summer period). Opposite to mass concentration, the UFPs composition was season-dependent for a large number of the detected species.

Total PAHs contribution was higher during wintertime compared to summertime (difference statistically significant at $p<0.05$); nevertheless, PAHs remained trace contributors accounting for less than 1‰ of the UFPs mass. Benzo(b)fluoranthene was the PAH compound giving the largest contribution in term of mass concentration as well as showing the highest winter to summer ratio. It is noteworthy that known tracers for wood burning emissions (i.e. levoglucosan and its isomers, K^+, and benzo(a)pyrene as reported in Lin et al., 2010) were among those species characterized by significant seasonal differences and very high (>8) winter to summer ratios between average concentrations. The wood burning tracers (levoglucosan and its isomers as well as K^+) accounted for about 8% of the total mass during wintertime, whereas they were nearly absent during summertime. Whereas the absence of anhydrosugars during summer does not definitively indicate the absence of wood burning contribution during summer - because of the possible degradation of such compounds due to hydroxyls radicals (Henningan et al., 2010) - K^+ behavior reinforces the indication of negligible wood burning contribution to the total mass during the hot season.

Focusing on PAHs showing larger seasonal differences on UFPs relative mass contributions, benzo(b)fluoranthene and benzo(a)pirene concentrations were about 11 and 5 times higher during winter than during summer (not shown), respectively. Emission data from small scale appliances burning wood and pellets showed that benzo(b)fluoranthene and benzo(a)pirene gave the highest contribution among the various PAH compounds (Ozgen et al., 2014), thus confirming the hypothesis that wood burning could be likely be the source for these compounds in Morbegno. Moreover, Perrone et al. (2012) reported that PAHs recorded at an high altitude site located in the same alpine valley as Morbegno could be largely (>75%) ascribed to biomass burning.

Total carbon was always the dominant component of UFPs mass but showed no seasonal difference. Most of the detected metals showed average concentrations (Table 2) not statistically different in the investigated periods (only exceptions were Ba and Mn) and seasonal differences on the relative contribution to UFPs mass were at most a factor 2 (not shown).

3.2 Ambient air UFPs induced dose- and season-related production of IL-8

Taking into account the UFPs chemical composition, the ability of ambient air UFPs in inducing the release of the proinflammatory cytokine IL-8 in two human cell lines - A549 and THP-1, used as surrogates of lung epithelial cells and alveolar macrophages - and in human peripheral blood leukocytes was investigated. Cells were exposed for 24 h to increasing concentrations of ambient UFPs (0-100 μg/ml) sampled in the winter and summer periods, and effects were compared to DEP (100 μg/ml). To avoid the variability in cellular response, samples collected within each sampling campaign were extracted and tested at the same time. As shown in Figures 1 and 2, both A549 and THP-1 cells showed a dose-related

response to ambient air UFPs with IL-8 release, with particles collected in summer being the most active (Figure 2D). In A549 cells, cell viability was assessed by MTT reduction (Figure 1A). Summer and winter UFPs (100 μg/ml) induced on average a 20 % reduction in cell viability, with no statistically significant difference on a seasonal basis. On a weight basis, when compared to DEP (100 μg/ml), in A549 cells the release of IL-8 induced by summer UFPs was similar (Figure 1B), while it was slightly lower ($p<0.05$) with winter UFPs (Figure 2C). Winter UFPs showed a lower IL-8 stimulation index compared to summer UFPs ($p<0.01$). The release of IL-8 was dose-related, and reached a statistical significance at 50 and 100 μg/ml (Figure 1D).

In THP-1 cells summer UFPs induced on average a higher release of IL-8showing high variability in the response among the different samples tested (Figure 2A). The release induced by winter UFPs was significantly ($p<0.01$) lower on a weight basis compared to DEP (Figure 2B). Also for THP-1, as reported for A549 cells, the release of IL-8 was dose-related and reached a statistical significance at 50 and 100 μg/ml (Figure 2C). Compared to DEP, winter UFPs on a weight base were less active in inducing IL-8 release, while UFPs collected in the summer period showed a similar or higher activity. The high activity observed in THP-1 cells with summer UFPs could be partially explained by an endotoxin contamination, as demonstrated by the use of polymixin B (Figure 2D), which significantly reduced ($p<0.05$) UFP-induced IL-8 production. The possible presence of endotoxin was investigated pooling together summer UFPs, which were pre-incubated with polymixin B sulfate (15 μg/ml final concentration) at room temperature for 1 h and then added to THP-1 cells for 24 h. Bacterial lipolysaccharide (LPS, 10 ng/ml) was incubated with polymixin B as described for UFPs and, as expected, it significantly reduced LPS-induced IL-8 production.

