Bubble CPAP Support after Discontinuation of Mechanical Ventilation Protects Rat Lungs with Ventilator-Induced Lung Injury

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Key Words

Bubble continuous positive airway pressure · Nitric oxide synthase · Ventilator-induced lung injury

Abstract

Background: Bubble continuous positive airway pressure (BCPAP) has been used in neonates with respiratory distress for decades, but its lung-protective effect and underlying mechanism has not been investigated. **Objectives:** To test the hypothesis that BCPAP use after extubation decreases lung injury and that alterations to lung nitric oxide synthase (NOS) 3 expression may be one of the underlying mechanisms. **Methods:** We compared gas exchange, lung injury severity, and lung NOS expression among rats with ventilator-induced lung injury (VILI) treated with either BCPAP or spontaneous breathing. After high tidal volume ventilation for 30 min, the rats were randomly divided to three groups: a control group underwent spontaneous breathing ($n = 7$), and two BCPAP groups were treated with the bubble technique with either a 2.5-mm-diameter ($n = 7$) or a 5.5-mm-diameter ($n = 7$) expiratory limb for 2 h. **Results:** The bubble technique

(2.5 and 5.5 mm diameter combined) resulted in a significantly higher PaO_2 , decreased alveolar protein levels (1.01 vs. 1.43 mg/kg, $p < 0.05$), a lower lung injury score (3.87 vs. 4.86, $p < 0.05$), and decreased NOS3 expression (1.99 vs. 3.32, $p < 0.05$) compared to spontaneous breathing in the control group. BCPAP with a 2.5-mm-diameter and with a 5.5-mm-diameter expiratory limb was not different with regard to gas exchange, alveolar protein levels, and lung injury scores, but there was a trend for lower NOS3 expression in the 5.5-mm group (1.41 vs. 2.56, $p = 0.052$). **Conclusions:** BCPAP decreases lung injury in rats with VILI after stopping mechanical ventilation. Attenuation of lung NOS3 expression may be one of the underlying mechanisms. © 2016 S. Karger AG, Basel

Introduction

Mechanical ventilation with high tidal volumes may initiate and/or exacerbate acute lung injury via a process known as ventilator-induced lung injury (VILI) [1, 2]. Shear forces generated during high tidal volume ventila-

tion induce alveolar-capillary barrier disruption and pulmonary inflammation. Several mechanisms have been implicated in the development of VILI, including neutrophil infiltration, an increased production of proinflammatory cytokines, and nitric oxide (NO) [1–3]. NO is produced by three NO synthase (NOS) isoforms: neuronal, inducible, and endothelial NOS (NOS1, 2, and 3, respectively) [4]. Several studies have shown that NOS2 contributes to VILI [5–7], whereas the role of NOS3 remains controversial. Transgenic mice overexpressing NOS3 and mice that were congenitally NOS3 deficient were both found to be protected from VILI [8, 9].

Nasal continuous positive airway pressure (nasal CPAP) is a method of noninvasive respiratory support which can facilitate extubation and reduces the risks of prolonged ventilation [10]. For more than 40 years, bubble CPAP (BCPAP) has been used to treat respiratory distress syndrome in newborn infants during the weaning period or acute stages [10–12]. BCPAP has also been shown to be associated with a significantly higher rate of successful extubation and reduced duration of CPAP support compared with Infant Flow Driver CPAP for infants ventilated for fewer than 14 days [13]. Pillow et al. [14] reported that treatment with BCPAP immediately after birth enhances gas exchange, lung mechanics, gas mixing efficiency, and lung volume compared with constant-pressure CPAP in an ovine model of preterm lung disease. However, the exact lung-protective effect comparing BCPAP and spontaneous breaths after discontinuation of mechanical ventilation had not been investigated.

Suki et al. [15] suggested that the alveolus and terminal airspace recruitment process may benefit from the superimposition of noise on the applied driving pressure, exploiting a phenomenon known as stochastic resonance. This feature might be essential for understanding the unique physiological benefits that BCPAP offers. The principles of stochastic resonance suggest that the amplitude and frequency of superimposed noise can be optimized to achieve the most favorable amplification (i.e., volume recruitment events). Recently, *in vitro* studies have shown that the applied bias flow and mechanical properties of the lung, the diameter of the bubble generator bottle, and the size and submergence depth of the underwater seal of the expiratory limb of the CPAP circuit influenced the magnitude and the frequency content of the noise transmitted to the lung [16, 17].

