# Indoor pollution: Dysregulations of mitochondrial functions induced by formaldehyde

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## Abstract

**Background**: People spend about 90% of their time indoors where they are exposed to chemicals, such as volatile organic compounds (VOCs). Among these agents, formaldehyde has been classified as priority indoor pollutants. These pollutants are known to have a negative impact on skin health especially on mitochondrial functions, leading or contributing to some disorders such as accelerated skin aging.

**Methods**: The effects of formaldehyde (250µM) on mitochondrial functions of primary keratinocytes have been investigated. ATP production, as well as mitochondrial membrane potential (JC-1 red-to-green fluorescence), have been evaluated. Modulations of MOTS-c, a mitochondrial-derived peptide involved in metabolism, were also explored by ELISA. SIRT3 (sirtuin 3) protein level and Lon protease (LONP) content were evaluated by immunofluorescence. Then, the protecting effects of SM3 (a multimineral active ingredient) against damage induced by formaldehyde has been investigated.

**Results**: Formaldehyde exposure induced mitochondrial dysfunctions: drops in ATP production (-43%, $p<0.001$) and mitochondrial membrane potential (-46%, $p<0.001$), as well as the decrease of MOTS-c protein level (-54%, $p<0.001$). Moreover, formaldehyde exposure induced the downregulation of SIRT3 (-93%, $p<0.05$) and alterations of mitochondrial proteasome such as LONP content (-98%, $p<0.01$) in keratinocytes. SM3 showed an interesting protection capacity on mitochondrial functions, protecting mitochondrial metabolism, its antioxidant activity and mitochondrial proteolysis.

**Conclusion**: Formaldehyde induced mitochondrial metabolism dysfunctions, down-regulated antioxidant activity and mitochondrial proteolysis. SM3, is a promising active ingredient for protecting skin from damage induced by indoor pollution.

**Keywords:** indoor pollution; formaldehyde; mitochondria; keratinocytes.

## Introduction.

As a physical barrier between the body and the external environment, skin is challenged everyday by atmospheric pollutants.
The indoor environment has always been considered free from health-damaging pollutants. However, indoor air quality may be far from perfect. Indeed, human activities such as cooking and the use of cleaning chemicals can introduce harmful particulate matter and volatile organic compounds (VOCs). Moreover, emission of VOCs also occurs from the use of organic solvents in paints, varnishes, vehicle refinishing products in repairing car paint, environmental tobacco smoke, stored fuels, exhaust from cars and from emissions from industrial facilities [1, 2].
So, VOCs are an important indoor source of air pollutants. Moreover, for most VOCs, the indoor concentrations are typically 2–5 times higher than outdoors [3]. These pollutants are known to have a negative impact on skin health especially on mitochondrial functions, leading or contributing to some disorders such as accelerated skin aging.

One of the most harmful and injurious VOC is formaldehyde (HCHO), which has been classified as priority indoor pollutants known to induce airway irritation and an inflammatory response [4-6].
Formaldehyde is used as adhesive in pressed wood products, building materials, a preservative in paints, coatings, and as a finish to coat paper products [1, 7, 8]. At room temperature, formaldehyde is a gas but is very soluble in water. So, exposure to formaldehyde can occur via inhalation, ingestion and dermal contact.
A major mechanism by which pollution exerts a detrimental effect on the skin is through the generation of oxidative stress. Moreover, VOC exposure has been described as a mitochondrial ROS inducer, leading to alterations in cutaneous cell homeostasis [9]. In this context, the aim of this study was to investigate the effects of formaldehyde on mitochondrial functions.

## Materials and Methods.

Mitochondrial metabolism

Normal human keratinocytes from a Caucasian donor of 41 years old were cultivated in 96-well plates in KGM-gold medium (Lonza). After 3 days of incubation at 37°C and 5% $CO_2$, SM3 at 0.005% or dexamethasone at 500 nM, as a positive reference, were added to the culture medium. After 24h, the media were replaced by an indoor pollution stress, formaldehyde at 250µM, for 24h.
Cells were lysed (PBS buffer and sonification) to analyze ATP (adenosine triphosphate) production.
For JC-1 staining, at the end of formaldehyde incubation, 20µM JC-1 (Dojindo) was added to the medium for 10 min at 37°C in the dark to label the mitochondria. Normal

mitochondrial potential showed red fluorescence (590 nm), and damaged mitochondrial potential showed green fluorescence (530 nm). The red-to-green fluorescence intensity was used to quantify the mitochondrial membrane potential.
MOTS-c was evaluated using a specific ELISA kit (RayBioTech).
All experimental conditions were performed in 4 replicates (n=4).