By comparing the IL-8 stimulation index, THP-1 cells appeared to be more sensitive than A549 cells and summer UFPs were more active compared to winter UFPs in both cell lines.

As UFPs can be systemically absorbed (Nemmar et al., 2002 and 2004; Mills et al., 2006), the capability of summer UFPs in inducing IL-8 production in peripheral blood leukocytes was investigated. Blood was obtained from three healthy female donors (mean age 35 y), diluted 1:10 in culture medium and stimulated with summer UFPs (50 μg/ml); finally, the response was compared to the one obtained with DEP (100 μg/ml) and LPS (10 ng/ml). As shown in Figure 2F, summer UFPs induced IL-8 production above control levels in all subjects, confirming their ability to induce an inflammatory response.

3.3 Ambient air UFPs induced genotoxic damage in A549 cells

Genotoxicity in A549 cells was assessed by the comet assay and the phosphorylation at serine 139 (γ) of histone H2AX. The comet assay is a simple and sensitive method for analyzing and quantifying DNA damage. In particular, the alkaline comet assay highlights single strand (SS) and double strand (DS) breaks as well as alkali labile sites, while the phosphorylation of H2AX is a sensitive marker for DNA double strand breaks.

In Figures 3A-B the percentage of non-damaged nucleoids (A) and nucleoids with progressive damage (BCD) are represented; in addition, Figure 3C reports the tail length (μm) after 24h of treatment with 50 μg/ml of summer and winter UFPs. DEP (50 μg/ml) was used for comparison. The dose used for the genotoxicity assessment was chosen based on the lack of cytotoxicity as assessed by the MTT test. At this concentration cell viability was higher than 80% for all samples. An increase in DNA damage - in terms of tail length and BCD nucleoids - compared to the control samples was observed with both summer and winter UFP samples (**$p<0.01$). Tests with winter UFPs also showed more DNA damage compared to summer UFPs (*$p<0.05$ and **$p<0.01$). The treatment with DEP at the same

concentration also leaded to a statistically significant damage compared to controls (**$p<0.01$), which resulted similar to UFP-induced DNA damage.

The quantification (100 cells for treatment) of foci (marked sites for γ-H2AX) following 24 h exposure to UFPs or DEP showed a significant increase (*$p<0.05$ and **$p<0.01$) in the percentage of cells with a number of foci between 6-10 and higher than 10, indicative of DNA damage (Figure 4). In control cells, the percentage of cells with 0-5 foci was between 80-90%. A trend in increased DNA damage in cells treated with winter UFPs compared to summer UFPs was observed although not statistically significant. A similar DNA damage was observed after the treatment with DEP. In Figure 4B representative images of the various treatments are shown.

3.4 UFP-induced ROS/RNS formation

The possible role of oxidative stress in UFPs-induced DNA damage was investigated treating A549 cells with summer and winter UFP samples (50 μg/ml) for 30 min, 60 min, and 24 h. A statistical significant increase in ROS was observed at earlier time point ($p<0.05$), which can support the DNA damage observed at 24 h. After a 24-h treatment, ROS production (Figure 5A) was similar in control and treated cells, while RNS production (Figure 5B) was higher in cells exposed to summer UFPs. RNS were not measured at earlier time points.

4. CONCLUSIONS

In this study, a multidisciplinary approach was applied to investigate UFPs physical-chemical properties and potential harmful effects with a focus on the impact of wood burning. Results showed a relationship between wintertime and summertime ambient UFPs chemical composition and different biological activities thus providing useful data to achieve a better knowledge of UFPs toxicity.

The sampling strategy adopted in this work aimed at comparing the biological effects induced by UFPs emitted during periods with wood burning on/ off while no seasonal difference was expected in other major sources active in the area (i.e. traffic and industry).

Ambient air UFPs were able to stimulate an inflammatory response, as shown by the release of IL-8 in several cellular models, including peripheral blood leukocytes. The latter may be relevant for systemically absorbed UFPs. The results obtained in the whole blood assay were similar to the one observed in THP-1 cells. UFPs collected in the summer were more active in inducing IL-8 release compared to winter UFPs in both cells lines, but the release was overall similar to the one observed with DEP. The intensity of toxicological responses clearly varied according to season. It is important to note that UFPs can be originated by gas-to-particle conversion processes in atmosphere (i.e. secondary aerosol production) and are also subject to photochemical processing, which is responsible for their oxidation in typical summertime conditions; indeed, the latter may explain the higher proinflammatory effect observed in THP-1 cells with summer UFPs.

