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List of abbreviations

EBC: exhaled breath condensate

EIA(s): enzyme immunoassay(s)

NP(s): nanoparticle(s)

RT: RTube

SB: SensAbues

Abstract

Background

In the field of nanoparticle exposure biomonitoring, oxidative stress biomarkers measured in exhaled breath condensate appear promising to detect early respiratory effects in workers handling nanomaterials. However, condensation is known for its poor efficiency in collecting non-volatiles in exhaled breath, leading to the low sensitivity of such measurements. Moreover, to be easily used in field studies on large groups of workers, the collection device must be disposable and convenient.

Objectives

In this study, we have tested a totally disposable commercial device that allows for the easy dry collection of exhaled air after filtration on a patented filter. The suitability and efficiency of the SensAbues (SB) device for collecting 8-isoprostane were evaluated and compared to the RTube (RT).

Methods

Seven healthy volunteers performed two 15-minute collections of exhaled breath, one with the SB and one with the RT. Blank devices were used to determine the background levels induced by each device. 8-isoprostane was measured in all samples using an EIA technique.

Results

The levels of 8-isoprostane in the exhaled breath of volunteers after collection with the SB were significantly higher than those after collection with the RT. Moreover, the levels obtained in volunteers with the SB were significantly higher than background levels obtained in blank devices, which was not the case for the RT.

Conclusions

This is the first study to report the ability of the SB device to collect and measure 8-isoprostane in exhaled breath. The proposed method offers better sensitivity than a classical collection with the RT device and should be further explored before future application in biomonitoring studies.

1. Introduction

Engineered nanoparticles (NPs) are increasingly widespread in everyday consumer products. The number of researchers and workers involved in nanotechnology should reach about 6 millions by 2020 [1]. Due to the suspected toxicity of NPs, potentially leading to early stage fibrosis and carcinogenesis following inhalation [2], it is now accepted that biomonitoring activities may play a key role in preventing respiratory adverse effects in NP workers [3]. Although no biological indicator predictive of toxicity in humans has yet been validated, the stepwise approach suggested for defining novel biomarkers for NPs has led to the identification of a panel of candidate biomarkers of exposure and early effects [4]. Available epidemiological studies highlight the quantitative variations of several of these biomarkers following exposure, which might indicate an association between early adverse health effects and NP exposure [5]. Among these effects, oxidative stress is well represented, along with the common propensity of NPs to generate toxicity through oxidative pathways [6]. To evaluate oxidative stress, the measurement of isoprostanes is of principal interest since these are stable molecules found in various tissues and biological fluids, and specific of lipid peroxidation [7-9]. More precisely, isoprostanes form a complex family of naturally occurring lipids originating from a radical metabolism of the essential fatty acid, arachidonic acid. 8-iso-prostaglandin $F_{2\alpha}$ (8-isoprostane), is the most studied, and is regarded as the gold standard for detection of excessive chemical lipid peroxidation in humans [10]. As a sign of the interconnection between oxidative stress and inflammation, 8-isoprostane has also been described as an inflammatory mediator in human macrophages [11]. To counterbalance the non-specificity of oxidative stress and inflammation biomarkers, their measurement in exhaled breath condensate (EBC) appears particularly promising to assess potential respiratory effects following the inhalation of NPs. Historically, EBC has been explored as a research tool in various respiratory disorders. It is a non-invasive

respiratory sampling that can also be used in the field of occupational health. It contains several molecules and non-volatile compounds issuing from the respiratory tract and potentially reflecting pulmonary pathobiology [12]. Very recent studies have shown an increase in 8-isoprostane in the EBC of workers handling NPs compared to controls [13-15]. Among others, 8-isoprostane is proposed as a robust marker associated with nano-exposure, as it is not associated with other covariates [15].

