Presentation of an experimental method to induce in vitro ("organ chambers") respiratory acidosis and its effect on vascular reactivity[1]

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ABSTRACT

PURPOSE: To create *in vitro* a model to generate acidosis by CO_2 bubbling "organ chambers", which would be useful for researchers that aim to study the effects of acid-base disturbs on the endothelium-dependent vascular reactivity.

METHODS: Eighteen male Wistar rats (230-280g) were housed, before the experiments, under standard laboratory conditions (12h light/dark cycle at 21°C), with free access to food and water. The protocol for promoting *in vitro* respiratory acidosis was carried out by bubbling increased concentrations of CO_2. The target was to achieve an ideal way to decrease the pH gradually to a value of approximately 6.6.It was used, initially, a gas blender varying concentrations of the carbogenic mixture (95% O_2 + 5% CO_2) and pure CO_2.

RESULTS: 1) 100% CO_2, pH variation very fast, pH minimum 6.0; 2) 90%CO_2 pH variation bit slower, pH minimum6.31; 3) 70%CO_2, pH variation slower, pH minimum 6.32; 4) 50% CO_2, pH variation slower, pH minimum 6:42; 5) 40 %CO_2, Adequate record, pH minimum 6.61, and; 6) 30 %CO_2 could not reach values below pH minimum 7.03. Based on these data the gas mixture (O_2 60% + CO_2 40%) was adopted,

CONCLUSION: This gas mixture (O_2 60% + CO_2 40%) was effective in inducing respiratory acidosis at a speed that made, possible the recording of isometric force.

Key words: Acidosis, Respiratory. Endothelium. Nitric Oxide. Rats.

Introduction

Acid-base shifts are caused by pCO_2 changes (respiratory mechanisms) and addition of acid or base (non-respiratory mechanisms).These shifts affect the vascular reactivity, and, besides the direct effect on vascular tone, may also alter vascular vasoconstrictor and/or vasodilatorsresponsiveness[1-3].

This study proposes to develop a method in which acidosis is induced *in vitro* by bubbling CO_2 in Krebs solution ("organ chambers" bath), in adequate interval of time to record the changes in isometric force. In other words, the present study was carried out to create an *in vitro* model to generate acidosis by CO_2 bubbling "organ chambers", which would be useful for researchers that aim to study the effects of acid-base disturbs on the endothelium-dependent vascular reactivity.

Methods

The experimental procedures and animal handling were reviewed and approved by the Institutional Animal Care Review Board (CETEA – Ethics Committees of Animal Experiments of the Ribeirao Preto School of Medicine, University of Sao Paulo. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Eighteen male Wistar rats (230-280 g) were housed, before the experiments, under standard laboratory conditions (12h light/dark cycle at 21°C), with free access to food and water.

The rats were anesthetized with isoflurane, followed by a laparotomy for exsanguination via abdominal aorta and a thoracotomy for thoracic aorta harvesting. The thoracic aorta was carefully dissected free of connective tissue and immediately immersed in Krebs or Hanks solution. For the vascular reactivity studies, Krebs solution with the following composition (mM) was employed: NaCl - 118.0, KCl - 4.7, $CaCl_2$ - 2.5, KH_2PO_4 - 1.2, $MgSO_4$ - 1.66, glucose - 11.1, $NaHCO_3$ - 25.0 (pH 7.4).

The thoracic aorta was cut into rings (4-5 mm in length). The endothelium was removed from some rings by gently rubbing the intimal surface of the blood vessel with a pair of watchmaker's forceps. This procedure removes the endothelium, but it does not affect the ability of the vascular smooth muscle to contract or relax. Then, these rings were placed in isolated organ baths (10 mL) filled with Krebs solution, maintained at 37ºC and bubbled with 95% O_2 / 5% CO_2 (pH 7.4). Each arterial ring was suspended by two stainless steel clips placed through the lumen. One clip was anchored to the bottom of the organ bath while the other was connected to a strain gauge for measurement of the isometric force with the aid of the Grass FT03 equipment (Grass Instrument Company, Quincy, MA, USA). Each ring was stretched to a resting tension of 1.5 g and allowed to equilibrate for 60 min. During this period, tissues were washed every 15 minutes. By means of a pilot study, the resting tension value was determined by construction of a curve expressing contraction (in response to KCl 45 mM) per tension (given by progressive ring stretching). The tension value that evoked the maximal contraction was elected as the optimal length-tension. The efficacy of the procedure for endothelium removal was confirmed by the lack of relaxant effects induced by acetylcholine (10^{-6} M) in rings pre-contracted with Phe (10^{-6} M – EC_{80}). Endothelium was considered to be present when the Ach-induced relaxation was at least 80%. Endothelium was deemed absent when its response was not developed. Then, each ring was washed and re-equilibrated for 30 minutes. Aortic rings were then pre-contracted with Phe (10^{-6} M), and pH-response curves were obtained after a stable plateau was reached.