It is interesting to note that UFPs collected in the winter period showed that, opposite to the inflammatory effect, genotoxic effects induced by UFPs sampled during wintertime were higher than those induced by UFPs sampled during summertime, indicating that seasonal differences in UFPs composition differently affected biological responses. Results also indicated that different cell types and toxicological parameters were differently triggered depending on the composition of ambient air UFPs. More in detail, the results obtained in this work suggested that – assuming exposure of cells to the same quantities of UFPs mass – exposure to wintertime ambient UFPs (with the presence of wood burning emissions) were more effective in inducing genotoxicity with limited pro-inflammatory responses compared to summer UFPs in which no wood burning contribution was expected. The presence of PAHs and transition metals in ambient air UFPs is likely to contribute to the genotoxicity

observed in A549 cells in this study as PAHs are known to cause DNA damage and transition metals may induce DNA strand breakage by inducing ROS.

Overall, results obtained in this work showed that intensity of toxicological responses varied according to season. Results indicated that ambient UFPs evoke a pulmonary inflammatory response by inducing IL-8 production and DNA damage, with different responses observed comparing UFPs collected in wintertime versus those sampled in summertime. They did not, however, support a higher reactivity of ambient UFPs compared to DEP.

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FIGURE CAPTIONS

Figure 1. Pro-inflammatory effects of summer and winter airborne UFPs in A549 cells. A) Effect of UFPs on cell viability. B) IL-8 secretion induced by summer UFPs; C) IL-8 secretion induced by winter UFPs; D) Dose-related release of IL-8 induced by summer UFPs. Each dot represents an UFPs sample; the bar is the mean value.

*p<0.05, ** p < 0.01 vs. control cells (Control), and §p<0.05 vs. DEP treated cells.

Figure 2. Pro-inflammatory effects of summer and winter airborne UFPs in THP-1 cells. A) IL-8 secretion induced by summer UFPs; B) IL-8 secretion induced by winter UFPs; C) Dose-related release of IL-8 induced by summer UFPs. D) Comparison of the IL-8 stimulation index for both summer and winter UFPs and both THP-1 and A549 cell lines; E) Endotoxic contamination of summer UFPs; F) IL-8 production in human peripheral blood leukocytes.

Each dot represents an UFPs sample; the bar is the mean value.

*p<0.05 and **p<0.01 vs. control cells (Control), and §p<0.05 or §§<0.01 vs. DEP or vs. summer UFPs alone or LPS treated cells.

Figure 3. DNA damage as assessed by the alkaline Comet assay. DNA damage is reported as A) Percentage of type A nucleoids (%, undamaged); ***B)*** Percentage of types BCD nucleoids Type BCD (% damaged); and C) Tail lenght (μm).

Each dot represents an UFPs sample; the bar is the mean value.

All samples are statistical significant versus Control (p<0.01) and *p<0.05,** p<0.01 winter UFPs vs summer UFPs.

Figure 4. DNA double strand breaks as assessed by γ-H2AX phosphorylation. DNA damage is reported as

A) percentage of cells with different number of foci. Each value represents the mean±SD of 3-6 independent samples.

Statistically significant differences from DEP controls are indicated at *$p<0.05$, **$p<0.01$, ***$p<0.001$.

B) representative γ-H2AX immunofluorescence staining (60x magnifications). a) Control cells; b) summer UFPs treated cells; c) winter UFPs treated cells; d) DEP treated cells.

Figure 5. Reactive species as assessed by ROS and RNS production in A549: A) ROS production after 30, 60 minutes and 24 hours of treatment; B) RNS production after 24 h of treatment. Each value represents the mean±SD of 3-6 independent samples.

The results are given as fluorescence units (FU)/mg of total cell protein.

*$p<0.05$ and **$p< 0.01$ vs control cells (Control).