Oxidative stress & mitoproteostasis

Normal human keratinocytes were cultivated in Labtek in KGM-gold medium (Lonza). After 3 days of incubation at 37°C and 5% $CO_2$, SM3 at 0.005% or dexamethasone at 500 nM, as a positive reference, were added to the culture medium. After 24h, the media were replaced by an indoor pollution stress, formaldehyde at 250µM, for 24h.
At the end of the incubation, the cells were rinsed, fixed and permeabilized. Studies of SIRT3 (Abcam) and LONP (Abcam) modulations were then carried out. Results were normalized by counting nuclei in parallel by Hoechst labeling.
Each condition was tested in triplicates (n=3).

Results analysis

For each evaluated parameter, mean and standard deviation were calculated. A percentage of effect versus formaldehyde-exposed cells was also calculated.
Statistical significance was assessed using a two-tailed, unpaired Student's t-test, with $p<0.05$ being considered significant.

**Results**.

Protection of mitochondrial metabolism

The primary function of mitochondria is to produce ATP for cellular metabolism.
Interestingly, ATP production was significantly reduced after exposure to formaldehyde by 43% ($p<0.001$). Positive reference (dexamethasone 500 nM) and SM3 (0.005%) induced a protection of ATP production by 20% and 29% respectively (Figure 1).

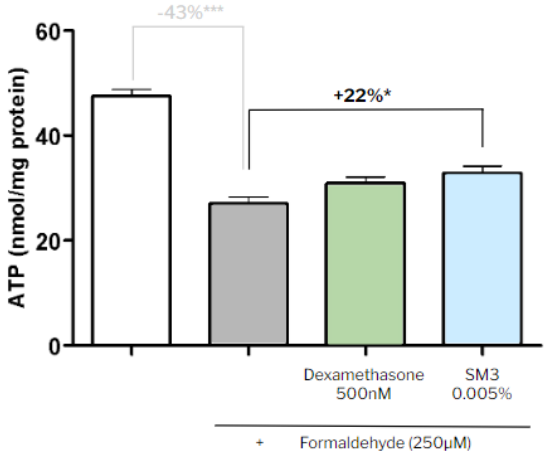

**Figure 1**: Effects of formaldehyde on ATP level in keratinocytes and evaluation of SM3 (0.005%)
Statistical significance: *: p<0.05, ***: p<0.001

At the molecular level, mitochondria convert the mitochondrial membrane potential into ATP with the help of the mitochondrial respiratory complex. So, mitochondrial membrane potential was observed using the JC-1 probe. The red-to-green fluorescence intensity was used to quantify the mitochondrial membrane potential.

