Exhaled breath condensate collection for nitrite dosage. A safe and low cost adaptation[1]

Coleta do condensado do ar exalado pulmonar para a dosagem de nitrito. Uma adaptação segura e barata

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ABSTRACT
Purpose: Standardization of a simple and low cost technique of exhaled breath condensate (EBC) collection to measure nitrite. **Methods**: Two devices were mounted in polystyrene boxes filled either with crushed ice/salt crystals or dry ice/crushed ice. Blood samples were stored at -70º C for posterior nitrite dosages by chemiluminescence and the Griess reaction. **Results**: a) The use of crushed ice/dry ice or salt revealed sufficient EBC room air collection, but was not efficient for patients under ventilation support; b) the method using crushed ice/salt collected greater EBC volumes, but the nitrite concentrations were not proportional to the volume collected; c) The EBC nitrite values were higher in the surgical group using both methods; d) In the surgical group the nasal clip use diminished the EBC nitrite concentrations in both methods. **Conclusions**: The exhaled breath condensate (EBC) methodology collection was efficient on room air breathing. Either cooling methods provided successful EBC collections showing that it is possible to diminish costs, and, amongst the two used methods, the one using crushed ice/salt crystals revealed better efficiency compared to the dry ice method.
Key words: Extracorporeal Circulation. Thoracic Surgery. Nitrites.

RESUMO
Objetivo: Padronizar técnica simples e barata de coleta do condensado do ar exalado pulmonar (CEP) para medir nitrito. **Métodos**: Dois dispositivos foram montados em caixas de isopor e preenchidos com gelo picado/sal grosso ou gelo picado/gelo seco. Amostras de sangue foram armazenadas a -70º C para dosagem de nitrito por quimiluminescência e pela reação de Griess. **Resultados**: a) a utilização de gelo picado/gelo seco ou sal foi eficiente para a coleta em respiração espontânea, mas ineficiente durante ventilação mecânica; b) o método gelo picado/sal coletou volumes maiores, sem aumento proporcional do nitrito; c) os valores do nitrito foram mais elevados no grupo cirúrgico utilizando os dois métodos; d) no grupo cirúrgico com clipe nasal ocorreu diminuição do nitrito em ambos os métodos. **Conclusões**: A metodologia do condensado do ar exalado pulmonar (CEP) foi eficiente na coleta respirando em ar ambiente. Os dois métodos de congelamento foram eficientes mostrando que é possível diminuir os custos, e, entre os dois métodos utilizados, o uso de gelo picado/sal mostrou melhor eficiência quanto ao volume da coleta do CEP em comparação com o uso de gelo seco.
Descritores: Circulação Extracorpórea. Cirurgia Torácica. Nitritos.

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Introduction

Until the 1980's, invasive techniques used for lung sampling, such as bronchoscopy and bronchoalveolar wash, were used as tools for complementary studies of respiratory functions and illnesses[1]. From there, Russian studies were pioneers on non-invasive techniques to study pulmonary inflammatory markers. They described the use of exhaled air – exhaled breath condensate (EBC), of which collection and posterior condensation allowed the study of volatile molecules (particularly nitric oxide - NO) proceeding from bronchi and alveoli[2,3]. Thus, many researchers started to use this technique as tool for the study of respiratory system biomarkers[4]. EBC collection and analysis were of negligent knowledge until the decade of 90 which is proven by the lack of publications (Pubmed/MEDLINE) regarding this subject. This world-wide technique arose through Kharitonov and Barnes studies performed in the 1990 decade[2]. Since then, the interest for the EBC technique grew sufficiently supported by a safe, simple and low cost method. Moreover, EBC collection does not influence airway function, thus it can be repeated as many times as demanded.

There are two main modalities for NO measurement in exhaled air: "online" and "offline"[1]. The term "online" relates to the real time NO measurement with data storage in the memory of proper device. In the "offline" modality, the exhaled NO is collected through a reservoir with a condensation system, allowing its storage for posterior analysis[2]. However, this technique is still not well standardized, presenting some limitations. Certainly, the most important limiting factor is the cooling method used for exhaled air condensation. In consulted literature, the majority of investigations use liquid nitrogen, a highly explosive and high cost gas that requires special care in transport and storage in hospital environments. Moreover, the dilution of the biomarkers, saliva contamination and the temperature of condensation also acts as limiting factors[5].

The present investigation was carried out to standardize and validate a safe, low cost and easy applicability method for EBC nitrite dosage.