Table 1 - summary of experimental methodologies

Sampling methodology	Gravimetric analysis	Chemical analysis	Biological tests
SDI with polycarbonate foils and PTFE as back-up filter	yes	Elements by ICP-AES	-
MOUDI 1 with quartz-fiber filters (also for the back-up filter)	no	Total carbon by TOT Anhydrosugars by HPAEC-PAD	-
		Polycyclic Aromatic Hydrocarbons by GC-MS	-
MOUDI 2 with aluminium foils and PTFE as back-up filter	yes	-	Cell viability
			Chemokine production
			Cytotoxicity
			Genotoxicity test (Comet assay and ϒ-H2AX)
			ROS and RNS production

SDI=Small Deposit Impactor; MOUDI=Micro Orifice Uniform Deposit Impactor

ICP-MS=Inductively Coupled Plasma Atomic Emission Spectroscopy; TOT=Thermal Optical Analysis;

HPAEC-PAD= High Performance Anion-Exchange Chromatography coupled with Pulsed Amperometric Detection;

GC-MS=Gas Chromatography Mass Spectrometry

Table 2 -Chemical composition of UFPs sampled during winter and summer campaigns

Chemical species	Unit	Winter campaign (n=11)	Summer campaign (n=5)
Total mass	$\mu g/m^3$	2.23±0.41	2.00±0.98
Levoglucosan (*)	ng/m^3	93.3±31.6	0.2±0.1
Mannosan (*)	ng/m^3	13.7±3.6	0.1±0.1
Galactosan (*)	ng/m^3	5.0±1.4	0.5±0.2
Na^+	ng/m^3	14.2±10.5	18.6±6.8
NH_4^+ (*)	ng/m^3	27.8±21.7	70.3±38.0
K^+(*)	ng/m^3	69.4±20.4	5.7±7.4
Mg^+	ng/m^3	0.6±0.5	0.8±0.4
Ca^{2+}	ng/m^3	30.6±22.1	34.1±19.1
Cl^-	ng/m^3	17.3±7.9	19.6±9.6
NO_2^-	ng/m^3	24.0±20.5	10.2±14.0
NO_3^-(*)	ng/m^3	76.5±25.8	13.8±11.8
$SO_4^=$	ng/m^3	39.5±21.8	22.4±18.4
TC	ng/m^3	1155±245	863±319
Benzo(a)anthracene(*)	pg/m^3	83±68	22±3
Benzo(b)fluoranthene (*)	pg/m^3	634±472	35±18
Fluoranthene (*)	pg/m^3	167±189	28±31
Fenanthrene	pg/m^3	114±95	56±13
Chrysene (*)	pg/m^3	295±295	38±9
Pyrene	pg/m^3	398±518	88±86
Benzo(a)pyrene (*)	pg/m^3	143±47	16±9
Anthracene	pg/m^3	39[§]	29±17

Table 2 (continued) -Chemical composition of UFPs sampled during winter and summer campaigns

Chemical species	Unit	Winter campaign (n=11)	Summer campaign (n=5)
Al	pg/m^3	2978±1257	3164±2921
As	pg/m^3	<LOD	53±10
Ba (*)	pg/m^3	150±66	72±48
Cd	pg/m^3	33±12	18±17
Co	pg/m^3	25±24	24[§]
Cr	pg/m^3	597±401	704±142
Cu	pg/m^3	1062±2036	424±219
Fe	pg/m^3	6661±6993	4260±3069
Mn (*)	pg/m^3	114±40	163±35
Mo	pg/m^3	633±449	405±183
Ni	pg/m^3	337±314	240±99
P	pg/m^3	812±407	1025±277
Pb	pg/m^3	532±291	402±163
Sr	pg/m^3	<LOD	49±23
Ti	pg/m^3	64±46	69±27
V	pg/m^3	<LOD	14±6
Zn	pg/m^3	7743±5989	3288±1600

Each value is expressed as mean±SD. Concentrations lower than the limit of detection are reported as <LOD. [§]Only 1 sample with concentration larger than LOD. (*) indicates those species for which the difference between means is statistically significant at $p<0.05$.