In the present study, we hypothesized that (1) BCPAP support after discontinuation of high tidal volume ventilation provides a lung-protective effect and alterations

to NOS3 expression may be one of the underlying mechanisms, and that (2) different settings of the BCPAP circuit will influence gas exchange efficiency, lung inflammation, and NOS3 expression in rat lungs with VILI. To test this hypothesis, we compared three groups of rats undergoing either spontaneous breathing or BCPAP with two variously sized expiratory limbs after high tidal volume ventilation by evaluating gas exchange efficiency, severity of lung injury, and NOS3 expression in these groups.

Materials and Methods

Animal Preparation

This study was approved by the Animal Care and Use Committee of Taipei Medical University. Twenty-one pathogen-free, adult male Sprague-Dawley rats weighing 250–300 g were maintained on a 12-hour light-dark cycle with free access to food and water. The animals were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg; Abbott, North Chicago, Ill., USA), weighed, and then placed supine on a heating pad. A polyethylene (PE-50; Becton Dickinson, Sparks, Md., USA) catheter containing heparinized, isotonic saline was placed in one carotid artery to sample blood for gas analysis. Blood gas tensions were measured with a blood gas analyzer (model 1620; Instrumentation Laboratories, Lexington, Mass., USA). A tracheostomy was performed, and a 14-gauge plastic cannula was inserted into the trachea. The endotracheal tube was connected to a volume-cycled small animal ventilator (model SAR-830/AP; CWE, Ardmore, Pa., USA), and all rats were ventilated for 30 min at a tidal volume of 40 ml/kg, zero positive end-expiratory pressure, a respiratory rate of 30 breaths/min, an inspiratory-to-expiratory time ratio of 1:1, and an inspiratory O₂ fraction of 0.21.

Experimental Protocol

After 30 min of high tidal volume ventilation, arterial blood gases were assessed and the rats were randomized to three groups: spontaneous breathing (*n* = 7), BCPAP using a bias flow of 6 liters/min with a 2.5-mm-diameter expiratory limb (*n* = 7) or BCPAP using a bias flow of 6 liters/min with a 5.5-mm-diameter expiratory limb (*n* = 7). During the experiment, anesthesia was maintained with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). The depth of the underwater seal in the BCPAP circuits was adjusted to 5 cm. Respiratory rates and arterial blood gases were assessed 0, 30, 60, 90, and 120 min after BCPAP had been applied.

Lung Processing

The rats were killed 2 h after BCPAP treatment, and the tracheal tube was clamped for 3 min to facilitate oxygen absorption and lung collapse. The thorax was opened and the lung was removed. Tissue from the right lower lobe was used for histological examination, and the rest was immediately frozen in liquid nitrogen for Western blot analysis. Three repeated saline lavages of the left lung were combined for the bronchoalveolar lavage fluid (BALF), and aliquots were saved for further analysis.

BALF Protein and Cytokine Analysis

The total protein concentration in BALF was measured with a bicinchoninic acid assay (Pierce Chemical, Rockford, Ill., USA). The levels of IL-6 and MIP-2 in the BALF were determined using the ELISA kit (Cloud-Clone Corp., Houston, Tex., USA) and the Rat ELISA kit (KRC1022; Invitrogen Life Technologies, Carlsbad, Calif., USA), respectively. The data are expressed in picograms per milliliter.