Methods

Subjects and ethics

After the approval of the Committee of Ethics and consent term acceptance, twenty eight patients of both genders, with age range between 26 and 71 years, were allocated in the following groups: a) Nonsurgical Group (NSG): formed by fourteen healthy volunteers, ages between 26 and 46 years, smokers, ex-smokers and non-smokers, that were not making use of any medicine. They were divided in 2 sub-groups: NSG I (7 volunteers using the two devices - ice/dry ice and ice/salt crystals - without nasal clip) and NSG II (7 volunteers using the two devices with nasal clip) (Table 1); b) Surgical Group (SG): formed by 14 patients, with ages between 42 and 71 years, submitted to cardiac surgery (7 patients were submitted to valve surgery and 7 patients to coronary artery bypass graft) with extracorporeal circulation. They were divided in 2 sub-groups: SG I (7 volunteers using the two devices without nasal clip) and SG II (7 volunteers using the two devices with nasal clip). EBC collection was performed preoperative, during the mechanical ventilation and 24 hours post operative (Table 2).

TABLE 1 - Profile of non surgical volunteers group

	NSGI	*NSGII*
Number of patients	07	07
Male sex	02	03
Female sex	05	04
Mean of age (years)	33,0	34,1
Smokers	1	2
Ex-smokers	3	2
Diabetics	0	1
Systemic hypertension	1	0

Disinfection of devices materials was made with water and liquid detergent Extran neutral MaO_2 (Merck), rinsed in current water, rinsed with Milli-Q water and dried with medical compressed air.

TABLE 2 - Profile of patients of surgical group

	SGI	*SGII*
Number of patients	07	07
Gender male	06	03
Gender female	01	04
Mean age (years)	55,4	53,0
Valve surgery	4	3
Myocardial revascularization	3	4
Smokers	3	2
Ex-smokers	2	3
Non-smokers	2	2
Diabetics	1	2
Systemic hypertension	7	5
Pulmonary hypertension	2	2
CPB time (min)	91,4	89,3
Temp. 34º C	5	6
Temp. 28º C	2	1
Mechanical ventilation (hours)	10,6	8,9

Exhaled breath condensate devices

To test the methodology, two different devices were mounted in polystyrene boxes for EBC collection. A mouth adaptor was added to the circuit, to aid the patients to breathe in their tidal volume (VT). After correct placement of each circuit, the plastic boxes were filled either with crushed ice and salt crystals (ratio of 6 crushed ice parts to 3 parts of salt crystals) or dry ice and crushed ice. Attention was given not to permit direct contact between the two types of ice. Measured temperature was around -10º C in the device with dry ice and crushed ice, and -15º C in the device with crushed ice and salt crystals.

Exhaled breath condensate collection

Room air breathing patients were asked to rinse their mouth prior to collection to minimize saliva contamination. They were instructed to breathe in their VT through the piece adapted to the mouth closing the lips around it. A calm respiratory pattern was requested during sampling which lasted 10 minutes for each device. As the main objective of this investigation was to standardize the collection technique, both devices were used in each volunteer with and without nasal clip.

EBC collection in the surgical group occurred at the following moments: a) Moment 0 (zero): pre-operatory spontaneously breathing patients; b) Moment 1: intubated patients (twenty minutes after the patient arrival in the post-operative unit - POi), under controlled ventilation with 100% of oxygen inspired fraction (FiO_2); c) Moment 2: spontaneously breathing patients 24 hours after the cardiopulmonary bypass (CPB) exit (PO 24hs). The collections of the volunteers who were in room air had duration of 10 minutes in each device, with an interval of 5 minutes between them.

Exhaled breath condensate sampling

The EBC samples were stored at -70º C and opportunely analyzed by two nitric oxide dosage methods: the Griess reaction and the NO/ozônio chemiluminescence (Sievers 280i NOA, Sievers, Boulder, CO, U.S.A.). The concentration of nitrite in the exhaled condensed air was measured using injecting 200ul of the sample in a reaction vase filled 20 ml of a reducing agent (constituted of acid ascetic glacial, potassium iodide and iodine flakes) and 15 ml of HCl 1N, that converted nitrite into NO, in equimolares amounts.

Statistical analysis

The parameters were expressed as mean ± standard deviation and N corresponding to the number of patients. Data distribution normality was tested using Shapiro-Wilk text, evidencing lack of normality. Thus, data were analyzed by Mann-Whitney and Wilcoxon tests, with using the GRAPHPAD PRISM 4.0 software. P was considered staticsally significant when $p<0.05$.