Figure 1

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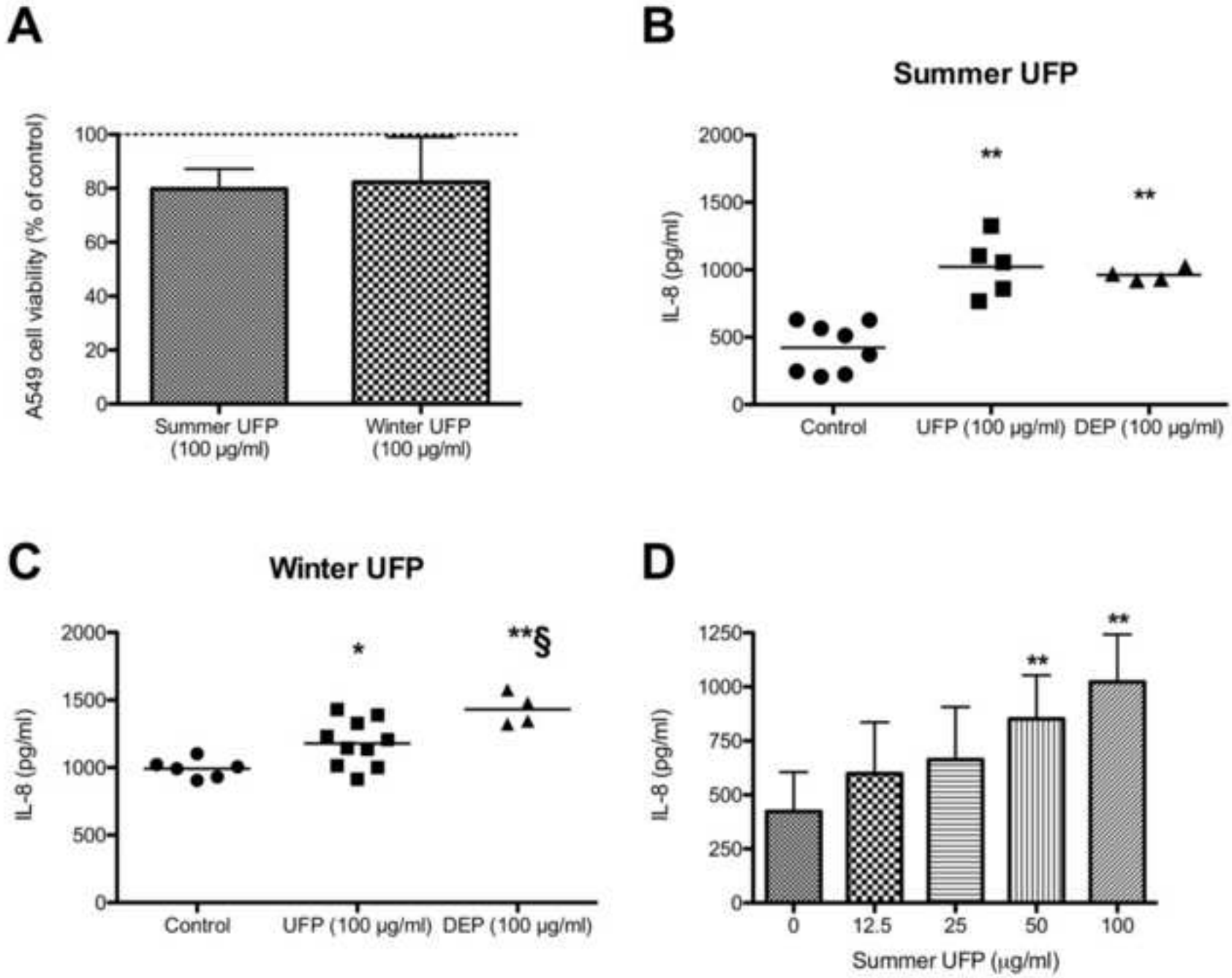