One major challenge with measurements of biological molecules in EBC is to reach sufficient sensitivity since the matrix is highly diluted and biomarkers are found at very low levels. The most commonly used device in published data related to 8-isoprostane is the Ecoscreen (Jaeger, Germany), which offers electronic stable cold condensation. However, this device is not easily implementable in field studies, mainly due to its low portability. Turbo DECCS (Medivac, Italy) might be better suited to field studies due to its greater portability and entirely disposable collecting system, but only one sample can be collected at a time (unless different apparatus is available, which has a significant cost). That is why the RTube (RT) from Respiratory Research seems more appropriate for field studies since it is entirely portable and disposable while offering the possibility of different samplings at the same time. This is often a significant practical issue in occupational studies on a large group of workers [16]. Each RT works with an aluminum sleeve which is cooled in a freezer before sampling. Associations between 8-isoprostane collected with the RT and pollution or respiratory disorders are mixed. While some studies fail to reveal respiratory impairment in the case of asthma [17-19] or exposure to polluted air [20, 21] when compared to healthy or non-exposed controls, some other studies demonstrate significant influence of various air pollution parameters on exhaled 8-isoprostane, especially in youth [22-25]. These variegated results might be related to low detection levels, since it is now admitted that condensation is not a very efficient method for collecting non-volatiles in exhaled breath [26]. The sampling of exhaled breath without any condensation step

might be a promising solution for the measurement of non-volatile compounds such as 8-isoprostane.

In the past few years, the SensAbues (SB) breathalyser with an electrostatic air filter developed by the Karolinska Institutet has been investigated for its suitability in the field of drug abuse testing. Methods were validated for various drugs collected with this disposable commercial device in exhaled breath with quantification limits ranging from 2 to 6 pg/filter using mass spectrometry [27, 28]. The collection is reported to be convenient, rapid, safe and well accepted by volunteers. Two feasibility studies have also demonstrated that phosphatidylethanols for testing alcohol consumption, along with phosphatidylcholines for surfactant analysis, could be analyzed in exhaled breath collected with the SB [29, 30]. From a chemical point of view, 8-isoprostane might be related to these hydrophobic lipid compounds. That is why we have aimed in this paper to evaluate the suitability and efficiency of the SB for collecting 8-isoprostane, in comparison with exhaled breath condensate collection using the RT.

2. Methods

2.1. Subjects and collection of EBC and exhaled air

Seven volunteers were recruited among the laboratory staff and each gave written informed consent. Each volunteer performed two collections of exhaled air, one with the RT and one with the SB, on two separate days less than three days apart, at the same time of the day. Prior to the collection, RT devices were washed seven times with ultrapure water as explained elsewhere [16]. Moreover, a filter (Virobac II, France Neir) was added to the inlet valve of each RT in order to prevent unwanted substances from entering the respiratory tract during the sampling. The sampling with the RT was performed for 15 minutes, with a nose-clip, at an initial condensation temperature of -20°C. The EBC was immediately frozen at -80°C after collection in the RT collection tube. The EBC samples were characterized by their total volume and total protein concentration (MicroBCA Assay, Protein quantitation kit, Uptima Interchim). The collection with the SB device was carried out in line with the manufacturer's recommendations, with inhalation through the nose and exhalation through the mouth. Each subject breathed tidally for 15 minutes inside the device for comparison with the RT. After the collections, the devices were capped and immediately frozen at -80°C.

In order to evaluate the analytical background induced by each device, blank RT and SB were prepared. The blank RT consisted in 1.5 mL of ultrapure water incubated in new devices for 15 min. The blank SB consisted in unused new devices. Both blanks were prepared in the same manner as real samples.

2.2. EIA

Samples collected with the RT were analyzed half diluted in the EIA buffer, as recommended for low salt concentrated samples to better mimic the standard matrix. Samples collected with the SB were prepared as prescribed by the manufacturer. Briefly, the filters were extracted three

times with methanol (total volume of 7 mL). The methanol was evaporated at 45°C in a light stream of nitrogen. Dry residues were taken up in 200 µL of EIA buffer.

The EIA analysis was performed with a commercial kit (Cayman Chemical) and according to the manufacturer's instructions, except that calibration standards were analyzed in triplicate instead of duplicate for greater accuracy. EIA-grade ultrapure water was used for the dilutions. The samples were analyzed in triplicate.

