

CXCL8 and CXCL11 chemokine secretion in dermal fibroblasts is differentially modulated by vanadium pentoxide

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Abstract. An increase in skin rashes or atopic dermatitis has been observed in individuals working with vanadium. However, to the best of our knowledge no *in vivo* or *in vitro* studies have evaluated the effect of exposure to vanadium in dermal fibroblasts. Cells viability and proliferation were assessed by WST-1 assay, cells were treated with increasing concentrations of V₂O₅ (1, 10 and 100 nM). CXCL8 and CXCL11 concentrations were measured in the supernatants using an ELISA assay. V₂O₅ was not observed as having a significant effect on dermal fibroblast's viability and proliferation. However, it was revealed that V₂O₅ was able to induce the secretion of CXCL8 and CXCL11 chemokines into dermal fibroblasts. V₂O₅ synergistically increased the effect of interferon (IFN) γ on CXCL11 secretion. In addition, V₂O₅ synergistically increased the effect of the tumor necrosis factor α on CXCL8 secretion and abolished the inhibitory effect of IFN γ . V₂O₅ induction of CXCL8 and CXCL11 chemokines may lead to the appearance and perpetuation of an inflammatory reaction into the dermal tissue. Further studies are required to evaluate dermal integrity and manifestations in subjects occupationally exposed, or living in polluted areas.

Introduction

Vanadium is a grey metal that exists in different states of oxidation (ranging from -1 to +5) of which vanadium pentoxide (V₂O₅) is the most usual form.

All vanadium compounds have been considered toxic. The exposure limit to V₂O₅ dust and fumes in workplace

air (8 h work day/40 h work week) has been fixed by the Occupational Safety and Health Administration in 0.05 and 0.1 mg/m³, respectively (1).

The National Institute for Occupational Safety and Health (NIOSH) sets to 35 mg/m³ the dose of vanadium exposure that may cause seriously health issues up to death (1).

Toxic effects of vanadium are reflected mainly on respiratory system, while the effect on the gastrointestinal system is less relevant because of the minimal gut absorption rate of the substance (2-4). Unfortunately, no sufficient data are available in order to determine the reference range of a subchronic or chronic inhaled dose.

Studies conducted on rat models showed the toxic effects (resulted from an oral, or inhaled, vanadium exposures) on serum parameters (5,6), liver (7), nervous (8) and other tissues development (9).

Vanadium workers (NIOSH 1983) showed an increased prevalence of skin rashes, such as atopic dermatitis.

Until now no *in vivo*, or *in vitro*, studies were carried out to evaluate the effect of exposure to vanadium in dermal fibroblasts.

Here, we evaluate the effect of V₂O₅ on viability and proliferation, and secretion of chemokine (C-X-C motif) ligand (CXCL)8, or CXCL11 [an interferon (IFN) γ dependent chemokine, of the same class of CXCL9, and CXCL10] in dermal human fibroblasts.

Materials and methods

Fibroblast cell cultures. We have obtained fibroblasts from derma of six patients who underwent an operation for thyroid nodular goiter (discard dermal material; all females, age range 57-76 years, euthyroid, without other disorders or diseases, and not treated with any kind of drugs).

Involved subjects gave their informed consent and the study was approved by the University of Pisa (Pisa, Italy) Ethics Committee. Tissue explants were firstly minced and then placed in culture dishes, allowing the fibroblasts proliferation (as previously described) (10). Fibroblasts were propagated in 199 medium [with 20% FBS (Gibco; Thermo Fisher Scientific,

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Waltham, MA, USA), gentamycin (20 $\mu\text{g/ml}$), penicillin (100 U/ml), in a 37°C humidified incubator with 5% CO₂; and maintained subsequently in a 199 medium with 10% FBS (and antibiotics) (11). The cells were all used at the 4th passage, and were tested for purity by immunocytochemistry (12).

Proliferation and viability. We have done the WST-1 (Roche Diagnostics, Almere, The Netherlands) assay (that uses 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, in the MTT assay) to evaluate cell viability and proliferation (13-16).

Firstly fibroblasts were seeded in each well of 96-well plates at a concentration of 35,000 cells/ml (in a final volume of 100 μl).

Subsequently V₂O₅ effect on fibroblasts viability and proliferation was determined exposing cells for 24 h with increased concentrations of the compound (1, 10, 100 nM).