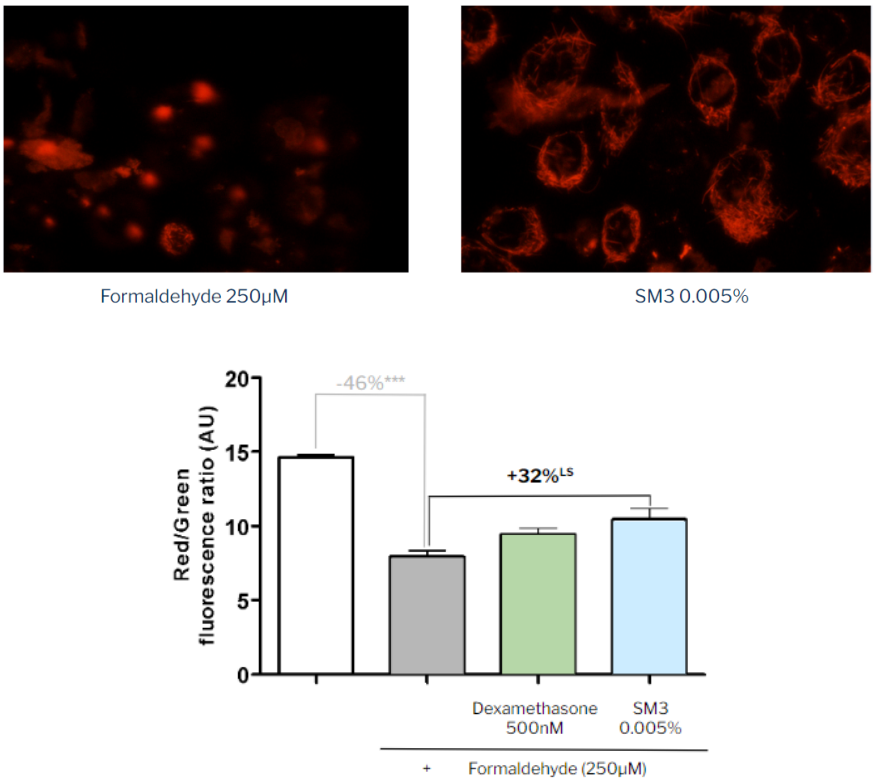


**Figure 2**: Effects of formaldehyde on mitochondrial membrane potential using JC-1 probe in keratinocytes and evaluation of SM3 (0.005%) - Statistical significance: LS (limit of significance): p<0.1, ***: p<0.001

As shown in Figure 2, formaldehyde demonstrated a significant decrease in JC-1 red-to-green fluorescence following treatment by 46% (p<0.001). So, as expected, the drop in ATP content was concomitant with the one of mitochondrial membrane potential.
Positive reference (dexamethasone 500 nM) induced a protection of the mitochondrial membrane potential by 22%. At 0.005%, SM3 protected keratinocytes from the drop in mitochondrial membrane potential induced by formaldehyde treatment by 38% (+32% *vs* formaldehyde-stressed cells, p<0.1).
Because MOTS-c (mitochondrial open reading frame of the 12S ribosomal RNA type-c) plays a role in metabolism regulation and can be activated by oxidative stress, we examined whether formaldehyde exposure resulted in a modulation of MOTS-c protein level.

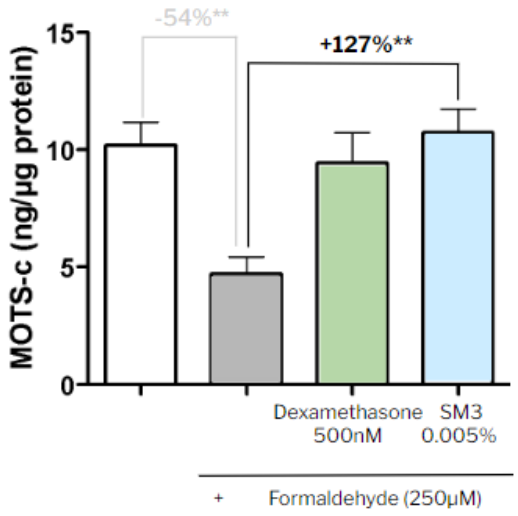


**Figure 3**: Effects of formaldehyde on MOTS-c protein content in keratinocytes and evaluation of SM3 (0.005%) - Statistical significance: **:p<0.01

Formaldehyde treatment induced a significant decrease in MOTS-c level in keratinocytes (-54%, p<0.001). Positive reference (dexamethasone 500 nM) showed a significant protection of MOTS-c by 86%. SM3 0.005% permitted the reverse of the downregulation of MOTS-c protein level induced by formaldehyde by 127% (p<0.01, protection of 110%) (Figure 3).

To see whether this metabolism collapse could induce alterations in mitochondrial ROS production, we measured SIRT3 protein level using immunolabeling. Indeed, SIRT3 has shown to be correlated with oxidative stress by deacetylation of SOD2, a mitochondrial antioxidant enzyme.