Results

EBC volumes collected by the two methods, did not present intragroups or intergroups significant differences. However, there was evidence that the crushed ice/ salt crystals method provided greater volumes of condensate compared with the method of the dry ice in both groups (SG, n=14, 2,73 ± 1,57 / 1,93 ± 1,06; NSG, n=14, 2,11 ± 1,17 / 1,48 ± 0,89) (Figure 1).

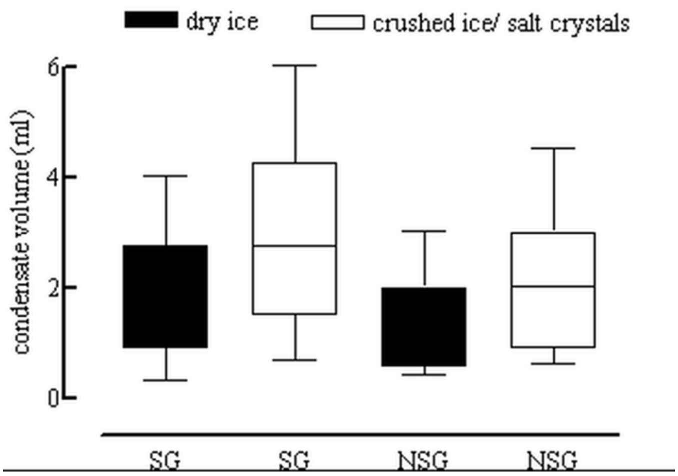

FIGURE 1 - Exhaled breath condensate (EBC) volume: surgical group versus nonsurgical group in preoperative day using the both methods

Collected volumes of EBC SG in the pre-operative (n=14) and 24 hours postoperative (n=14) did not present significant differences for both collection methods. However, crushed ice/salt crystals collection provided greater volumes of condensate compared with the method of the dry ice (2,73 ± 1,57 / 1,93 ± 1,06 in the pre-operative group and 2,40 ± 0,94 / 1,75 ± 0,70 in the post-operative group) (Figure 2).

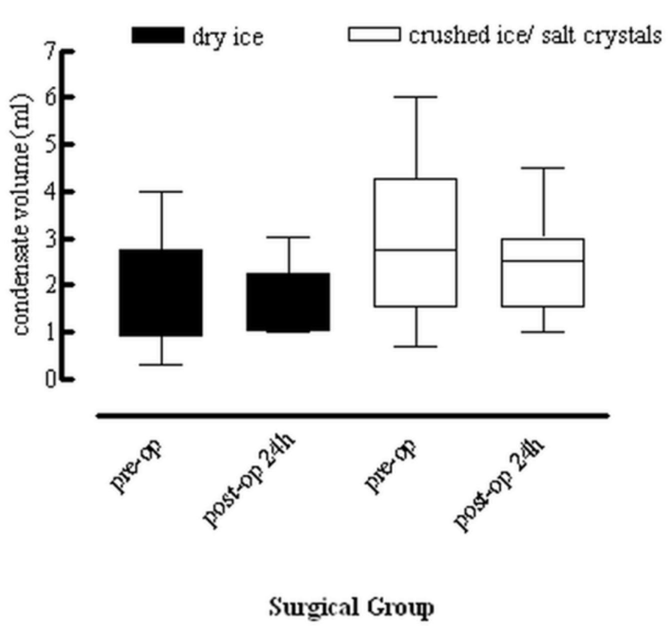

FIGURE 2 - Exhaled breath condensate (EBC) volume of surgical group: preoperative day versus 24 hours postoperative day

Comparison of NO_2- values by the chemiluminescence method for both groups, revealed significant greater concentration in the SG EBC, for dry ice (4,28 ± 3,43) and crushed ice/salt crystals (3,55 ± 3,15) methods (Figure 3). Asterisk indicates $p<0,05$, between groups (Mann Whitney and Wilcoxon tests).

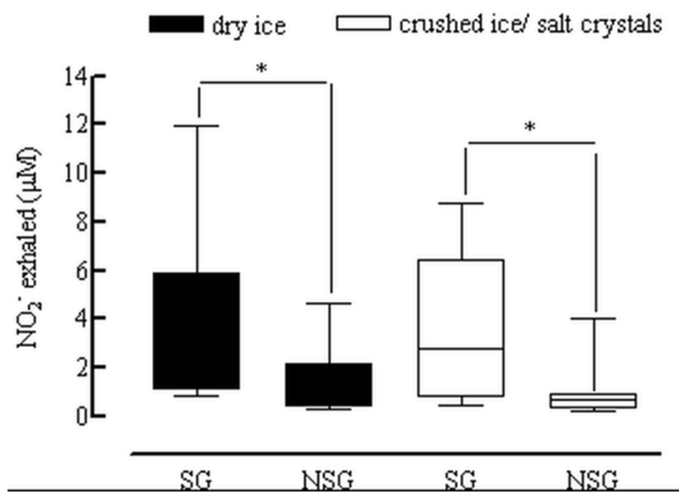

FIGURE 3 - Chemiluminescence exhaled nitrite (NO_2-): surgical group versus non surgical group in the preoperative day