Fibroblasts were plated and treated with V₂O₅ or with its vehicle alone (for 24 h), performing all experiments in triplicate for each cell preparation.

As the cell viability and proliferation WST-1 assay may have limitations on evaluating cellular proliferation (17), fibroblasts proliferation was determined also by cell number counting (13-16).

Chemokine secretion assay and ELISA. To perform the CXCL8 and CXCL11 secretion assays, 30,000 cells/ml were seeded in 96-well plates, in a final volume of 100 μl per well, in growth medium, that was removed after 24 h. After cells were washed in PBS, and incubated (24 h) in phenol red and serum-free medium containing IFN γ (500, 1,000, 5,000, 10,000 IU/ml) and/or 10 ng/ml TNF α (all R&D Systems, Minneapolis, MN, USA), alone or in combination (10). The TNF α concentration to obtain the highest secretion was selected in preliminary experiments. After 1 day the supernatants were collected and then kept frozen at -20°C (until chemokine assay).

We treated fibroblasts, for 24 h, with increasing concentrations of V₂O₅ (1, 10, 100 nM), in presence/absence of IFN γ (1,000 IU/ml), and/or TNF α (10 ng/ml), in order to evaluate the effect of V₂O₅ on the chemokine secretion induced by IFN γ .

CXCL8 and CXCL11 concentrations were measured in the supernatants using the ELISA assay. The experiments were carried out three times, for each different cell preparation.

Chemokines levels were measured in culture supernatants, using commercially kits (R&D Systems). The mean minimum detectable dose was 2.7 pg/ml for CXCL8 and 3.2 pg/ml for CXCL11; the intra- and inter-assay coefficients of variation were 3.5 and 6.5% for CXCL8, 4.7 and 8.5% for CXCL11. Quality control pools of low, normal, or high concentration for all parameters were included in each assay.

Statistical analysis. For normally distributed variables values are given in text as mean (\pm SD), or mean (\pm SEM) in figures, otherwise as median [and interquartile range]. Mean group values are compared by one-way analysis of variance (ANOVA) for variables normally distributed, or with the Kruskal-Wallis test, or Mann-Whitney U test. Proportions are compared by the Chi-Square. We have used the Bonferroni-Dunn test for post hoc comparison of normally distributed variables.

Results

Cell proliferation of dermal fibroblasts. Cell counting shows that V₂O₅ (1, 10, 100 nM) does not change viability or proliferation of dermal fibroblasts (Fig. 1). The results of WST-1 assay in dermal fibroblasts with V₂O₅ (1, 10, 100 nM) confirmed the cell counting data: with V₂O₅ 1 nM it was 99% with respect to the control; with V₂O₅ 10 nM it was 97% with respect to the control; and with V₂O₅ 100 nM it was 98% with respect to the control.

Fibroblast secretion of CXCL8. In basal conditions, the secretion of CXCL8 (range, 51-213 pg/ml) was measured in all preparations of cultured dermal fibroblasts (Fig. 2).

CXCL8 secretion increased in a dose-dependent manner using different concentrations of TNF α (1, 5, 10 ng/ml), with the highest response reached with 10 ng/ml TNF α (basal 156 \pm 46 pg/ml vs. TNF α 1154 \pm 321 pg/ml; P<0.01) (Fig. 2).

The basal CXCL8 secretion was significantly inhibited by IFN γ in a dose-dependent manner (CXCL8: 84 \pm 37, 34 \pm 25, pg/ml; respectively, with IFN γ 500 or 1,000 IU/ml; ANOVA, P<0.05), while TNF α alone (10 ng/ml) significantly stimulated the CXCL8 secretion (P<0.01) (Fig. 3). Combining IFN γ with TNF α led to a significant reversal of the stimulating effect of TNF α (TNF α +IFN γ 661 \pm 176 pg/ml vs. TNF α 1154 \pm 321 pg/ml; P<0.05) (Fig. 3). However, the stimulating effect of TNF α on the secretion of CXCL8 was not completely reversed by IFN γ , because the concentration of this chemokine was still significantly higher than in basal conditions (TNF α +IFN γ vs. basal; P<0.01).

When fibroblasts were treated with increased V₂O₅ concentrations (1, 10, 100 nM) the CXCL8 release was dose-dependently stimulated (P<0.0001, by ANOVA) (Fig. 4).

