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Effect of Sample Storage Temperature on ciliary Beat Frequency of Flow Subjected Cilia

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Abstract

The aim of this study was to determine the optimal temperature for storage and subsequent measurement of the ciliary beat frequency (CBF) of cells brushed from human nasal epithelium. In each case, human nasal epithelial cells were stored at 4, 20 or 32°C and CBF measured at 20 or 32°C. Ciliated cells were placed in a perfusion chamber and exposed to flow of normal tissue medium (M199) for 20 minutes to simulate a dynamic insult, thereby exposing cells to perfusion stress. CBF was measured in a pre-flow (20 minutes) and post-flow (60 minutes) period. A significant decrease in CBF was noted for all cells stored at 32°C, both pre- and post-flow. However, post-flow CBF cells displayed an even greater decrease, and results obtained at 40 and 100 minutes showed a 37 and 44% decrease in CBF for cells stored at 32°C and measured at 20°C and 32°C respectively. Storage of ciliary cells at 32°C renders them more sensitive to fluid flow stress. Cells stored at 4 and 20°C show no significant difference in CBF when measured at 20 or 32°C. We propose standardization of ciliary cell storage and measurement protocols.

Key words: Ciliary beat frequency, mucociliary clearance, storage temperature, post-flow stress

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Introduction

Mucociliary clearance (MCC) is one of the respiratory system's primary defence mechanisms. Ciliary beating provides the driving force for MCC [1], but the regulation of ciliary beat frequency (CBF) is not fully understood. CBF varies with temperature, but there is no consensus as to the optimal temperature to use for *ex vivo* measurements of CBF. Some suggest that 32°C, the normal physiological temperature for human nasal epithelial cells (HNE), is best for CBF studies [2], while others prefer to measure CBF at core body temperature (36.7°C) [3, 4] or at room temperature (20°C) [5, 6]. M199 is often used for the storage of respiratory epithelium in such studies. In our laboratory, practice has evolved that samples collected for studies on ciliary cell signalling cascades are stored in medium 199 (M199) on ice and warmed to room temperature prior to CBF measurement. This protocol was developed to standardise the initial state in biopsy samples that are often not available at predictable times from volunteers or may have to be sent to a specialist centre from long distances. However, other laboratories follow alternative storage protocols [7] prior to measurement, and it is unclear what impact such differences may have upon CBF. These differences might be important because the diagnosis of ciliary dyskinesia is often made based on an abnormally low CBF, highlighting the importance of accurately measuring CBF under experimental conditions [3]. Many studies have looked at variables that may affect CBF[8], however to date the effect of storage temperature on ciliated cells has not been reported in detail. If CBF measurements are affected by storage conditions, there is a potential for misinterpretation. Hence, this study focuses on the effects of storage temperature on subsequent CBF measurement.

First, immediately post nasal brushing, we determined whether a difference exists when brushings are stored on ice (4°C), stored at room temperature (20°C), or stored at nasal physiological temperature (32°C). In each case, subsequent CBF measurements were performed at either 20 or 32°C. The study also investigated a second problem that could confound analysis of CBF. Measurement of CBF is most often undertaken statically, using a sealed coverslip where cilia are not subject to fluid flow. However, dynamic studies are also possible whereby ciliated cells are placed in a perfusion chamber. Fluid flow is known to alter CBF [9], and this variable was also investigated. We pragmatically chose a perfusion rate that we had previously found not to alter CBF significantly in cells stored on ice but studied at room temperature[9].

Materials and Methods

Human nasal epithelial cells were obtained from non-asthmatic, non-atopic patients undergoing routine operations immediately after induction of anaesthesia (age range, 10-40) as previously described in detail [10, 11]. Only patients without respiratory infections for at least one month prior to the date of the operation were included in the study.

Ciliated epithelial cells were obtained by passing a 2 mm cytology brush over the inferior turbinate, as described elsewhere [8, 11, 12]and dislodged into Eppendorf tubes containing 1 ml of tissue culture medium (M199; ICN Biochemicals Limited, Oxfordshire, UK). The tubes were then either placed on ice at 4°C or kept at a constant temperature of either 20 ± 0.5°C or 32 ± 0.5°C in a water bath containing appropriately warmed medium. During each study, temperature was varied accordingly[13] and samples collected on a particular day were used within an hour of collection in a thermally controlled environment.