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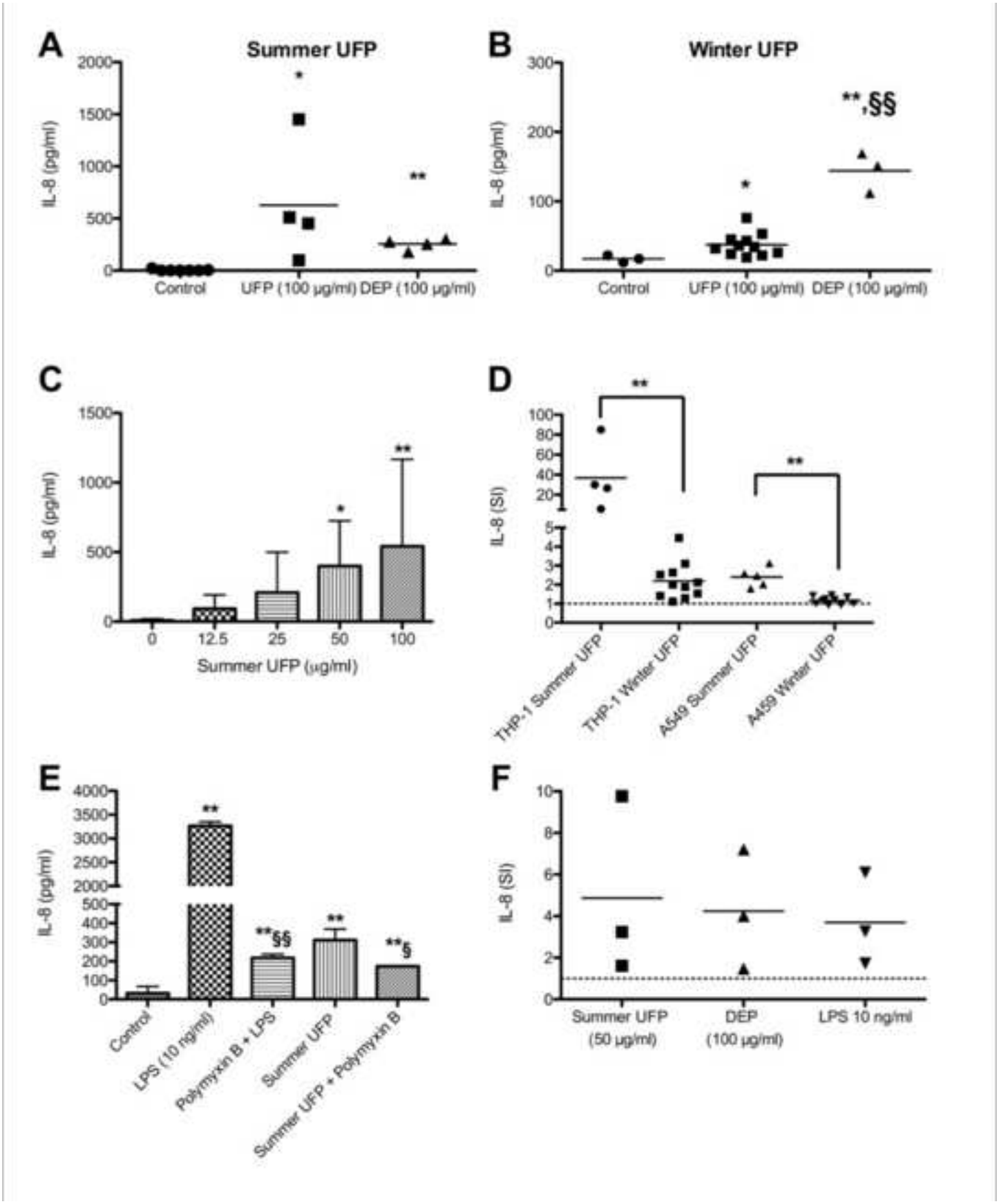

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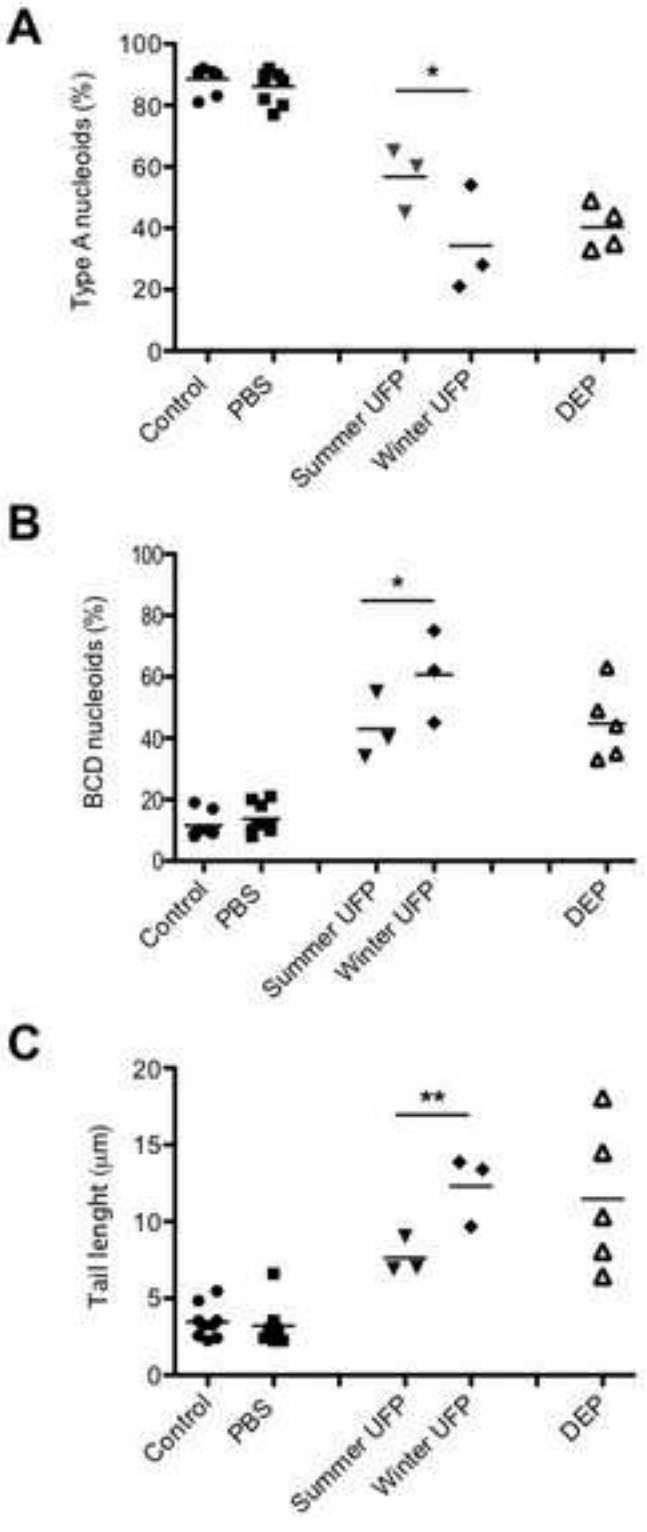