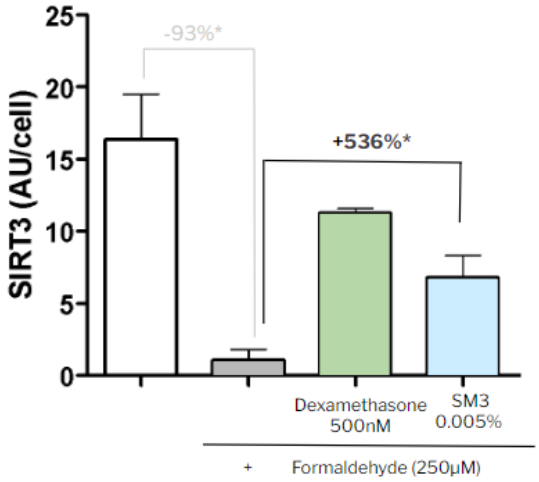

**Figure 4**: Effects of SM3 (0.005%) on SIRT3 protein content in kératinocytes
Statistical significance: *: p<0.05

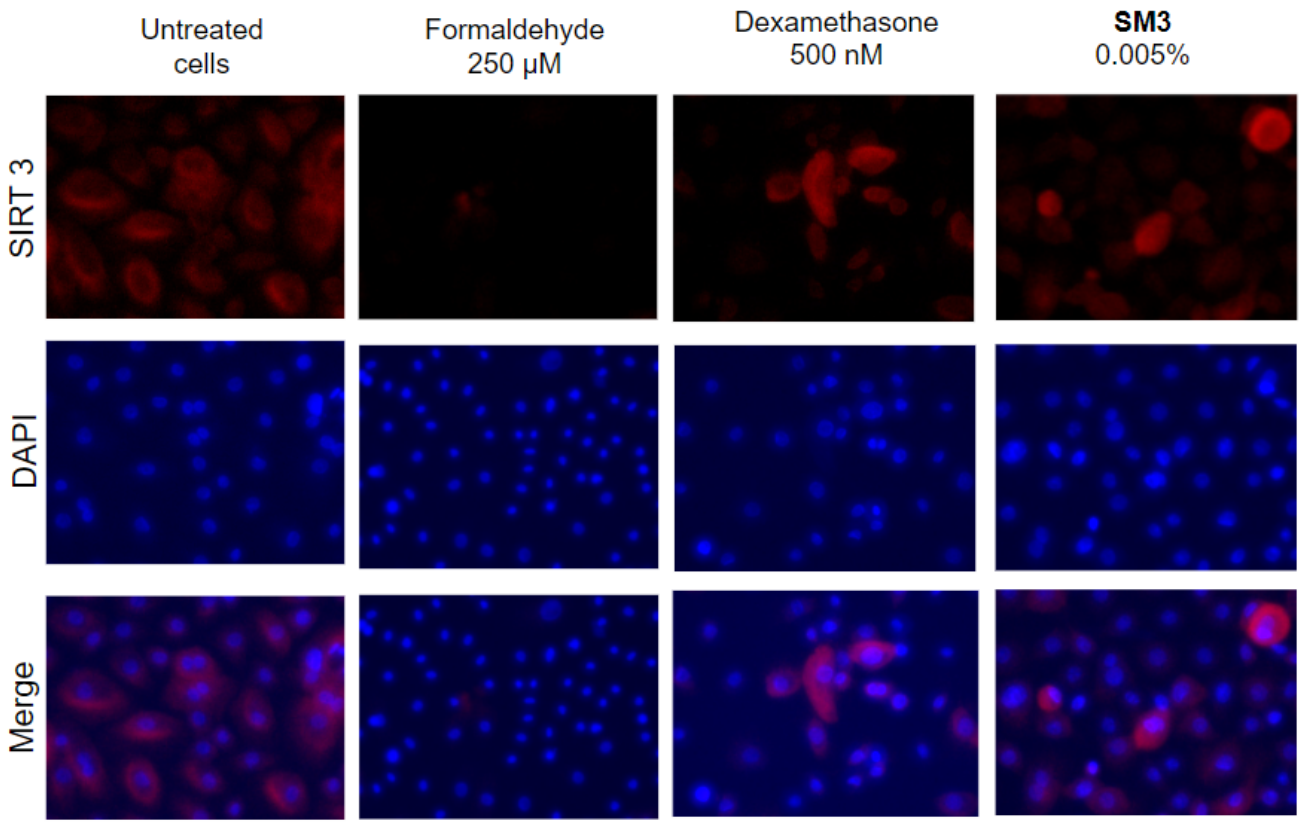


**Figure 5**: Immunofluorescence stainings of SIRT3.
Effects of SM3 (0.005%) in keratinocytes (red: SIRT3, blue: nuclei).