Western Blot Analysis of NOS

Lung tissue (0.1 g) was homogenized in 1 ml of ice-cold lysis buffer containing 1% Nonidet P-40 (NP-40), 0.1% SDS, 0.01 M deoxycholic acid, and a complete protease cocktail inhibitor (Sigma, S8830). The samples were then centrifuged at 13,000 rpm for 20 min at 4°C, and the supernatant was aliquoted and stored at -20°C. Proteins (30 µg) were resolved in 10% SDS-PAGE in reduced condition and electroblotted to a polyvinylidene difluoride membrane (Immobilon-P, IPVH00010; Millipore, Bedford, Mass., USA). After blocking with 5% nonfat milk, the membranes were incubated with polyclonal anti-NOS3 antibody (1:800, GT50505; GeneTex Inc., San Antonio, Tex., USA), anti-NOS2 antibody (1:1,000, PA3-032A; Thermo Fisher Scientific, Vernon Hills, Ill., USA), and anti-NOS1 antibody (1:1,000, PA3-030A; Thermo Fisher Scientific). β -Actin rabbit monoclonal antibody (1:5,000, 13E5; Cell Signaling Technology, Danvers, Mass., USA) was used as an internal control. Goat anti-rabbit IgG (H+L) secondary antibody, HRP linked (1:10,000, 31460; Pierce Biotechnology Inc., Rockford, Ill., USA), was used as a secondary antibody. Densitometric analysis was used to measure the intensity of the target proteins and β -actin using Image J software. The data were normalized to β -actin for each protein.

Histological Examination

Lung tissue was fixed in 4% paraformaldehyde in phosphate buffer, embedded in paraffin, stained with hematoxylin and eosin, and examined by a pathologist who was blinded to the protocol and experimental groups. The lung injuries were scored according to the following four criteria: (1) alveolar congestion, (2) hemorrhage, (3) infiltration of neutrophils into the air space or vessel wall, and (4) thickness of the alveolar wall. Each item was graded according to a 5-point scale as follows: 0 for minimal (little) damage, 1 for mild damage, 2 for moderate damage, 3 for severe damage, and 4 for maximal damage.

Immunohistochemical Staining for NOS3

Immunostaining was performed on 5-µm paraffin sections with immunoperoxidase visualization. After routine deparaffinization, heat-induced epitope retrieval was performed with slides immersed in 0.01 M sodium citrate buffer (pH 6.0). To block endogenous peroxidase activity and nonspecific binding of antibody, sections were first preincubated for 1 h at room temperature in 0.1 M phosphate-buffered saline containing 10% normal goat serum and 0.3% H₂O₂ before being incubated for 20 h at 4°C with rabbit polyclonal anti-NOS3 (1:200 dilution; Bioss Inc., Boston, Mass., USA) as primary antibodies. The sections were then treated for 1 h at room temperature with biotinylated goat anti-rabbit IgG (1:100; Jackson ImmunoResearch Laboratories Inc., West Grove, Pa., USA), followed by reacting them with the reagents from an ABC kit (avidin-biotin complex, VECTASTAIN ABC KIT, pk4000; Vector Laboratories Inc., Burlingame, Calif., USA) ac-

cording to the manufacturer's recommendations; the reaction products were visualized by diaminobenzidine solution (Vector Laboratories). The sections were dehydrated by increasing concentrations of ethanol, cleared by xylene, and coverslipped with Permount (Fisher, Pittsburgh, Pa., USA).

Statistical Analysis

Results are shown as means ($\pm$ SD) unless specified otherwise. Statistics were analyzed using SPSS version 17.0 (SPSS Inc., Chicago, Ill., USA). Two-way repeated-measures analysis of variance was used to detect potential differences between groups with time, and Bonferroni adjustments were used as a post hoc analysis. One-way analysis of variance was used for all other statistical comparisons between the three groups. Significance was accepted at $p < 0.05$.

Results

Time Course of Changes of Arterial Blood Gas Values

A comparison of pH, PaO₂, and PaCO₂ from the start of high tidal volume ventilation to the end of BCPAP between the BCPAP group and the control group is shown in figure 1a–c. Arterial blood gases were comparable at the beginning of the study between the BCPAP and the control group. Rats receiving BCPAP treatment exhibited significantly lower pH values at 90, 120, and 150 min compared to control rats (fig. 1a). Rats undergoing BCPAP treatment after extubation had significantly higher (or showed a trend for higher) PaO₂ values between 60 and 150 min compared to control rats (fig. 1b), and they had significantly higher (or showed a trend for higher) PaCO₂ values between 90 and 150 min compared to control rats (fig. 1c). The BCPAP groups formed according to their varying expiratory limb diameters (2.5 and 5.5 mm) exhibited comparable arterial blood gas values (fig. 1d–f). The mean respiratory rate of the BCPAP group at 0, 30, 60, 90, 120, and 150 min was 93, 80, 69, 72, 75, and 69 breaths/min, respectively. The mean respiratory rate of the control group at 0, 30, 60, 90, 120, and 150 min was 105, 119, 118, 124, 138, and 108 breaths/min, respectively. The mean respiratory rates of the BCPAP group were lower than those of the control group, though the differences did not reach statistical significance.