When treating dermal fibroblasts with V₂O₅ (100 nM), together with IFN γ , CXCL8 release was not significantly changed with respect to the basal condition, and IFN γ suppressed the V₂O₅ stimulating effect, but it stills increased it compared to IFN γ alone (Fig. 5).

V₂O₅ (100 nM) plus TNF α elicited a synergistic effect on CXCL8 secretion (P<0.0001, by ANOVA), compared to TNF α alone (Fig. 6).

The CXCL8 release synergistically increased (P<0.0001, by ANOVA), when fibroblasts were treated with V₂O₅ (100 nM) with the combination of IFN γ and TNF α , abolishing the inhibitory effect of IFN γ (Fig. 7).

Fibroblast secretion of CXCL11. CXCL11 release was induced by IFN γ in a dose-dependent manner (CXCL11: 0, 31 \pm 17, 87 \pm 35, 123 \pm 47, 187 \pm 52 pg/ml; respectively, with IFN γ 0, 500, 1,000, 5,000, 10,000 IU/ml; ANOVA, P<0.001).

TNF α alone had no effect (chemokine remaining undetectable), while the combination of IFN γ and TNF α had a significant synergistic effect on the CXCL11 secretion (CXCL11, 1724 \pm 252 vs. 87 \pm 35 pg/ml with IFN γ alone, ANOVA, P<0.0001).

When fibroblasts were treated with increased V₂O₅ concentrations (1, 10, 100 nM) the CXCL11 release was dose-dependently stimulated (ANOVA, P<0.0001) (Fig. 8).

CXCL11 release was not significantly changed treating cells with V₂O₅ (100 nM), together with TNF α , with respect to V₂O₅ alone (data not shown).

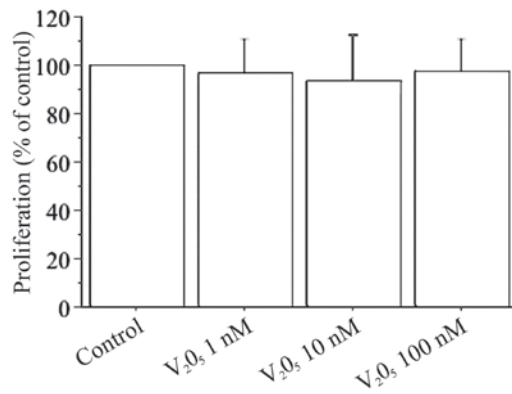


Figure 1. V₂O₅ treatment does not significantly change the proliferation of dermal fibroblasts (mean group values are compared using one-way analysis of variance; the Bonferroni-Dunn test was used for post-hoc comparison; P>0.05, for all comparisons). V₂O₅, vanadium pentoxide.

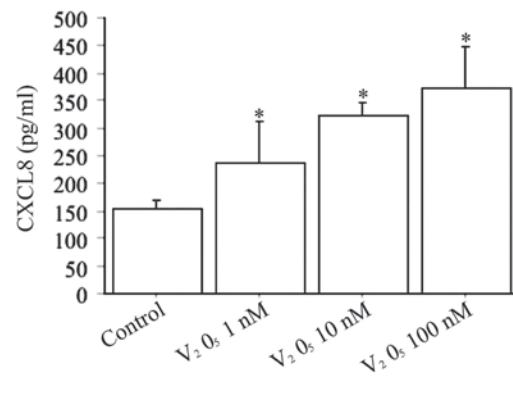


Figure 4. CXCL8 release was dose-dependently stimulated by treating dermal fibroblasts with V₂O₅ (1, 10 and 100 nM). *P<0.05 vs. control (the Bonferroni-Dunn test was used for post-hoc comparison). V₂O₅, vanadium pentoxide.

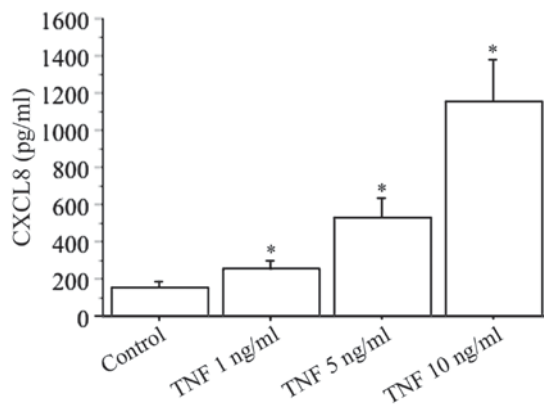


Figure 2. CXCL8 secretion was measured in all cultured dermal fibroblasts preparations in basal conditions (control); its secretion increased significantly and dose-dependently with different concentrations of TNF α (1, 5 and 10 ng/ml) (the Bonferroni-Dunn test was used for post-hoc comparison). *P<0.05 vs. control. TNF, tumor necrosis factor.