As shown in Figures 4 and 5, formaldehyde drastically reduced SIRT3 protein level in keratinocytes by 93% ($p<0.05$). Positive reference (dexamethasone 500 nM) as well as SM3 (0.005%) induced a protection of SIRT3 by 67% ($p<0.001$) and 37% ($p<0.05$) respectively.

Protection of mitoproteostasis

The degradation of oxidized proteins in mitochondria is essential to maintain mitochondrial homeostasis. To characterize the effects of formaldehyde exposure on proteasome activity, the effect of formaldehyde exposure on Lon protease (LONP) activity was studied. As

LONP is an ATP-stimulated protease and formaldehyde induced a significant decline in ATP synthesis in keratinocytes, formaldehyde exposure should also decrease LONP level.

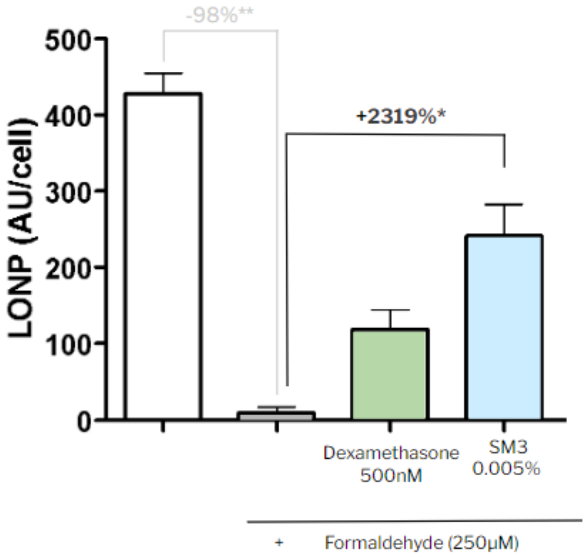


**Figure 6:** Effects of formaldehyde on LONP protein content in keratinocytes and evaluation of SM3 (0.005%) - Statistical significance: *: p<0.05; **:p<0.01

As expected, formaldehyde treatment induced a drastic significant decrease in LONP level in keratinocytes (-98%, p<0.01). Positive reference (dexamethasone 500 nM) showed a significant protection of LONP by 26%. SM3 also induced a significant protection of LONP protein level by 55% (Figures 6 and 7).