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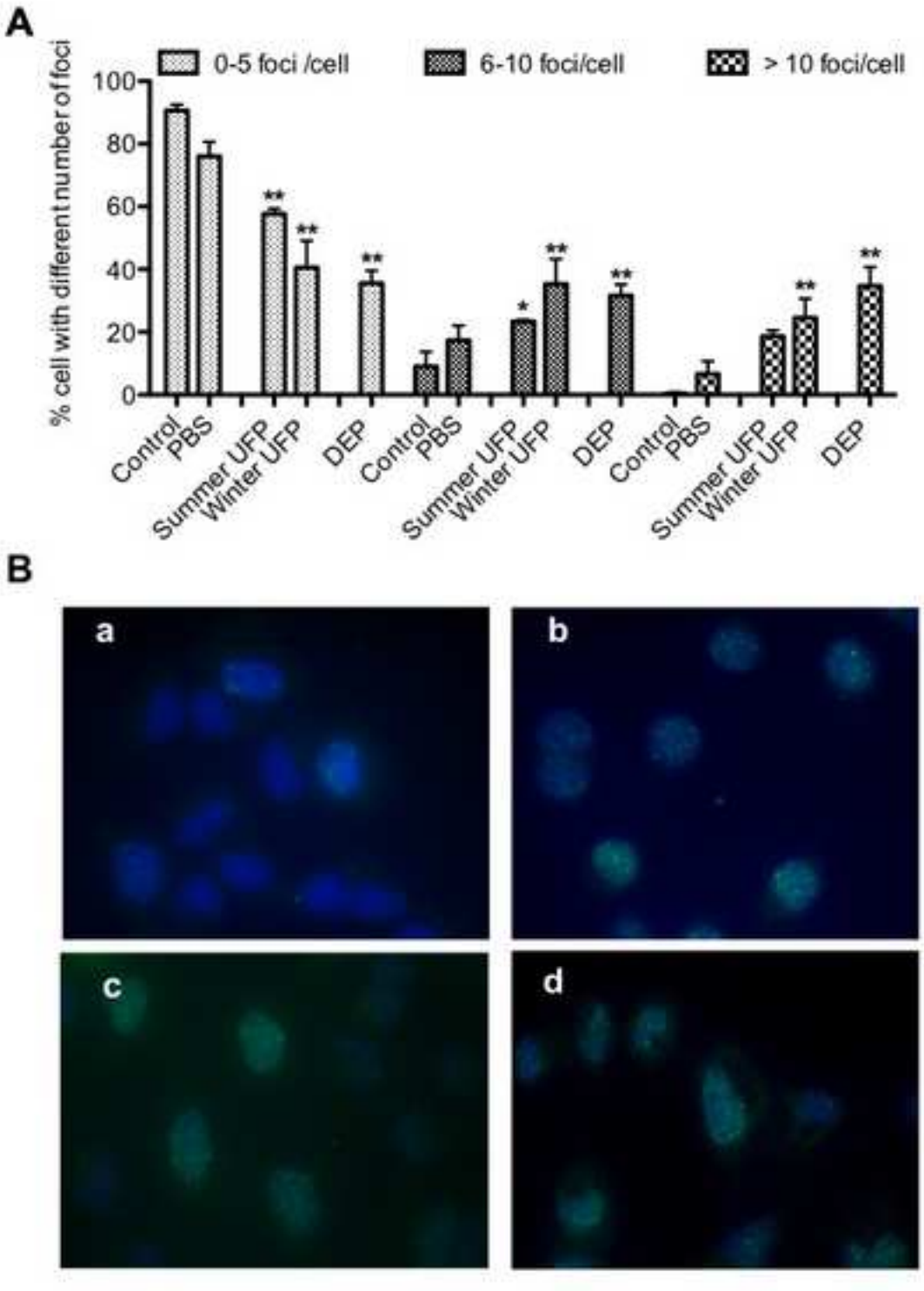