Comparison EBC NO_2 - values for both groups using the Griess reaction, revealed statiscally greater concentration in SG group for the dry ice method (1,84 ± 1,66). In addition, there are significant evidences that EBC NO_2- levels in the NSG group were greater by comparing the dry ice (3.87 ± 1,01) with the crushed ice/salt crystals methods (0,82 ± 0,54). Asterisk indicates $p<0.05$, between groups (Mann Whitney and Wilcoxon tests) (Figure 4).

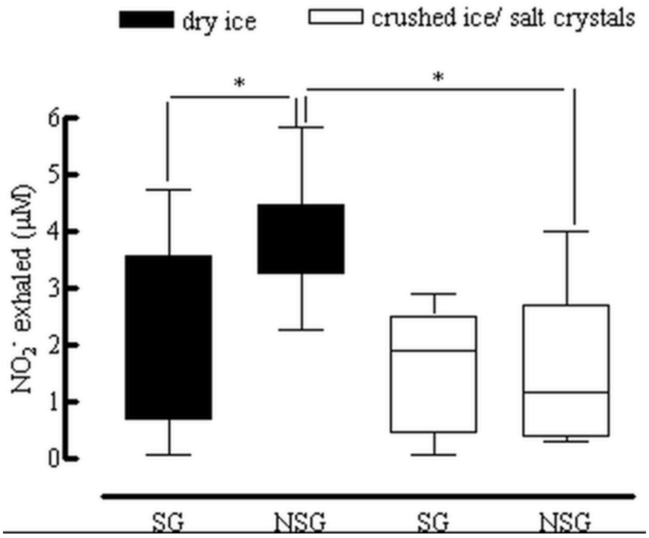

FIGURE 4 - Griess reaction exhaled nitrite (NO_2-): surgical group versus non surgical group in the preoperative day

Comparing the techniques chemiluminescence versus Griess reaction used for the EBC NO_2- dosage in both groups, statically greater concentration of NO_2- was observed through Griess (3,87 ± 1,01) reaction for the dry ice method in the NSG group compared to the same samples dosed through the chemiluminescence technique (1.34 ± 1.35). Asterisk indicates $p<0.05$, between groups (Mann Whitney and Wilcoxon tests) (Figure 5).

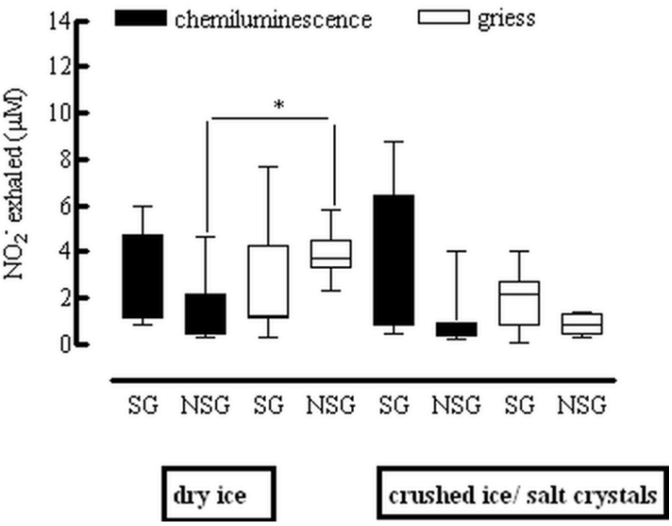

FIGURE 5 - Exhaled breath nitrite (NO_2-): chemiluminescence versus Griess reaction in the preoperative day of surgical and nonsurgical groups

In collections performed with nasal clip, exhaled NO_2- levels provided by SG did not present significant difference when comparing both methods. However, in the dry ice method, statiscally significant differences in the chemiluminescence exhaled NO_2- values was observed when comparing the individuals using nasal clip (2.19 ± 1.87) to those that had not made correct use (6.26 ± 3.93). Asterisk indicates $p<0.05$ (Figure 6).