According to the manufacturer, the assay detection limit was 0.8 pg/mL. For each assay, the quantification limit was calculated and corresponded to 80% of B/B0. The standard curve is a plot of the %B/B0 values versus concentrations, where B is the absorbance of standard wells and B0 the maximal binding well. In practice, on the two assays performed in this study, the quantification limits were 1.77 pg/mL and 1.70 pg/mL, which is highly comparable.

2.3. Statistics

Mann-Whitney non-parametric tests were used to compare the levels of 8-isoprostane between the different groups. Additionally, the Wilcoxon signed-rank test was used to compare the levels of 8-isoprostane collected either with the RT or the SB for each volunteer. The *p* value $p < 0.05$ was considered statistically significant.

3. Results

3.1. Description of the volunteers and EBC characteristics

Volunteers were mainly women (six women and one man), non-smokers (5 non-smokers and 2 smokers) and aged 37 ± 12 years. All of them were healthy with no respiratory inflammation or fever reported at the time of the collections.

The EBC mean volume and mean total protein concentration obtained with the RTube over 15 min were 1254 ± 157 µL and 1.4 ± 0.5 µg/mL respectively, which is consistent with previous data obtained on more than 400 subjects [16].

3.2 Comparison of 8-isoprostane levels obtained with each device in volunteers compared to blank devices

All the levels of 8-isoprostane measured in blanks or on volunteers, with both devices, were above the assay detection limit. However, five out of seven volunteers gave 8-isoprostane levels below the assay quantification limit with the RT, while all volunteers had 8-isoprostane levels above the assay quantification limit with the SB.

In order to compare the results obtained with the RT and the SB devices, they were expressed as total 8-isoprostane collected during the sampling period (15 min) in pg. For the SB this amount corresponded to the total quantity of 8-isoprostane eluted from the filter after collection, and for the RT it was calculated from the EBC concentration and the total volume of EBC collected for 15 minutes. The results are presented in Figure 1.

With the RT, the levels found in volunteers were not significantly different from those found in RT blanks (4.1 ± 0.6 *vs* 6.1 ± 1.9 pg). This background level was also similar to that obtained in SB blanks (5.2 ± 1.6 pg). However, the levels found in volunteers after collection with the SB (16.0 ± 3.2 pg) were significantly higher than the levels in the SB blanks (Mann-Whitney, $p < 0.05$) and in volunteers collected with the RT (Mann-Whitney, $p < 0.01$).

When the levels of 8-isoprostane collected with both devices for each volunteer were studied in pairs, those after collection with the SB were still significantly higher than those with the RT (Wilcoxon signed-rank test, $p < 0.05$) (Figure 2). The mean ratio between the SB level

and the RT level for each volunteer was 3.9 ± 0.7, which corresponds to a variation coefficient inferior to 20%.

4. Discussion

In this feasibility study we have tested the suitability of the SB disposable commercial device for the collection and measurement of 8-isoprostane in exhaled breath, in comparison with the RTube. The collections were performed on healthy volunteers in order to identify the sensitivity of the method and its ability to detect background levels. When seeking to identify early effects on workers, the method should be as sensitive as possible. Moreover, blank devices were used to determine the total analytical background of the method and compare it to the levels obtained in the study volunteers.

With the RT, most levels found in volunteers fell between the EIA detection limit and its quantification limit, which is usually interpreted as very low levels of 8-isoprostane. However, these levels are not significantly higher than the levels detected in blank devices, indicating that this background level might be of non-specific origin. While EIAs are of widespread use, their specificity and selectivity can be challenging [31]. This casts light on the importance of ascertaining the detection limit of the method taking the collection devices into account, a rare consideration in practice.

Apart from the comparison with the blanks, our results on the low levels found with the RT device agree with available published data. Higher levels in EBC are generally obtained with the Ecoscreen device. It has been reported that the levels of different biomarkers measured in EBC, including 8-isoprostane, tend to be lower after collection with the RT than after collection with the Ecoscreen [32]. According to the authors, this difference in the collection efficiency may be due to the collection temperature, which is stable with the Ecoscreen but not with the

RT. Indeed, the temperature of the sampling system is one of the key parameters governing condensation efficacy and sampling reproducibility [26].