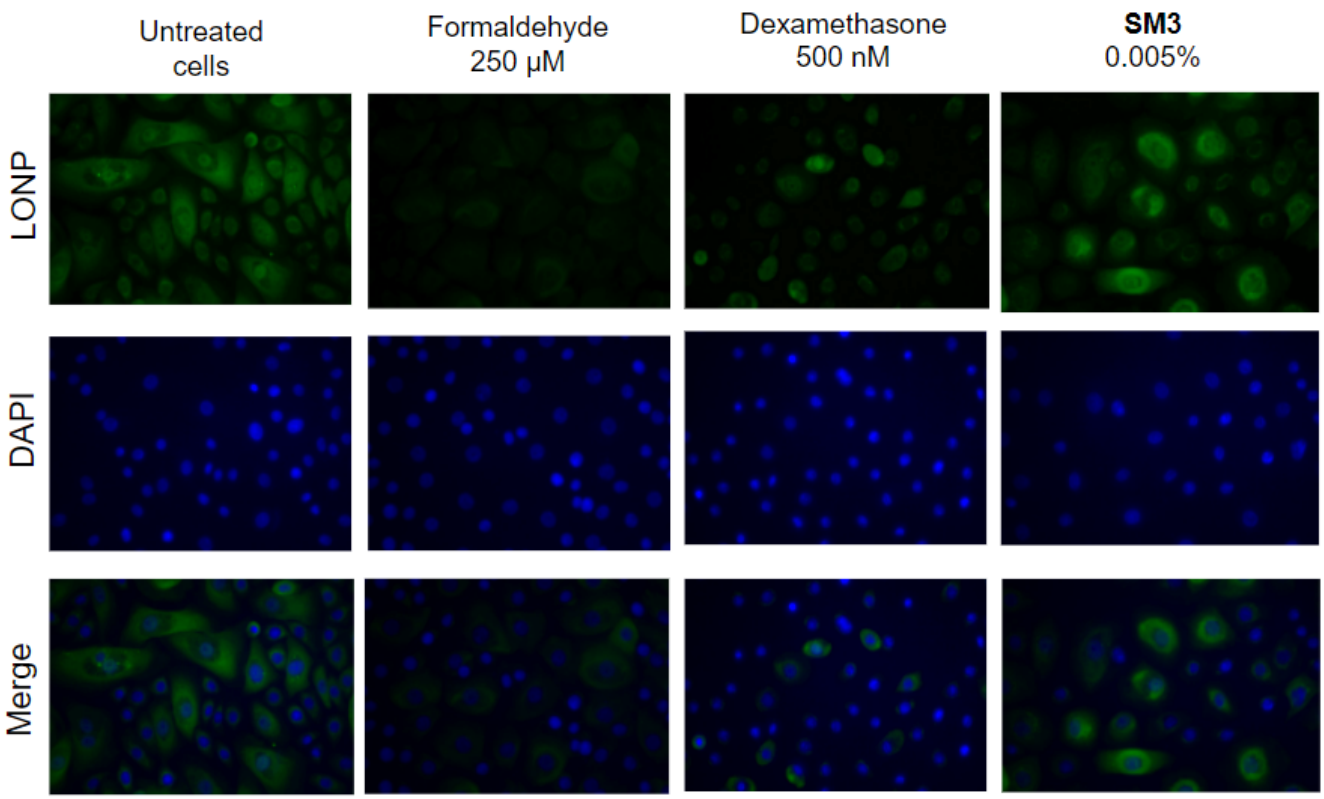


**Figure 7**: Immunofluorescence stainings of LONP.
Effects of SM3 (0.005%) in keratinocytes (green: LONP, bleu: nuclei)

**Discussion.**

This study evaluated the effects of formaldehyde exposure on keratinocytes. A major mechanism by which pollution exerts a detrimental effect on the skin is through the generation of oxidative stress. This oxidative stress overwhelms the skin's defenses by quickly depleting the enzymatic (glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase) and non enzymatic (vitamin E, vitamin C and glutathione) anti-oxidant capacity. Moreover, free radicals and reactive oxygen species (ROS) generated can act at different cellular levels inducing damage to proteins, lipids and DNA. ROS contribute also to the induction of an inflammatory state by the release from skin cells of pro-inflammatory mediators such as cytokines [2, 10, 11].

Alterations of normal functions of lipids, DNA and/or proteins in the human skin via oxidative damage, lead to extrinsic skin aging and inflammatory or allergic conditions such as contact dermatitis, atopic dermatitis, psoriasis, acne and skin cancer [2].

Moreover, VOC exposure has been described as a mitochondrial ROS inducer, leading to alterations in cutaneous cell homeostasis [9].