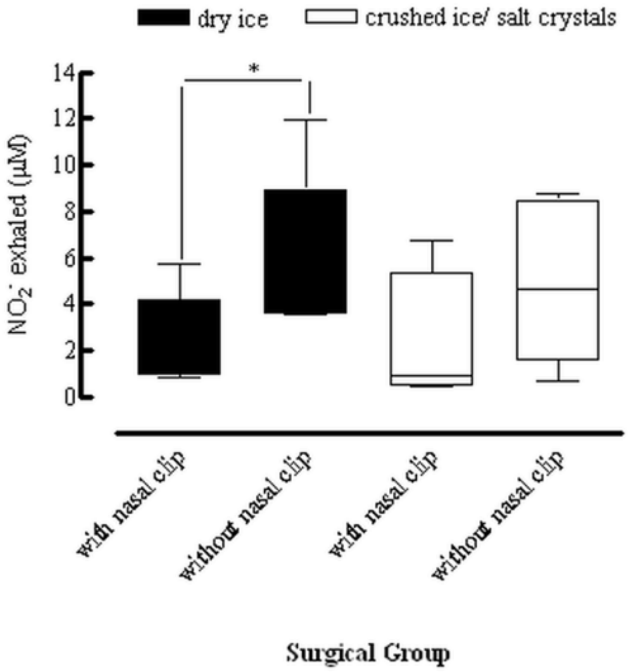

FIGURE 6 - Chemiluminescence exhaled breath nitrite (NO_2-): surgical group with nasal clip versus without nasal clip in the preoperative day using both collection methods

Exhaled NO_2- levels did not present significant differences in both collection methods in respect to the use of nasal clip in the preoperative and the 24 hours postoperative days. However, in preoperative day the dry ice method had statiscally significant difference of exhaled NO_2- values, comparing individuals that carried out the collections using nasal clip (2.19 ± 1.87) with those that had not made use of it (6.26 ± 3.93). Asterisk indicates $p<0.05$ (Figure 7).

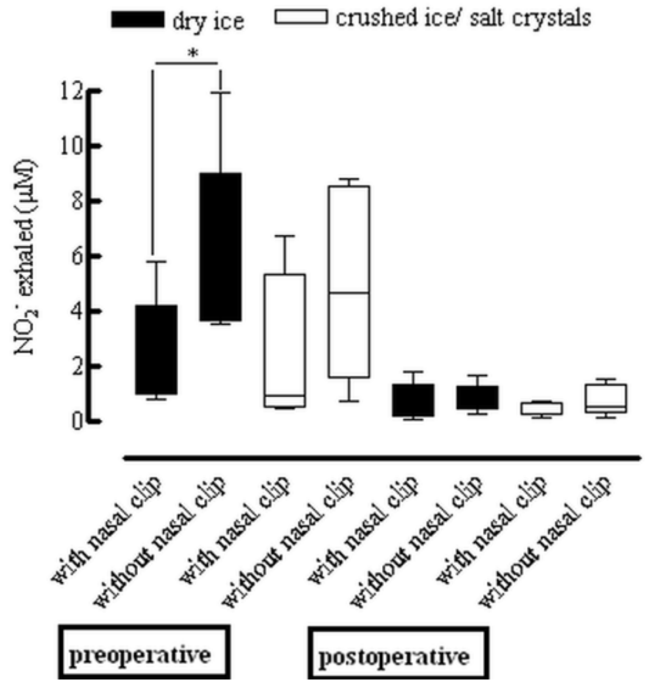

FIGURE 7 - Chemiluminescence exhaled breath nitrite (NO_2-): surgical group with nasal clip versus without nasal clip in the 24 hours postoperative using both collection methods

With the use of nasal clips, exhaled NO_2- values provided by the NSG group did not present significant differences when comparing both EBC collection methods. However, in the method of the crushed ice/salt crystals, exhaled NO_2- values had statically significant difference when comparing individuals that made collections using nasal clip (0,98 ± 0,60) to those that had not made use of it (0.33 ± 0.15). Asterisk indicates $p<0.05$ (Figure 8).