Dry collection appears appealing as a way to overcome the condensation issue, especially since collection efficiency might be improved for non-volatile compounds. In the study by Larsson et al. [33], surfactant protein A and albumin were detected in 100% of dry samples collected with the PExA method, against 21% and 0% respectively of liquid samples collected with the Ecoscreen. The disposable SB device, which is also based on dry collection, thus appears interesting for its collection efficiency for 8-isoprostane measurement. In our study, the levels obtained in volunteers were almost four times higher than those obtained with the RT, and these levels were significantly higher than the background level obtained in blank devices. Since the SB is performed without the use of a nose-clip and the RT is used with a nose-clip, the question is raised of whether the better efficiency of the SB could be due to the different mode of inhalation. However, although oral *versus* nasal inhalation is suspected to have an influence on the composition of EBC, it has been reported that the levels of thromboxane B2, which is produced by the metabolism of arachidonic acid such as 8-isoprostane and has a very close chemical structure, are not significantly different in EBC collected with or without a nose clip. Moreover, no significant difference is found between the levels of thromboxane B2 in nasal lavages and EBC samples [34]. This might indicate that the higher levels of 8-isoprostane obtained with the SB in our study might well be attributable to the collection mode rather than the inhalation mode. To confirm the specificity of 8-isoprostane collection with the SB, tandem mass spectrometry analyses coupled with high performance liquid chromatography should be envisioned in the future.

From a practical point of view, collection with the SB is better accepted by volunteers than collection with the RT, since nasal inhalation is less restrictive than oral inhalation.

To enlarge the scope of this study, the adequacy of the SB device for collecting other biomarkers should now be studied in order to determine what kind of panel might be used for the detection of early respiratory effects. Interestingly, the fact that the SB is suitable to collect phosphatidylethanols and phosphatidylcholines opens the way to the possibility of collecting and measuring peroxidised phospholipids for lipidomics approaches. Indeed, pulmonary peroxidised phospholipids are produced in specific relative proportions following carbon nanotubes inhalation in mice, which could be a means of overcoming the non-specificity of oxidative biomarkers in the case of NP exposure [35].

However, from a technical point of view, only hydrophobic compounds are susceptible to being efficiently collected by the patented filter included in the SB. Therefore, the application of the SB would not be contradictory to classical EBC collection, which is more suitable for soluble compounds such as metals.

Finally, the potential standardization of the collection using the SB needs to be further assessed. Because the measurement of soluble components that can be proposed for EBC standardization are not prone to be trapped by the SB filter, we have based our comparison on the amount of 8-isoprostane exhaled during a fixed duration of collection for both types of sampling. Although no consensual reference method for EBC standardization is now available, this approach is one of the possibilities to reduce the confounding influence of dilution of EBC samples [26]. Additionally, the possibility of measuring total exhaled air during the collections should be explored since this is now considered as a key criterion for the standardization of exhaled breath samplings. In the future, the already described measurement of the main surfactant lipid component, dipalmitoylphosphatidylcholine [30], could also be evaluated as a way to standardize the levels of biomarkers measured with the SB.

Conclusion

This is the first study to report the collection and measurement of 8-isoprostane in exhaled breath using the SB device. The collection efficiency is better than with the RT and more data are now required to further explore the possibilities offered by this device.