The protocol is described in detail elsewhere [10, 11]. Briefly cells collected from nasal brushings were placed into a perfusion chamber at the desired temperature. The chamber was placed on the platform of an inverted microscope (Nikon TMS Model 301052, Japan) which was connected to a video camera visual display unit (VDU). A modified computer-based Hoffmann contrast technique (Brian Reece Scientific, Newbury, Berkshire, UK) was used to monitor changes in screen contrast. The signal was then digitized, and the frequency of the cilia beating displayed on the computer screen both in a wave form and as a numerical value.

The perfusion protocol has been described elsewhere [11]. In brief, the Rose perfusion chamber (0.5 ml) was connected to a syringe-operated non-peristaltic infusion pump (Medfusion Model 2010; Medex Medical, Lancashire, UK) which delivered 2.5 ml of test solution at 0.125 ml minute^{-1}. The protocol for taking CBF readings was identical for all samples irrespective of storage temperature. Within an hour of sample collection and storage at 4°C, 20°C or 32°C, CBF were started at both 20°C and 32°C. A total of 5 sample measurements were carried out for each storage temperature at both 20°and 32°C. For each aliquot of cells, different cell borders were chosen randomly over 5 minutes, and a baseline period (time=0) was established where after CBF readings were taken every 5 minutes as percentages of the baseline for 20 minutes. This was followed by a 20 minutes period in which M199 solution were perfused at 0.125 ml/minute, and no readings were taken during flow. Within 30 seconds after end of perfusion, CBF measurements were taken as percentages of the baseline every 5 minutes for 60 minutes.

Study was approved by the Tayside Committee on Medical Research Ethics and informed consent was obtained from the patients or from parents of minors in all cases.

Statistical Analysis: The average CBF was computed from four different cell borders for each of 5 subjects (n number in figures). The averaged results were expressed as mean ± SEM in absolute values (Hz) and percentages of the baseline (t=0). Linear regression analysis compared the slopes obtained from post-flow data obtained from percentage of baseline CBF. Statistical differences between mean values were determined by a nonparametric Wilcoxon test. Significance was accepted when $p<0.05$.

Results

'Pre-flow' CBF measurements at 20 and 32°C (baseline=0-20 minutes Fig. 1)

Cilia of human nasal epithelia cells stored and observed at 4°C do not beat in M199 alone [14]. Therefore, following storage of ciliated cells at 4°, 20° and 32°C, CBF was measured at 20° and 32°C in each case (Fig. 1, and Table 1). No significant difference existed between samples measured at 20°

or at 32°C throughout the flow-free baseline period, following storage at 4°, 20° and 32°C (p>0.1).

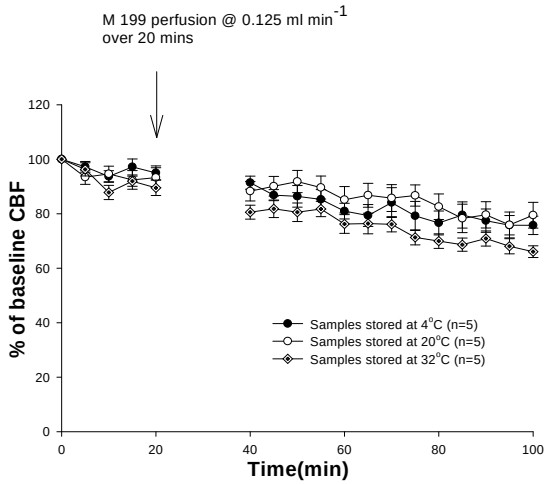

Figure 1: Effect of storage temperature on CBF at 20°C. Effect of sample storage temperature (4, 20 and 32°C) on CBF measured at 20°C. Time 0 minutes is the baseline and time 0-20 minutes is the pre-flow period. Cells were perfused during time 20-40 minutes. Time40-100 minutes represents the post-flow period.