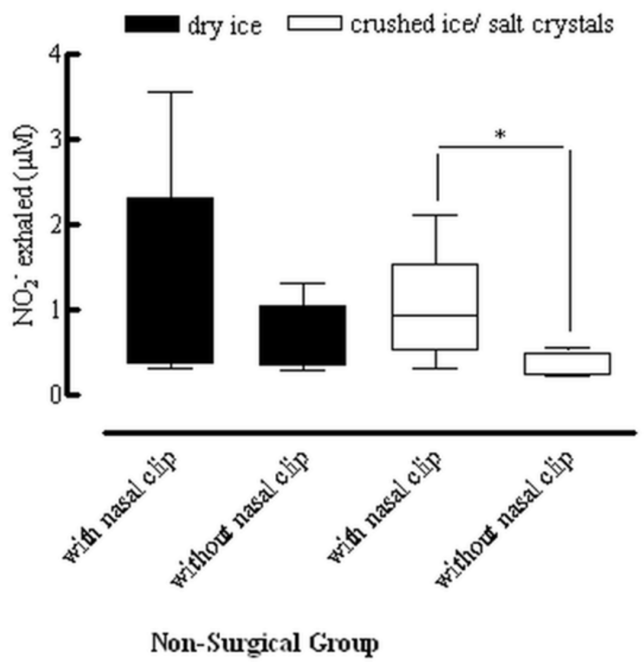

FIGURE 8 - Chemiluminescence exhaled breath nitrite (NO_2-): nonsurgical group with nasal clip versus without nasal clip in the 24 hours postoperative using both collection methods.

The use of dry ice for EBC collection in both groups, did not exhibit significant difference for the exhaled NO_2- levels in relation to the use of the nasal clip. On the other hand, collections made without the use of the nasal clip in both groups, we notice that it had statiscally significant differences between the groups (SG: 6.26 ± 3.83; NSG: 0.62 ± 0.38). Asterisk indicates $p<0.05$, between groups (Mann Whitney and Wilcoxon tests) (Figure 9).

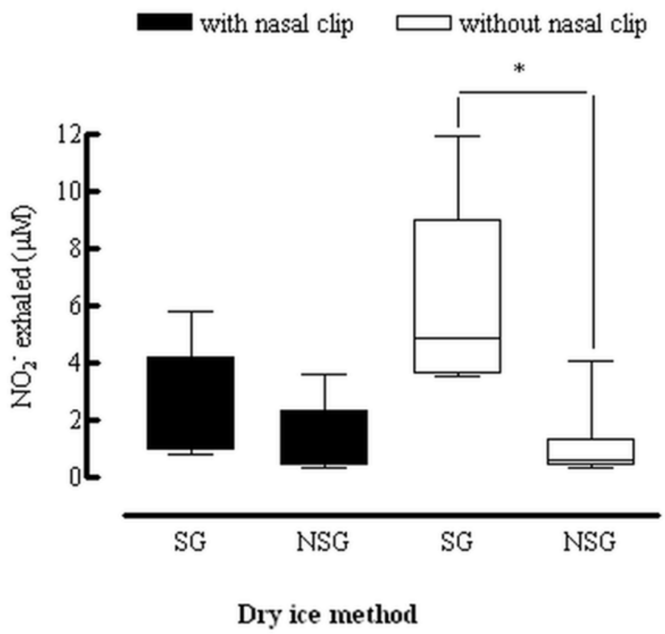

FIGURE 9 - Chemiluminescence exhaled breath nitrite (NO_2-): surgical group versus non surgical group with nasal clip in the preoperative day using the dry ice method

The use of crushed ice/salt crystals method in both groups for EBC collection did not have significant difference in the exhaled NO_2- levels in respect to the use of the nasal clip. On the other hand, for both groups, collections made without the use of nasal clip had statically significant differences (SG: 4.88 ± 3.30; NSG: 0.33 ± 0.15). Asterisk indicates $p<0.05$, between groups (Mann Whitney and Wilcoxon tests) (Figure 10).

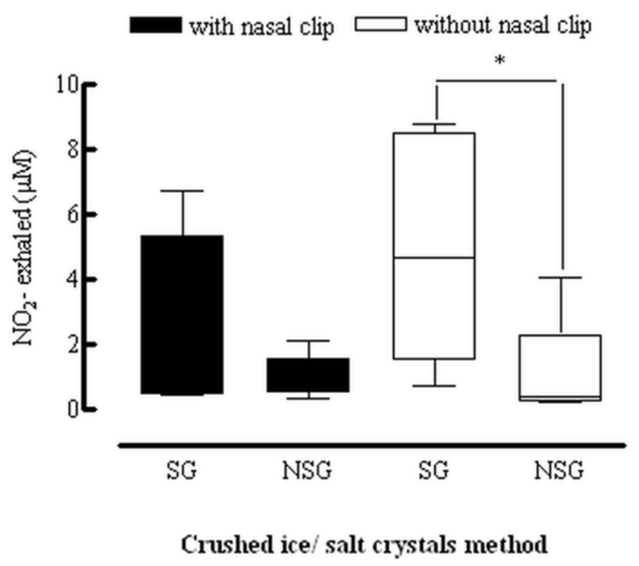

FIGURE 10 - Chemiluminescence exhaled breath nitrite (NO_2-): surgical group versus nonsurgical group with nasal clip in the preoperative day using the crushed ice/ salt crystals method