BALF Protein and Cytokines

Rats receiving BCPAP treatment (2.5 or 5.5 mm) exhibited a significantly lower BALF protein content compared to control rats (fig. 2a). The BALF protein content was not significantly different between the two BCPAP groups with varying expiratory limb diameters. Rats re-

Fig. 1. a–c Time course of changes of arterial blood gas values in the BCPAP and control groups. **d–f** Time course of changes of arterial blood gas values in the BCPAP groups with varying expiratory limb diameters. ‘0 min’ denotes the start of high tidal volume ventilation; ‘30 min’ denotes the start of BCPAP or spontaneous breathing. * $p < 0.05$ compared to the control group at each time point.

ceiving BCPAP treatment showed significantly lower BALF IL-6 levels compared to control rats undergoing spontaneous breathing (fig. 2b). The BALF IL-6 levels were not significantly different between the two BCPAP groups with varying expiratory limb diameters. Rats receiving BCPAP treatment exhibited a trend for lower BALF MIP-2 levels compared to control rats undergoing spontaneous breathing ($p = 0.067$; fig. 2c).

Lung Injury Score

Rats receiving BCPAP treatment (2.5 or 5.5 mm) exhibited significantly lower lung injury scores compared to control rats ($p = 0.04$; fig. 3). Lung injury scores were not significantly different between the two BCPAP groups with varying expiratory limb diameters. Typical images for each lung injury score are shown in the online supplementary figure (for all online suppl. material, see www.karger.com/doi/10.1159/000443528).

Fig. 2. BALF total protein (a), IL-6 (b), and MIP-2 (c) concentrations in the control and BCPAP groups.

NOS Expression

NOS1 and NOS2 protein expression was comparable between the three groups (fig. 4a, b). NOS3 expression was significantly decreased in rats treated with BCPAP when compared to rats undergoing spontaneous breath-

Fig. 3. Lung injury scores in the control and BCPAP groups.

ing (fig. 4c). The BCPAP 5.5-mm-diameter expiratory limb group exhibited a trend for lower NOS3 expression compared to the 2.5-mm-diameter expiratory limb group (1.41 vs. 2.56, $p = 0.052$).

Lung NOS3 Immunohistochemical Staining

NOS immunoreactivity was expressed prominently in bronchiolar epithelial cells and endothelial cells (fig. 5). Compatible with the Western blot findings, the control rats expressed the highest NOS3 immunoreactivity and the BCPAP group (5.5 mm) exhibited the lowest NOS3 immunoreactivity.

Discussion

Nasal CPAP is frequently used in preterm or term babies with respiratory disorders in the acute stage and post-extubation period [10–12]. This noninvasive respiratory support has many physiological benefits such as increasing transpulmonary pressure and functional residual capacity, preventing pharyngeal wall and alveolar collapse, decreasing intrapulmonary shunt and improving lung compliance, conserving surfactants, stabilizing the chest wall, and increasing the airway diameter and splinting the airway and the diaphragm [18]. Proper use of CPAP decreased the intubation rate immediately after birth in the delivery room [19] as well as the reintubation rate after weaning ventilator support in sick babies [13]. However, few studies have investigated the protective effects of CPAP on lungs with VILI during the post-extu-

bation period. In this study we demonstrated that after discontinuation of high tidal volume ventilation, rats on BCPAP treatment (2.5-mm-diameter and 5.5-mm-diameter expiratory limb combined) exhibited significantly lower BALF total protein levels (1.01 vs. 1.43 mg/kg, $p < 0.001$), lower lung injury scores (3.87 vs. 4.86, $p = 0.04$), and lower IL-6 levels (0.75 vs. 2.87 pg/ml, $p = 0.03$) compared to spontaneously breathing rats. These findings suggest that in addition to the previously described physiological benefits, BCPAP support after weaning ventilator support in mechanically ventilated patients can provide lung-protective effects as well as decrease lung inflammation. Although the BALF IL-6 and MIP-2 levels of the rats receiving BCPAP treatment with a 5.5-mm-diameter expiratory limb were higher than those in the 2.5-mm BCPAP group, which was inconsistent with our hypothesis on NOS3, the difference was small and statistically insignificant. In addition, it is still unknown what the most optimal amplitude of the BCPAP circuit is for lung recruitment and lung protection in lungs with VILI. Further research with larger numbers of subjects and greater amplitude variations are warranted to clarify the impact of different settings of BCPAP on lung protection in VILI.