'Post-flow' stress CBF measurements at 20°C (40-100 min Fig. 1)

CBF measurements at 20°C, from cells stored at either 4 or 20°C, showed no significant physiologically relevant difference (p= 0.3) up to 100 minutes following exposure to a flow rate of 0.125 ml/minute (Fig. 1 open and closed circle). A small rate of decline in post-flow CBF was noted; at 100 minutes the CBF was 76 ± 3 % and 80 ± 5 % for the 4and 20°C stored samples respectively (Table 1), while their slopes were parallel. In contrast, prior storage of ciliary cells at 32°C followed by cooling to 20°C did significantly affect measured CBF. Compared with cells stored on ice or at room temperature, CBF of cells stored at 32°C decreased throughout the post-flow period up to 100 minutes (p<0.002) (Fig. 1) (final CBF 63 ± 2 %). This contrasts with CBF measurements at 20°C from cells first stored on ice or at room temperature, which were unaffected. Thus, warming cells to 20°C yields a different CBF at 100 minutes (~ 5 Hz) compared to cooling cells to 20°C (CBF ~ 4 Hz; see Table 1).

'Post-flow' stress CBF measurements at 32°C (40-100 minutes Fig. 2)

The post-flow CBF of cells both stored and measured at 32°C declined sharply compared to equivalent measurements performed on cells stored at either 4° or 20°C and subsequently warmed to 32°C (Fig. 2 compare diamonds to the rest). The 32°C stored cells showed a significant, immediate and sustained (up to 75 minutes) post-flow fall in CBF compared to cells stored on ice or at room temperature (p <0.001), although all cells manifested a significant decline in CBF over time (see Table 2). Comparison of the CBF of cells stored on ice with those stored at room temperature prior to measurement at 32°C, showed no significant difference (p=0.2) (see first two rows of Table 2). By the end of the experiment (100 minutes), there was no longer a significant difference between the three storage conditions (p=0.5) (Fig. and Table 2). At 40 minutes, 4°, 20° and 32°C stored samples had baseline CBF values of 88.4 ± 2.7, 82.8 ± 1.8 and 69.1 ± 5.1 % of the baseline respectively, indicating a significant difference (p<0.001) between storage at 4°C or 20°C and storage at 32°C. At 100 minutes, the percentages of the baseline were 65.1 ± 3.6, 56.4 ± 1.4 and 56.0 ± 4.1 % respectively**,** indicating a significant difference (p=0.03) between storage at 4° or 20°C and storage at 32°C.

Sample storage Temp.	*CBF measured at 20°C*			
	Baseline 0 min Units (Hz)	**20 min Units (Hz)**	**40 min Units (Hz)**	**100 min Units (Hz)**
4°C	5.8 ± 0.1	5.5 ± 0.2 Hz (95.0 ± 2.5 %)	5.3 ± 0.2 Hz (91.4 ± 2.4 %)	4.4 ± 0.2 (75.7 ± 3.4 %)
20°C	6.1 ± 0.2	5.7 ± 0.2 Hz (93.3 ± 3.6 %)	5.4 ± 0.2 Hz (88.3 ± 3.6 %)	4.8 ± 0.3 (79.5 ± 4.8 %)
32°C	5.3 ± 0.1	4.7 ± 0.2 (89.5 ± 2.8 %)	4.2 ± 0.1Hz (80.6 ± 2.5%)	3.3 ± 0.1 (63.4 ± 1.8 %)

Table 1: Shows CBF at 20°C in M199 at specified time points on samples that had been stored at 4,20and 32°C. Top figures are the raw CBF values in Hz and below in brackets are percentages of the baseline CBF. Time 0 CBF is 100% of the baseline.

Discussion

There is no agreement as to the optimal conditions under which nasal brushings should be stored for clinical studies. Decisions made based on the observed frequencies could be affected by the storage conditions used. We report the effects of the temperature at which cells are stored prior to investigation of CBF. When CBF is measured at 20°C, irrespective of flow, the profile of CBF is similar for cells stored on ice compared to those

stored at room temperature throughout. This is not the case where CBF measurements at 20°C are undertaken with prior storage at 32°C where storage temperature has the greatest impact on the decrease in post-flow (but not pre-flow) CBF. Therefore prior storage of ciliary cells at 32°C affected the outcome of measurement, irrespective of measurement temperature. Samples stored at 32°C prior to measurement displayed a more rapid decline in CBF immediately after exposure to flow stimuli, whilst samples stored on ice or room temperature displayed a slow gradual decline.