The protocol for promoting *in vitro* respiratory acidosis was carried out by bubbling increased concentrations of CO_2. The target was to achieve an ideal way to decrease the pH gradually to a value of approximately 6.6.It was used, initially, a gas blender varying concentrations of the carbogenic mixture (95% O_2 + 5% CO_2) and pure O_2 (Figure 1).

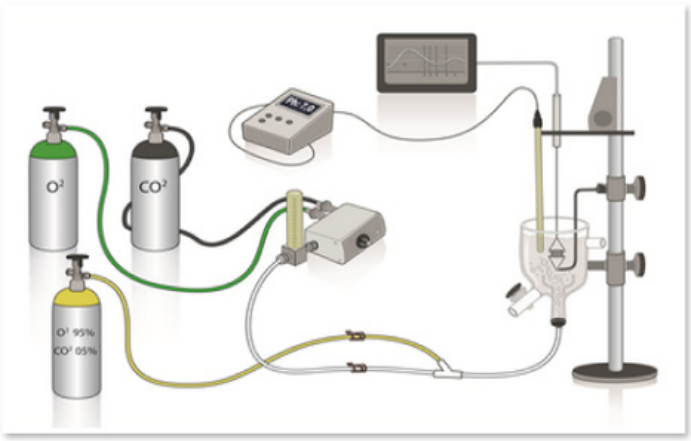

FIGURE 1 - Schematic experimental setting up test the gas concentrations.

Results

The test results are shown in Tables 1 and 2.

TABLE 1 - Test bubbling CO_2 in the Krebs solution at 37°C temperature. The gas mixture containing 40% CO_2 and 60% O_2 was sufficient to shift the pH from 7.5 to 6.6 gradually.

% CO_2 rating	pH variation	pH minimum
100	Very Fast	6.00
90	Bit slower	6.31
70	Slower	6.32
50	Slower	6.42
40	Adequate record	6.61
30	Could not reach values below	7.03

TABLE 2 - Values of blood gases in samples of Krebs harvested at the lowest pH achieved by mixing CO_2/O_2 - 40/60.

Sample	pH	pO_2 mmHg	pCO_2 mmHg
1ª	6.88	421.4	118.8
2ª	6.86	437.7	122.0
3ª	6.77	423.1	152.8

With these results, it was decided to test a commercially ready, with a cylinder gas mixture produced by the company Praxair with 40% CO_2 and 60% O_2 to start the experiment protocols reactivity (Figure 2). This gas mixture was effective in inducing respiratory acidosis at a speed that made possible the recording of isometric force (Figure 2).The final experimental setting up is presenting in Figure 3.

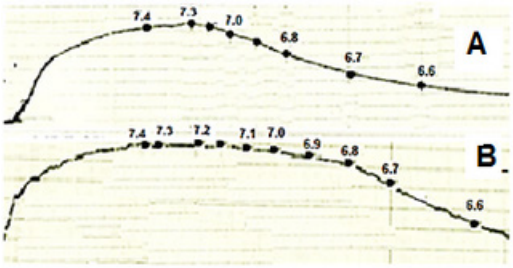

FIGURE 2 - Representative image of a pH-response curves in isolated rat aorta with endothelium (A) and without endothelium (B) with Phe pre-contracted rings. The curve was produced by bubbling CO_2 in a mixture 40/60 with O_2 and pH drop was annotation every 0.1 unit. The pre-contraction was induced with Phe (10^{-6} M).

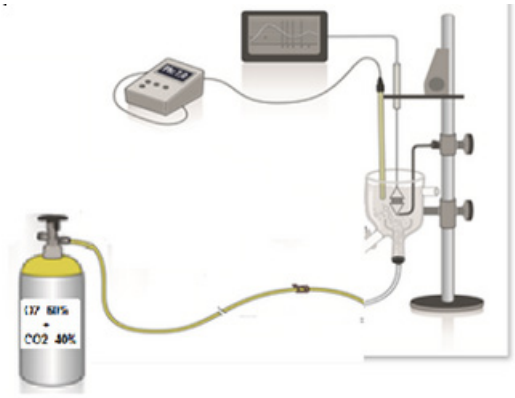

FIGURE 3 - Final experimental setting up (O_2 60% + CO_2 40%).