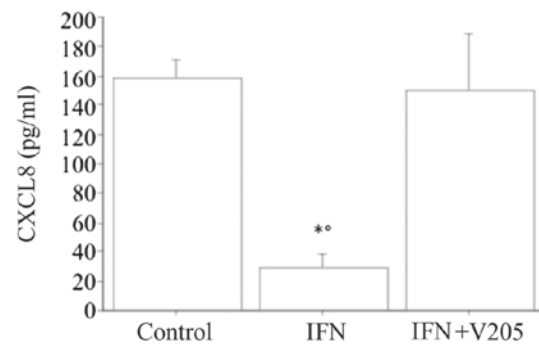


Figure 5. Treatment of dermal fibroblasts with V₂O₅ (100 nM) abolished the inhibitory effect of IFN γ (the Bonferroni-Dunn test was used for post-hoc comparison; *P<0.05 vs. Control; °P<0.05 vs. IFN+V₂O₅). V₂O₅, vanadium pentoxide; IFN, interferon.

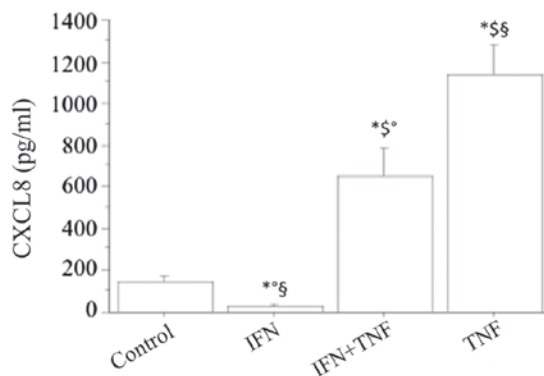


Figure 3. IFN γ (1,000 IU/ml) significantly inhibited the basal CXCL8 secretion. The stimulating effect of TNF α was significantly reversed after the addition of IFN γ (the Bonferroni-Dunn test was used for post-hoc comparison; °P<0.05 vs. Control; *P<0.05 vs. TNF; §P<0.05 vs. IFN+TNF; §P<0.05 vs. IFN). IFN, interferon; TNF, tumor necrosis factor.

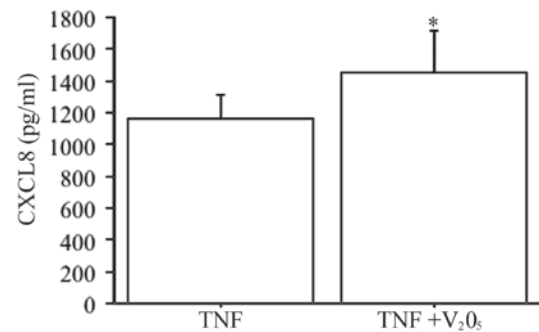


Figure 6. CXCL8 release was significantly increased by treating dermal fibroblasts with V₂O₅ (100 nM) and TNF α . *P<0.05 vs. the TNF group. V₂O₅, vanadium pentoxide; TNF, tumor necrosis factor.

When treating fibroblasts with V₂O₅ (100 nM), *plus* IFN γ , CXCL11 release synergistically increased (P<0.0001, by ANOVA), compared to both IFN or V₂O₅ alone (Fig. 9).

CXCL11 release was synergistically increased (ANOVA, P<0.0001) when fibroblasts were treated with V₂O₅ (100 nM), together with IFN γ and TNF α stimulation, compared to IFN γ +TNF α (Fig. 10).