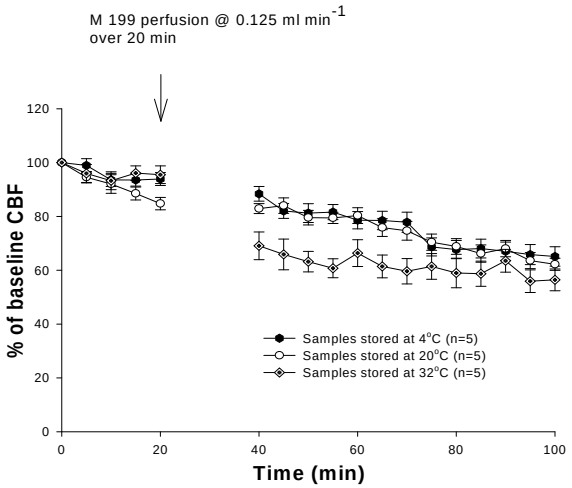

Figure 2: Effect of storage temperature on CBF at 32°C. Effect of sample storage temperature (4, 20 and 32°C) on CBF measured at 32°C. Time 0 minutes is the baseline and time 0-20 minutes is the pre-flow period. Cells were perfused during time 20-40 minutes. Time40-100 minutes represents the post-flow period.

Sample storage	*CBF measured at 32°C*			
Temp.	**Baseline 0 min Units (Hz)**	**20 min Units (Hz)**	**40 min Units (Hz)**	**100 min Units (Hz)**
4°C	9.7 ± 0.2	9.1 ± 0.2 (93.9 ± 2.4 %)	8.4 ± 0.2 (88.4 ± 2.7 %)	6.1 ± 0.3 (65.1 ± 3.6 %)
20°C	10.4 ± 0.2	8.8 ± 0.2 (84.7 ± 2.3 %)	8.6 ± 0.2 (82.8 ± 1.8 %)	5.8 ± 0.2 (56.4 ± 1.4 %)
32°C	9.9 ± 0.3	9.4 ± 0.2 Hz (96.0 ± 3.3 %)	6.7 ± 0.4 (69.1 ± 5.1 %)	5.5 ± 0.3 (56.0 ± 4.1 %)

Table 2: Shows CBF at 32°C in M199 at specified time points on samples that had been stored at 4,20and 32°C. Top figures are the raw CBF values in Hz and below in brackets are percentages of the baseline CBF. Time 0 CBF is 100% of the baseline.

Our results also suggest that the storage of ciliary cells at nasal physiological temperature renders the cilia more sensitive to fluid flow rate. We chose a constant flow rate of 0.125 ml/minute because our previous findings showed that perfusing nasal epithelial cells with higher flow rates, resulted in an acute reduction of CBF at 20°C which we called the acute dip response, followed by a partial recovery to a lowered plateau (post recovery plateau) [9]. This sharp decline is in contrast to earlier observations which showed acute increments in CBF immediately after mechanical stimulation of cultured airway cells [15, 16]. Nevertheless, we found that when post-flow CBF is measured at 32°C, there is a significant decline in CBF with poor recovery up to 100 minute.

Lower storage temperatures seem to have a stabilizing effect on CBF. Hypothermia slows all metabolic processes (thus conserving energy) by affecting enzymatic reactions in the cells [17]. That's why it is used for the preservation of isolated tissues and organs[18]. This probably can explain why there was rapid deterioration in CBF at both 20°C and 32°C when cells were stored at 32°C because storage at 32°C would speed enzymatic reactions to a much greater degree than storage at a lower temperature. The result of this would be reduced energy levels of the sample stored at 32°C than at either 4 or 20°C; hence the faster reduction of CBF.

Conclusion

In conclusion, we can say that the temperature at which nasal ciliary samples are stored prior to measurement can affect the CBF following fluid perfusion. Cells stored at 4 and 20°C show no significant difference in CBF when measured at 20 or 32°C, irrespective of flow. Cells stored at 32°C do however show a significant difference when measured at both 20° and 32°C immediately post-flow, with the latter temperature inducing an immediate decline in CBF. This decline has implications for dynamic studies of nasal CBF, since the cell storage protocol followed may affect the results obtained. It appears reasonable to have confidence that CBF measured at room temperature in cells either first stored at 4 or 20°C will yield similar results. We suggest that standardization of this protocol is necessary in order to obtain consistently reliable and meaningful results in the context of flow and temperature.

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Conflict of interest: Nil

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