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Figures

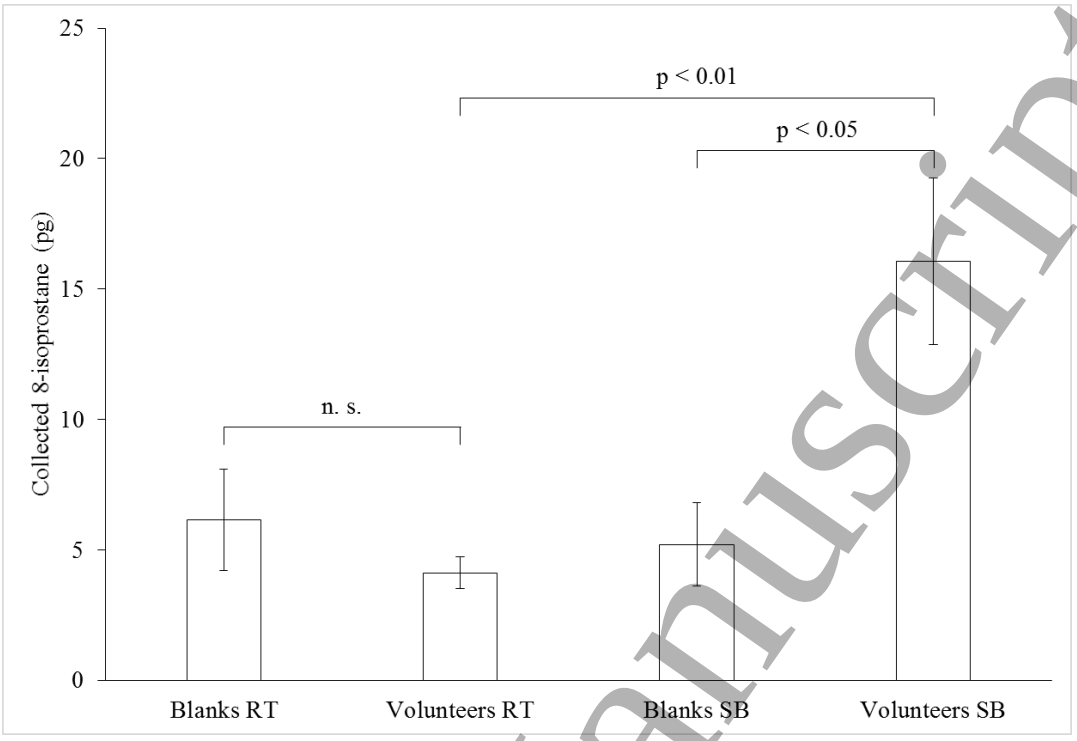

Figure 1: Comparison of the quantity of 8-isoprostane collected in volunteers (n = 7) for 15 minutes either with the RTube (RT) or with the SensAbues (SB), with background levels found in blank devices (n = 3). n.s.: non-significant.

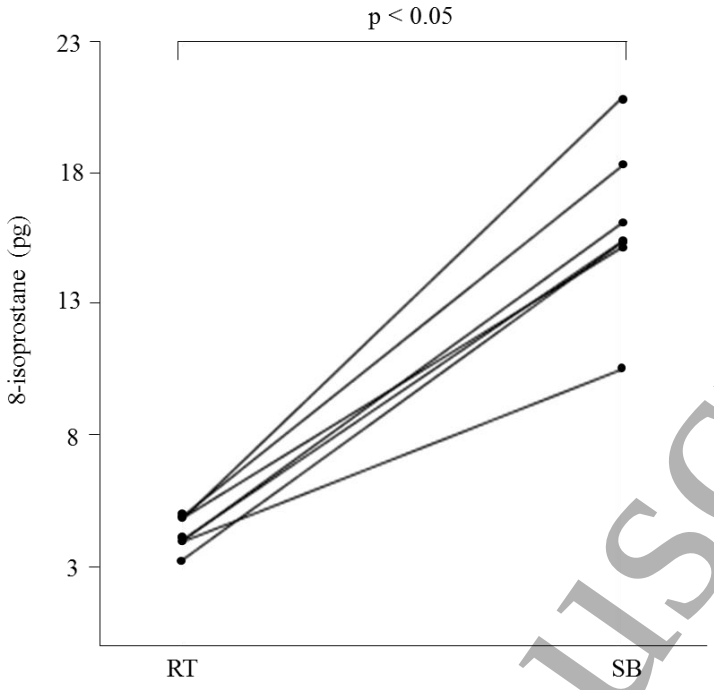

Figure 2: Amount of 8-isoprostane (pg) collected in 7 volunteers after a 15-minute collection with the RTube (RT) device and with the SensAbues (SB) device.