Discussion

This investigation was carried out considering the American Thoracic Society (ATS)/European Respiratory Society (ERS) Task Force on EBC which establishes the following general recommendations for oral sample collection: collect during tidal breathing using a nasal clip and a saliva trap; define cooling temperature and collection time (10 minutes is generally sufficient to obtain 1–2 mL of sample and is well tolerated by patients); use inert material for condenser; do not use resistor and do not use filter between the subject and the condenser. It is clear that EBC is an exciting new approach to monitoring lung diseases that represent great potential in the future. These guidelines provides the first step in standardizing measurements and encouraging research in this new field, therefore the following discussion was based on their principles[6].

Devices for exhaled breath condensate collections

Most researchers have been using custom devices, assembled in individual laboratories, consisting of jacketed cooling pipes, tubes in buckets of ice, or glass chambers in ice with inhalation and exhalation ports. The least expensive and simplest technique involves exhaling through a tube that is suspended in a bucket of ice. This can require a collection time of 15 minutes or longer, because the sample is fixed to the condenser wall by means of surface tension until enough fluid volume is collected so that gravity and/or airflow will propel the larger drops distally. After testing two sizes of vial devices we decided to try an adaptation similar to the one presented by Hunt[7].

Collections time

Collection time used in most published studies lies between 10–30 min with only a few studies using very short (3 min) or rather prolonged (60 min) collection time[8]. In most studies 10 min has been used and is perfectly suitable for the present study because of two practical reasons: 1) this collection time resulted in 1–2 mL of condensate from adult subjects and most children above 4 years of age; and 2) subjects usually tolerate this period of sampling without fatigue (although loss of interest may still occur in children). However, different study set-ups may require other collection times (i.e. shorter when time course of changes is assessed or longer when a larger amount of sample is needed). Direct comparison of different collection times has only been published regarding pH value, showing no effect of different time collections between 3–20 min on EBC pH of healthy subjects[8]. Although no direct comparison is available regarding other mediators, no difference can be found in the concentrations of H_2O_2, NO_2-/NO_3-, 8-isoprostane, adenosine and MDA among studies using 10, 15 or 20 min for EBC sampling[6].

Nasal clip

EBC collection with oral inhalation-oral exhalation may be performed with or without the use of a nasal clip. Nasal clip securely prevents any accidental inhalation-exhalation through the nose during sample collection. When tidal breathing is used for sample collection without resistance, the soft palate is not closed and air and mediators present in the nose and sinuses can be incorporated to the sample. The use of nasal clip may well minimise the aerosolisation of particles from the nasopharynx. On the other hand, volatile gases formed in the nasopharynx (such as NO) may be entrained to a greater extent in the exhaled air when nasal clips are employed (as is indeed the case for NO)[2]. At the moment, data on EBC collected with and without nasal clips are not available. The consensus of the ATS/ERS expert panel was that nasal clip use is advisable to ensure that no sample is lost through the nose and that inspiration bypasses the nose. The most important recommendation is that investigators should consider the potential effect of nasal airflow on the EBC levels of the compound of interest, and perform appropriate control experiments when feasible. In manuscripts, precise description of sampling method (route of inhalation and exhalation, use of nasal clip) is required and any ongoing upper airway disease should be mentioned. In addition, the consensus emphasized, as areas for further research, that the comparison of data reproducibility between EBC samples obtained with and without nasal clips (oral inhalation and exhalation) is needed for mediators detectable in EBC before making explicit recommendation on this issue[6].

In our experiment the use of nasal clip on the SG diminished the concentration of NO_2- exhaled in both methods, but the increase of exhaled NO_2- concentrations was not seen in the NSG when using the nasal clip.

Temperature of condensation

Condensation of EBC can be achieved at a temperature around 0°C using wet (salty) ice or even in lower temperatures using different techniques (dry ice, liquid nitrogen, placing cooling sleeve to required temperature, cooling air to preset temperature, etc.), resulting in the collection of frozen material. Regarding temperature of condensation, the collecting surface warms up from the exhaled air and this influences the collecting temperature. The solubility of volatile mediators in the collected samples may be influenced by temperature, and data reveal that concentration of ammonia is lower if the condensate is collected in ice rather by water. Condensing temperature and time is also important for those mediators that are unstable, such as leukotrienes and purines[6,8].