The main features of BCPAP that differ from constant-pressure CPAP are the stochastic resonance phenomenon and the high-frequency oscillatory ventilation effect. A key feature of stochastic resonance is that the most optimal amplitude has the best effect on lung volume recruitment [15], which is similar to the finding that the best balance between lung overinflation and collapse was achieved on the positive end-expiratory pressure at the lowest ratio of alveolar dead space to alveolar tidal volume and the phase III slope of volumetric capnography found in a surfactant-depleted piglet model [20]. Dibiasi et al. [21] used BCPAP with different amplitudes and found that high-amplitude BCPAP provides greater respiratory support than conventional BCPAP in lavaged juvenile rabbits. However, Pillow et al. [14] compared various amplitudes of BCPAP with different inspiratory flows and did not find any statistically significant difference in gas mixing efficiency immediately after birth in an ovine model of preterm lung disease. In our study as well, we did not find a statistically significant difference in gas exchange efficiency between the BCPAP

Fig. 4. Lung NOS1 (a), NOS2 (b), and NOS3 (c) protein expression in the control and BCPAP groups.

Fig. 5. Representative immunohistochemistry of NOS3 in the control (a), BCPAP 2.5 mm (b), and BCPAP 5.5 mm (c) groups. NOS3 immunoreactivity was expressed prominently in bronchiolar epithelial cells (arrows) and endothelial cells (arrowheads).

groups applying 2.5-mm expiratory limbs and 5.5-mm expiratory limbs. We speculated that this discrepancy may be due to varying lung compliances and BCPAP amplitudes in different laboratory settings, since the amplitude of the pressure waveform transmitted to a model lung markedly decreased while the lung compliance increased [16, 17].

Theoretically, rats with VILI receiving BCPAP support should have higher PaO_2 , lower PaCO_2 , and higher pH values of arterial blood gas compared to control rats without respiratory support. In this study, we indeed found that rats on BCPAP support had better oxygenation than control rats, but instead of lower PaCO_2 and higher pH values, the BCPAP group had higher PaCO_2 and lower pH values than the control group. We thought the reason might be that the CPAP level (5 cm H_2O) applied to the BCPAP group was too high, since increasing CPAP may lead to an increase in PaCO_2 when the pressure is too high, especially in a compliant lung [18]. Besides, we found that the average respiratory rate of the BCPAP group was lower than that of the control group from discontinuation of mechanical ventilation to the end of the study, although the differences did not reach statistical significance. Because the initial VILI process was the same, the lung compliance and tidal volume should have been similar in the BCPAP and the control group; thus, a lower respiratory rate could lead to higher PaCO_2 and lower pH values. The CPAP level might influence the result of the comparison between the BCPAP groups with different expiratory limb diameters, but it should not change the finding made in this study that BCPAP applied after discontinuation of mechanical ventilation decreases lung injury and inflammation compared to spontaneous breathing. We speculated that with more proper CPAP support, in rat lungs with VILI the lung-protective

effects might be more prominent than in the control group.

Another finding of our study was that BCPAP support after discontinuation of mechanical ventilation decreased lung NOS3 expression in rats with VILI. The role of NOS3 in VILI is still controversial. Schmidt et al. [9] found that NOS3 deficiency reduces high tidal volume ventilation-induced pulmonary edema and lung injury in isolated-perfused mouse lungs. Vaporidi et al. [22] found that NOS3 contributes to VILI via an increased production of superoxide in a mouse model. However, Takenaka et al. [8] observed that transgenic overexpression of NOS3, leading to increased pulmonary NO levels, protected mice from VILI. Vaporidi et al. [22] supposed that the conflicting results may be due to different degrees and a different consistence of lung injury in each study. In our study, we used the same tidal volume of 40 ml/kg as in the study by Vaporidi et al. [22] and found that the spontaneous breathing group had higher NOS3 expression and showed a statistically significantly higher degree of lung injury than the BCPAP group (4.86 vs. 3.87, $p = 0.04$), suggesting that NOS3 may contribute to VILI.