In this study we focused on formaldehyde effects on mitochondrial function. Indeed, mitochondria are essential double membrane cellular organelles which are closely associated with cell homeostasis. Mitochondrial homeostasis has been reported to be at the center of cell fate. Properly functioning mitochondria produce ATP and modulate the cell redox balance to ensure cell metabolism [12].
Exposure to formaldehyde resulted in a decrease of ATP production and in a drop in mitochondrial membrane potential (JC-1 red-green fluorescence). These results indicated that formaldehyde exposure induced injuries in mitochondria that have a lower mitochondrial potential (JC-1) and then cannot convert energy substrates into ATP.
As damaged mitochondria are an additional source of ROS and considering that MOTS-c can be activated by oxidative stress, we examined whether formaldehyde exposure resulted in a modulation of MOTS-c protein level. MOTS-c (mitochondrial open reading frame of the 12S ribosomal RNA type-c) is a mitochondrial-derived peptide that promotes metabolic homeostasis and plays a role in regulation of metabolism [13, 14].
Formaldehyde exposure induced a significant decrease in MOTS-c level in keratinocytes, confirming that VOCs can cause mitochondrial dysfunctions/damage.
To see whether this metabolism collapse could induce alterations in mitochondrial ROS production, we next measured sirtuin 3 (SIRT3) protein level using immunolabeling. SIRT3 is the major mitochondrial NAD+-dependent deacetylase, and its activity was shown to be correlated with oxidative stress [15, 16]. Of note, SIRT3 plays a crucial role in SOD2 activity [17, 18]. Moreover, SIRT3-mediated deacetylation of FOXO3 (forkhead box O3) also reduces levels of cellular ROS by upregulating the antioxidant enzymes manganese superoxide dismutase and catalase and protects mitochondria against oxidative damage [19]. Results obtained clearly showed that formaldehyde drastically reduced SIRT3 protein level in keratinocytes.

Dysfunctional mitochondria and elevated production of oxidants has been associated with numerous diseases and with the aging process itself [20].
Irreversible oxidative damage can impair or completely inactivate enzymes and structural proteins which, if not rapidly removed, can aggregate, cross-link, and cause significant cellular toxicity. Proteases contribute to the degradation of misfolded and damaged proteins and/or the maintenance of mitochondrial genome stability.
There are 3 known proteases that have been shown to degrade damaged proteins in the mitochondria, all of which are ATP-stimulated: the AAA protease, the Clp-like protease and the Lon protease (LONP) [21].
The AAA protease is localized to the mitochondrial inner membrane, while Clp and Lon are both found in the matrix. These proteases contribute to the degradation of short lived, misfolded, or damaged proteins. LONP has been shown to be the main protease for degradation of oxidized proteins [22]. Moreover, it has been shown that LONP downregulation leads to impaired mitochondrial proteolysis [23].

VOCs, such as formaldehyde, produce ROS that lead to the production of oxidized proteins which are preferentially degraded by the proteasome. Recently, Dezest *et al*. have demonstrated that VOCs exposure induced alterations in LONP activity in keratinocytes [9]. Experiments carried out clearly demonstrated that LONP level was drastically reduced in keratinocytes exposed to formaldehyde.
All together, these results indicated that formaldehyde induced mitochondrial metabolism dysfunction and down-regulated antioxidant activity and mitochondrial proteolysis.

As SM3 is a multimineral active ingredient increasing cell metabolism (ATP production) and cell regeneration, we evaluated its effects on alterations induced by formaldehyde exposure on mitochondrial functions.
Not only SM3 protected mitochondria from drops in ATP production and mitochondrial membrane potential induced by formaldehyde exposure but also from the decrease of MOTS-c protein level. SM3 also protected mitochondria from the downregulation of SIRT3 induced by formaldehyde treatment and thereby protected antioxidant activity of mitochondria. Finally, SM3 protected from alterations of mitochondrial proteasome and then cellular homeostasis by limiting formaldehyde deleterious effects on LONP activity. Then, SM3 is a promising active ingredient for protecting skin from damage induced by indoor pollution.

**Conclusion**.
In summary, our results provide support for the hypothesis that indoor pollution induced skin damage. Formaldehyde exposure induced mitochondrial dysfunctions: drops in ATP production and mitochondrial membrane potential induced by formaldehyde exposure as well as the decrease of MOTS-c protein level, a mitochondrial-derived peptide involved in metabolism. Moreover, formaldehyde exposure induced the downregulation of SIRT3 and alterations of mitochondrial proteasome as LONP content. Data on formaldehyde-stressed keratinocytes showed an interesting protection capacity of SM3 on mitochondrial functions, protecting mitochondrial metabolism, its antioxidant activity and mitochondrial proteolysis. Then, SM3 is a promising active ingredient for protecting skin from damage induced by indoor pollution.

**Acknowledgments.** NONE

**Conflict of Interest Statement**. NONE.

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