After characterizing the vascular profile triggered by the change of pH (Figure 3), it was investigated the three endothelium-dependent mechanisms (cGMP/NO, AMPc/PGI_2 and hyperpolarization) (Figure 4).

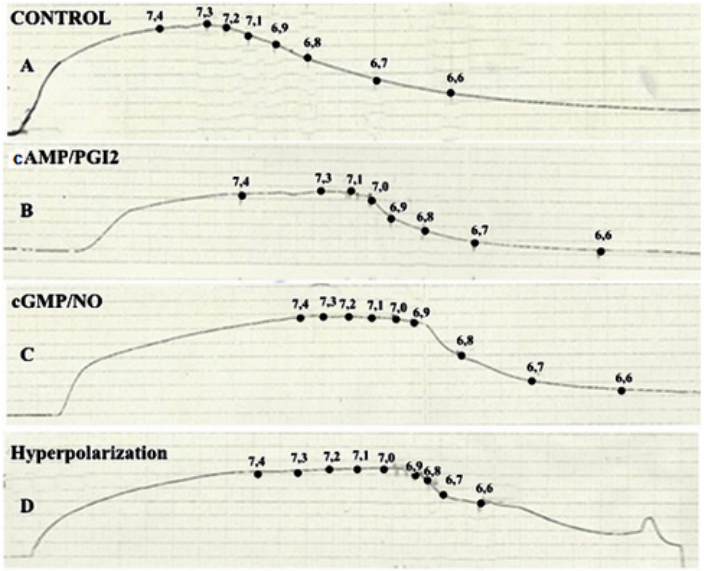

FIGURE 4 - pH response curves induced by bubbling $O_2$60%/CO_2 in vessels (rat aorta) with endothelium pre-contracted with Phenylephrine (10^{-6}M). A. Control vessel; B. Presence of Indomethacin (10^{-6}M) (phosphodiesterase blocker) in the organ bath; C. Presence of L-NAME (10^{-4}M) (NO synthase blocker) in the organ bath, and; D. Presence of tetraethylammonium (10^{-3}M) (potassium channel blocker) in the organ bath.

Discussion

The mechanisms by which pH influences vascular tone or their response to specific agonists are not yet fully understood, but there is some evidence to suggest the involvement of nitric oxide (NO), prostacyclin (PGI_2), channels for potassium and calcium flux[3].

Isolated vessel techniques were used in this study. Thus, the influences of the local control mechanisms (such as shear stress) and neurohumoral tone were eliminated by ensuring that the vascular responses observed could be attributed specifically to acidification[3].

The literature presents well-designed methods to produce metabolic acidosis *in vitro*[3-5]. However, methods to induce respiratory acidosis are rare. Stokke *et al.*[5] provoked *in vitro* acidification with different mixtures of oxygen and carbon dioxide, reaching partial pCO_2pressures up to 108 mmHg bubbling a mixture of 18.4% CO_2/O_2 in saline solution and reaching pH of 6.5. Another study used bubbling anesthetic substances *in vitro*[6].

Preliminary results were presented In: Experimental Biology 2014 - FASEB, 2014, San Diego - CA -EUA. Assays were carried out organ baths bubbling the herein mixture of CO_2/

(40%)/02 (60%), for the construction of pH-response curves (pH 7.4 at 6:6) registering in isometric pre-aortic rings contracted with phenylephrine (10^{-6}M). As main results: 1) Relaxations were seen only in endothelial rings; 2) Endothelium-dependent relaxations were inhibited by incubation for 30 min with indomethacin (10^{-5} M), L-NAME (10^{-4} M) and tetraethylammonium (10^{-3} M). These data allow concluding by the suitability of the method and the dependence of endothelial vascular response induced by extracellular respiratory acidosis, including the three known mechanisms of vasodilation: cAMP, cGMP and hyperpolarization[7].

Conclusions

The gaseous mixture of 40 % CO_2 and 60 % O_2 was sufficient to shift the pH from 7.5 to 6.6 gradually (pCO_2=118.2/152.8).This gas mixture was effective in inducing respiratory acidosis at a speed that made possible the recording of isometric force. Pilot studies demonstrating the effectiveness and reproducibility of the method, which has nowadays been routinely used in the Laboratory of Cardiovascular Research and Endothelium Function from the Department of Surgery and Anatomy, Ribeirao Preto School of Medicine-USP.

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