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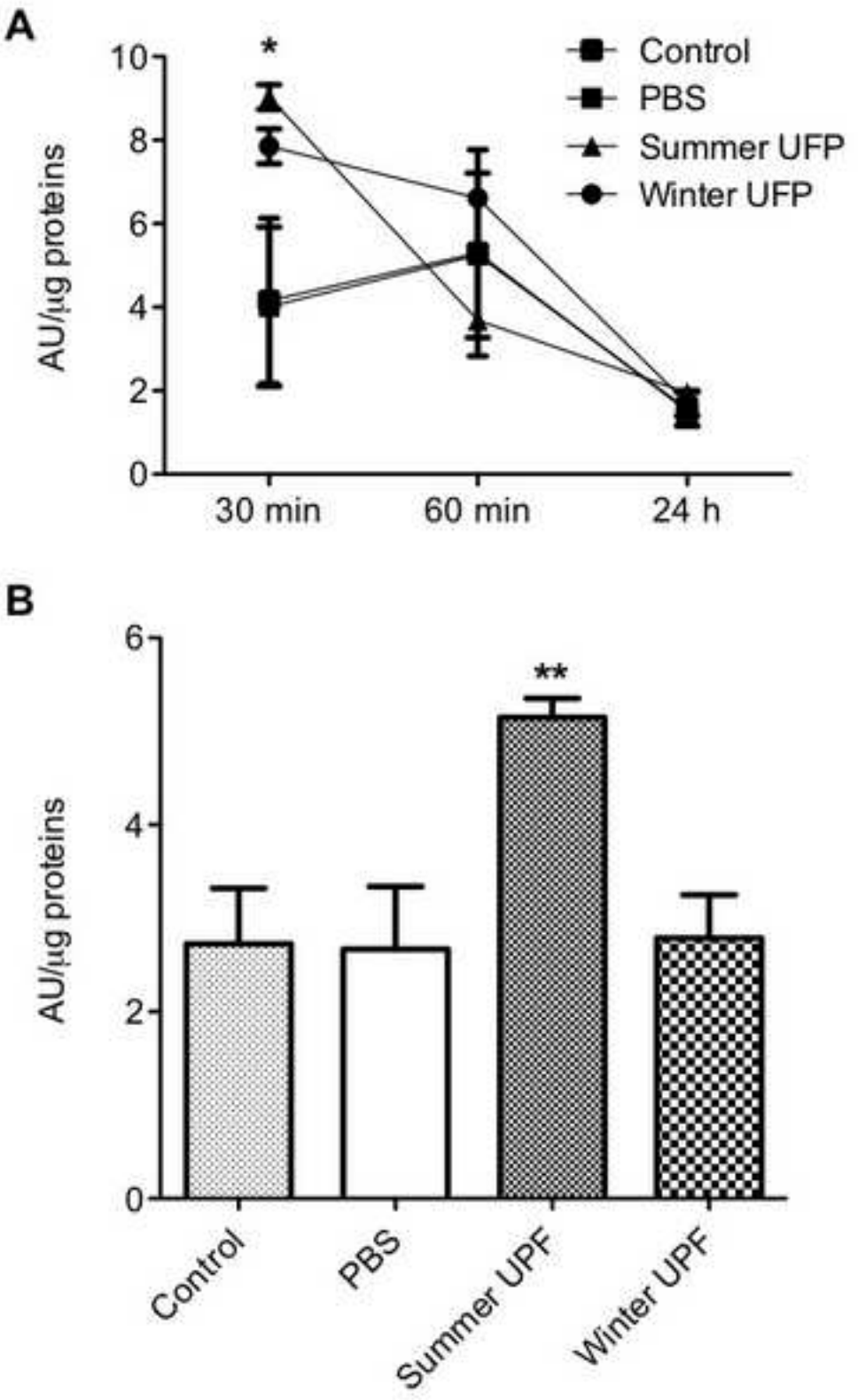

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