Discussion

Our results demonstrate that V₂O₅ stimulates the secretion of the CXCL8 chemokine, and of the IFN γ dependent chemokine CXCL11, in dermal fibroblasts, without altering their viability

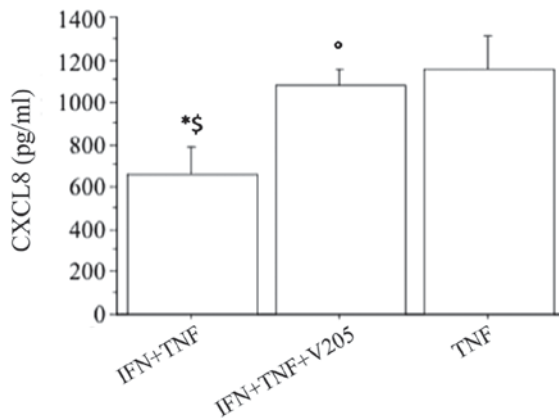


Figure 7. Treatment of dermal fibroblasts with V_2O_5 (100 nM) + $IFN\gamma$ + $TNF\alpha$ stimulation significantly increased CXCL8 release (the Bonferroni-Dunn test was used for post-hoc comparison; * $P < 0.05$ vs. $IFN+TNF+V_2O_5$; $^{\$}P < 0.05$ vs. TNF ; $^{\circ}P < 0.05$ vs. $IFN+TNF$). V_2O_5 , vanadium pentoxide; TNF , tumor necrosis factor; IFN , interferon.

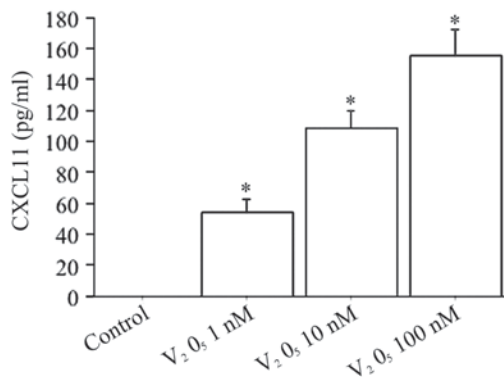


Figure 8. CXCL11 release was dose-dependently stimulated by treating dermal fibroblasts with V_2O_5 (1, 10 and 100 nM). * $P < 0.05$ vs. the control (the Bonferroni-Dunn test was used for post-hoc comparison). V_2O_5 , vanadium pentoxide.

and proliferation. Moreover, our study confirms that $IFN\gamma$ and $TNF\alpha$ stimulated in a different way, the secretion of CXCL8, or CXCL11, chemokines as expected (18). Interestingly, V_2O_5 can synergize with $IFN\gamma$ and $TNF\alpha$, furtherly increasing CXCL11 secretion. In addition, V_2O_5 combined with $TNF\alpha$, elicited a synergistic influence on CXCL8 chemokine production, abolishing the inhibitory effect of $IFN\gamma$.

These results, on the whole, agreed with the view that V_2O_5 is able to induce and perpetuate an inflammatory disorder in the dermal tissue inducing inflammatory chemokines secretion (13).

Our findings regarding $TNF\alpha$, and $IFN\gamma$ effect in fibroblasts are in line with the results of another study in a different type of cells. In fact, it has been recently investigated if CXCL8 and CXCL10 chemokines secretion by normal human thyrocytes is dependent upon specific proinflammatory stimuli. CXCL8, but not CXCL10 (an $IFN\gamma$ inducible chemokine, of the same class of CXCL11), was detected in basal conditions. The two chemokines showed differences in their response to proinflammatory cytokines.

Actually, $IFN\gamma$ induced a significant CXCL10 secretion, not obtained with $TNF\alpha$; whereas CXCL8 was secreted in response to $TNF\alpha$, being instead inhibited by $IFN\gamma$. The

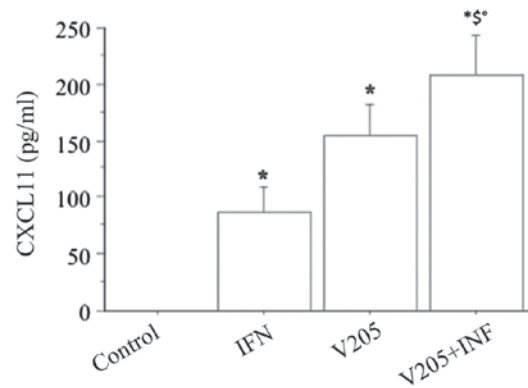


Figure 9. When dermal fibroblasts were treated with V_2O_5 (100 nM) + $IFN\gamma$ CXCL11 release was significantly increased * $P < 0.05$ vs. the control group. $^{\$}P < 0.05$ vs. the IFN group. $^{\circ}P < 0.05$ vs. the V_2O_5 group (the Bonferroni-Dunn test was used for post-hoc comparison). V_2O_5 , vanadium pentoxide; IFN , interferon.