The use of the dry ice and the crushed/thick ice were both highly effective in the collection of the EBC in both groups, when carried out in room air. Also, the crushed/thick ice method collected greater intragroup and intergroups EBC volumes. This easy way of achieving temperature of condensation led us to abandon observations using liquid nitrogen that is more difficult to obtain and may be hazardous.

Temperature was around -10º C in the device with dry ice and crushed ice, and -15º C in the device with crushed ice and salt crystals, attesting the salt utility in getting lower temperatures.

Room air

Room air contains molecules, which may influence EBC composition through several possible mechanisms. Atmospheric compounds can: 1) directly contribute to EBC levels; 2) react and, therefore, change or consume molecules trapped in EBC; and 3) lead to inflammatory and biochemical changes in the airway that are subsequently reflected by changes in EBC composition. It has been demonstrated that atmospheric NO reduces exhaled H_2O_2 levels[5]. Room air that has not interacted with the respiratory system (not been inhaled) can be excluded by a device designed with unidirectional flow through the condenser with the intention of minimizing direct contact with room air. EBC samples can also interact with room air if samples are left exposed to room air after collection. This may result in important changes in mediator concentrations if an unstable mediator or a volatile compound is of interest or when the measured molecule or a reactive precursor molecule is present in room air. Finally, velocity, temperature and humidity of inhaled air may all influence the volume and content of EBC, but data are lacking on this issue[9].

As ATS/ERS recommendation, the control experiments in which subjects inhale air that does not contain the compounds (or their precursors) that will be measured in EBC should be considered by each investigator. Furthermore, unless proven unequivocally to be acceptable for a given mediator, EBC samples should not be left out at room temperature after collection (the latter is important not only because of the interaction with room air, but also because substances may be degraded or formed in EBC more readily at this temperature). From the inspired room oxygen fraction point of view, areas for further research certainly have to include studies with inhalation of known gas mixtures to establish the effect of changes in ambient air on EBC characteristics[6].

Nitrite assays

Many NOx assays reported to date are not specific for one compound. Assays may be for nitrate, nitrite, nitrosothiols, nitrotyrosine, NO and other higher oxides of nitrogen. Chemiluminescence analysis after reduction by various methods, when carefully performed, provides sufficient sensitivity for most NOx in unconcentrated EBC. Spectrophotometric tests are less sensitive and NOx are commonly near the limits of detection, especially for nitrite and nitrosothiols, thus, these assays should be used cautiously[6]. We chose to test the chemiluminescence and Griess reaction nitrite assays. Why the Griess reaction? Because it is widely used in basic research laboratories, but it is few referenced in clinical investigation[10].

The chemiluminescence technique is sensitive in the nM range and NO_2-/NO_3- have been detected by this method. Caution must be taken when interpreting NO_2- assays, as the compound cannot be considered stable at low pH, and even at neutral pH. NO_3- is considered stable. NO_3- levels tend to be roughly 5–10 folds higher than NO_2- levels, although this may vary in part because of oxidative conditions changes in airway. Since most combined NO_2-/NO_3- assays rely on the formation of NO_2- from NO_3-, variability of data may arise partially from differences in the efficacy of the reduction method chosen. In this respect, enzymatic reduction was for NO_3- determination in EBC than cadmium reduction[11].

NOx are present on every laboratory surface, including glassware and pipette tips. Therefore, investigators should take great precautions to avoid contamination of the sample. Experience suggests that it is necessary to thoroughly rinse with highly pure (distilled/de-ionized) water any material that might come in contact with EBC, including devices used for collection, processing and assaying EBC. It is best to perform this rinsing immediately before use, because readily diffusible ambient NO becomes oxidized and contaminates surfaces rapidly[6].

In our experiments three main conclusions were possible: a) The EBC NO_2- absolute values in the two collection groups, did not present statically significant difference, in relation to the use of the Griess reaction or the chemiluminescence technique; b) The crushed/thick ice method was efficient enough to collect greater EBC volumes; however, the levels of EBC NO_2- in absolute values were not larger for the group where collection was made using this method and; c) The EBC NO_2- levels in patients that had been operated in a normothermia (34ºC) diminished in the postoperative 24-hour when compared with the preoperative days values. Such fact was observed for both methods of collection.

Conclusions

The proposal of this work was to standardize and validate a method for exhaled NO_2- dosage, using material and vehicles for EBC collection and condensation, that proved to be safe, cheap and of easy application. Besides collecting the minimum volume necessary for sample analysis, both methods provided condensed volumes which were equal and even superior to the collections using liquid nitrogen, reflecting better cost-benefit. It is important to emphasize that between methods, the crushed ice/ salt crystals revealed more efficiency for the condensed volume collection over the method using dry ice.

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