Acute respiratory distress syndrome results almost uniformly in a need for mechanical ventilation. Although the lung-protective ventilator strategy is widely used at present, the heterogeneity of ventilation in patients with poor lung compliance and marked pulmonary inflammation as seen in acute respiratory distress syndrome still frequently leads to the risk of superimposed VILI [23]. In endothelium, mechanical stretch has been shown to increase reactive oxygen species (ROS) production, leading to the upregulation of cell adhesion molecules and chemokines. Although few in number, there are studies demonstrating an increased ROS production by stretched alveolar or airway epithelial cells. Cyclic stretch stimulated

ROS production via an increased expression of the ROS-generating enzymes NADPH oxidase and NOS3. Recently, Vaporidi et al. [22] found that the source of ROS in VILI was mainly in NOS3 expression in the uncoupling condition. Uhlig [24] described that in pulmonary circulation, pathological overdistension of the lung may induce inflammatory processes triggered by mechanical activation of macrophages and epithelial and endothelial cells, which may cause alveolar and endothelial barrier dysfunction and vascular leakage and may culminate in VILI syndrome or pulmonary edema [24]. We think that the mechanisms described above can support our findings and the proposed mechanisms of BCPAP and NOS3 expression, that is, BCPAP can decrease uneven regional lung distension and increase homogenous alveolar recruitment and physiological pulmonary endothelial and epithelial stretch in rat lungs with VILI with pulmonary edema after discontinuation of mechanical ventilation. As a result, it prevents the pathological cyclic stretch-induced ROS production and signaling pathway contributing to lung injury and alveolar-capillary barrier disruption; then, NOS3 expression and uncoupling in rat lungs with VILI decrease. BCPAP, standard CPAP, or a high-flow nasal cannula are widely used after extubation in full-term or preterm neonates, and currently no sufficient evidence exists to indicate which one is best. If our hypothesis that BCPAP has a better effect on homogenous alveolar recruitment and resolution of lung injury is true, BCPAP support after discontinuation of mechanical ventilation might be the best choice in neonatal intensive care units to decrease respiratory morbidity, and a currently unavailable BCPAP device designed for children or adults with acute lung injury might also be beneficial during the post-extubation period.

One limitation of this study is that we did not compare the lung-protective effects and gas exchange efficiency of constant-pressure CPAP to those of BCPAP, since no available ventilator was capable of providing standard CPAP in rats. However, we designed two BCPAP groups with different diameters of the expiratory limb, which indicate different amplitudes transmitted to the rats' lungs according to the law of stochastic resonance. We found that the 2.5-mm-diameter BCPAP group (lower amplitude) tended to show higher lung NOS3 expression than the 5.5-mm-diameter BCPAP group (larger amplitude; 2.56 vs. 1.41, $p = 0.052$). Therefore, we speculated that constant-pressure CPAP with a zero amplitude may exhibit higher NOS3 expression than BCPAP. However, the actual difference in lung-protective effects in lungs with VILI after discontinuation of mechanical ventilation be-

tween BCPAP and standard CPAP still needs to be confirmed by further study. The other limitation is that we used adult rather than newborn rats in this study. Kuebler et al. [25] demonstrated that circumferential stretch activates NO production in pulmonary endothelial cells by a signaling cascade involving phosphatidylinositol-3-OH kinase, Akt, and NOS3 in intact and isolated-perfused mouse lungs. This response is independent of the mechanical factors causing vascular distension. We speculated that these biochemical and genetic responses of the pulmonary endothelial cells to mechanical stretch may be similar in adult and newborn rat lungs; considering the vulnerability of newborn or premature lungs, the degree of lung injury due to pathological overdistension of the alveoli may be more severe than in adult lungs.

Conclusions

BCPAP decreases lung injury after weaning rats with VILI from ventilation, and attenuation of lung NOS3 expression may be one of the underlying mechanisms. This study indicates the lung-protective effect of BCPAP use after extubation in mechanically ventilated patients, as well as the need for further research on BCPAP with different settings, and compares the lung-protective effect between different respiratory support devices after extubation.

Financial Disclosure and Conflicts of Interest

None.

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