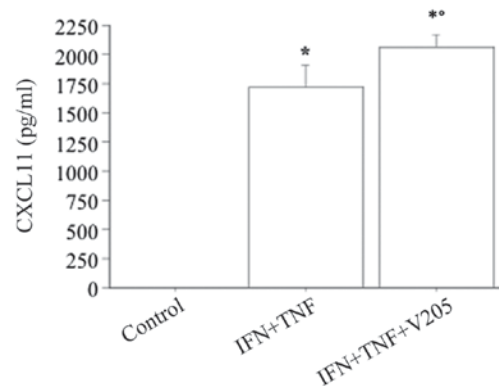


Figure 10. CXCL11 release was significantly increased by treating dermal fibroblasts with V_2O_5 (100 nM) + $IFN\gamma$ and $TNF\alpha$ * $P < 0.05$ vs. the control group. $^{\circ}P < 0.05$ vs. the $IFN+TNF$ group (the Bonferroni-Dunn test was used for post-hoc comparison). V_2O_5 , vanadium pentoxide; TNF , tumor necrosis factor; IFN , interferon.

combination of $TNF\alpha$ plus $IFN\gamma$ synergistically increased the $IFN\gamma$ -induced CXCL10 secretion, while reversed the $TNF\alpha$ -induced CXCL8 secretion (19).

$IFN\gamma$ -inducible CXC chemokines can be produced by several types of normal mammalian cells, such as thyrocytes, fibroblasts, colon epithelial cells, islet cells, and others (10,13,14,19-25). However, these cells are not able to produce the CXC chemokines in basal condition, but only when stimulated by cytokines, such as $IFN\gamma$ and $TNF\alpha$, that are released in a T-helper 1 (Th1) type inflammatory site, such as the thyroid at the beginning of Graves' disease, by Th1 activated lymphocytes. It has been suggested that this process can be involved in the initiation and the perpetuation of the inflammation in several autoimmune diseases (10,13,14,19-25), and considering our results it can be applied to the thyroid, too.

Our findings about vanadium stimulation of chemokines agree with those of other studies conducted in different cell types. V_2O_5 exposure is a cause of occupational bronchitis; a study evaluated gene expression profiles in human lung fibroblasts (in cultures) after V_2O_5 exposure with the aim to identify genes that could be implicated in the bronchial inflammation, repair, and fibrosis in the pathogenesis of bronchitis. Among

the 10 genes overexpressed by V_2O_5 , also *CXCL8*, *CXCL9* and *CXCL10* were induced (26).

Another study reports that fibroblasts have a role in the innate immune response to vanadium-induced oxidative stress through the synthesis of $IFN\beta$ and the activation of STAT-1 that cause an increase of *CXCL10* levels (27).

Interestingly vanadium can increase chemokine secretion in a dose range, from 1 to 100 nM. It could be observed that normal blood levels of vanadium are ranging from 0.45 to 18.4 nM, and that 100 nM is a dose that might mimic an abnormally high exposure (28). So we could hypothesize that the induction of an inflammatory reaction into the dermal tissue could predispose to the appearance of skin rashes, or atopic dermatitis.

In conclusion our study shows that V_2O_5 can induce *CXCL8*, and *CXCL11* chemokines secretion into the dermal fibroblasts. Interestingly, V_2O_5 synergistically increased the effect of the $IFN\gamma$ on *CXCL11* secretion. Moreover, V_2O_5 synergistically increased the effect of the $TNF\alpha$ on *CXCL8* secretion, abolishing the inhibitory effect of $IFN\gamma$. Overall *CXCL8*, and *CXCL11* chemokines induction by V_2O_5 could lead to the appearance and perpetuation of an inflammatory reaction into the dermal tissue. Further studies are needed to evaluate dermal integrity, and manifestations in subjects occupationally exposed, or living in polluted areas.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

PF, SB, AA and SMF made substantial contributions to the conception and design of the study and to the acquisition of data. All authors analyzed the data. PF, SB, AA and SMF drafted the manuscript. AA revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the study are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all study participants and the study was approved by the University of Pisa Ethics Committee.

Consent for publication

Written informed consent was obtained from all participants for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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