Abstract: We examined the use of surgical eye spears for saliva collection and recovery of secretory immunoglobulin A (s-IgA). We established a protocol for recovering s-IgA at concentrations equivalent to those present in the control samples (untreated by surgical eye spears). Four different types of eye spears were submerged in the salivary fluid. Saliva was recovered from each spear by centrifugation. Novel ultra-sensitive enzyme-linked immunosorbent assay was used to determine s-IgA concentration in each sample. The results revealed that Weck-Cel eye spears recovered s-IgA at concentrations not significantly different from s-IgA concentrations in control samples as long as the appropriate protocol was followed. We demonstrated that the use of surgical eye spears for saliva collection is a viable method for recovering s-IgA. (J Oral Sci 58, 205-210, 2016)

Keywords: immunoglobulin A; secretory; eye spears; saliva; enzyme-linked immunosorbent assay.

Introduction

Collection of oral fluid specimens is considered safe, fast, minimally invasive, economical, and acceptable to most patients in comparison with other methods of biological specimen collection, such as drawing blood (1-4). This approach has various benefits: it allows self-collection of specimens and reduces transmission of infectious diseases (4). Moreover, salivary biomarkers are an abundant source of information on the status of various physiological systems, making the collection of saliva essential for a wide range of research activities (1-2).

Several different methods and devices for saliva collection are currently available, such as the collection of passive drool, utilization of cotton absorbent materials, filter paper and, more recently, surgical eye spears (3,5-10). However, the samples obtained using these techniques and devices do not have the same volume and/or biomarker composition. This is a consequence of difficulties in separating the absorbed fluid sample and its components from the absorbent material (3,10). This also applies to one of the salivary biomarkers of particular interest, the secretory immunoglobulin A (s-IgA). s-IgA is the predominant secreted antibody found in the saliva at high concentrations (11). Enzyme-linked immunosorbent assay (ELISA) results have shown that cotton, used as the absorbent material (e.g., cotton Salivettes), interferes with IgA quantification; the results underestimate the s-IgA levels (9-10). Using cellulose fiber materials does not have this effect (10). Chang et al. have reported that devices with different absorbent materials (i.e., cotton, cellulose, or polystyrene), recover varying amounts of IgA, although the IgA in question was not derived from the salivary fluid (5). There is no consensus on the most appropriate absorbent material for collecting the saliva for accurate and reproducible recovery of s-IgA.

Surgical eye spears are used for sponging the ocular area during ocular surgical procedures (3,7,10).
They consist of a plastic applicator shaft joined to an arrowhead-shaped sponge. Some absorbent sponges are composed primarily of polyvinyl alcohol (PVA), while others contain mainly cellulose. Because of their excellent absorbency, the eye spears have recently been promoted as promising alternative devices for saliva collection. In particular, the cellulose eye spears absorb greater volumes of saliva than traditional absorbent cotton devices. They have been rated as more comfortable, pleasant, and easier to use than traditional methods of passive collection and other absorbent devices, such as cotton Salivettes (3,8,10). These preferences apply to small children as well as to adults (3,7). Saliva collection from infants and young children can be hampered by insufficient specimen volume and non-cooperation of the subjects and/or caregivers. Sample collection from the elderly might be difficult because of their impaired cognitive status, pharmacological side effects (e.g., xerostomia), immobility, and fatigue (3,7,12). Employing the eye spears should ensure that the volume of salivary fluid collected is adequate for biomarker analysis. This method might be the most effective and convenient approach to collecting the saliva samples from uncooperative subjects who might be incapable of simply expectorating the salivary fluid when prompted.

Here, we examine the efficacy of various currently available surgical eye spears in the collection and recovery of salivary IgA. We set out to determine which, if any, of these eye spears can effectively collect the saliva for s-IgA reconstitution, and establish the optimal protocol for s-IgA recovery.

**Materials and Methods**

**Collection devices**

Four different types of surgical eye spears were used: Visispear (Beaver-Visitec International, Waltham, MA, USA), Weck-Cel (Beaver-Visitec International), Merocel (Beaver-Visitec International), and Keraspear (Beaver-Visitec International) (Fig. 1). Each spear consisted of a plastic applicator shaft joined to a triangular absorbent sponge. The absorbent sponges varied in their composition. The absorbent portions of the Visispear and Weck-Cel eye spears are composed of cellulose, while the Merocel and Keraspear eye spears are made of PVA. No further information regarding the composition was available from the manufacturers.

**Sample collection and preparation**

Saliva was initially collected from one healthy male adult. The subject expectorated saliva into a collection vial. The saliva was aliquoted into centrifuge tubes at 300 µL per tube. One tube of untreated saliva was used as the reference saliva sample. Each type of spear was placed in a tube of saliva for 1 h. Afterward, the sponges were detached from their stems and centrifuged. The centrifugation radius was 11.3 cm in all cases. One set was centrifuged at 10,000 rpm for 5 min, and the second set was centrifuged at 2,000 rpm for 5 min, to recover the absorbed saliva. To prevent reabsorption of the fluid immediately after centrifugation, all the sponges were placed in plastic pipette tips, which allowed the fluid to seep down into the centrifuge tubes. All samples were treated at room temperature.

To examine the effect of centrifugation on the final IgA concentration measurements, the eye spears that initially performed best in the IgA recovery were tested at centrifugation speeds of 2,000, 4,000, 8,000, and 10,000 rpm for 5 min. The resultant samples were compared with untreated saliva samples centrifuged at the same speeds. As before, centrifugations were performed 1 h after submerging the spears into the saliva.

To assess the effect of storage time on IgA measurements, the best-performing eye spears were centrifuged at the optimum centrifugation speed (defined in the previous experiment) after submersion lasting for 1, 2, or 4 h. The concentration of recovered s-IgA was compared with its concentration in the reference sample (where s-IgA was assayed immediately after the collection of saliva). Each centrifuge tube remained uncovered at room temperature. A second trial was performed using almost identical protocol; however, the tubes remained covered. To rule out inter-individual differences in the composition of saliva, total IgA was then measured in two sets of saliva samples collected from 11 healthy individuals.
(data not shown). One set of samples was tested after simple expectoration while the second set was collected using the best-performing eye spear. Both sets of samples were initially centrifuged under the optimum conditions (centrifugation speed and time) identified in the previous experiments.

The Institutional Review Board of the Mount Sinai Medical Center approved this study (11-0112), and all subjects consented to having their saliva collected and examined.

Immunoassay protocol

An ELISA, slightly modified from the method described by Konstantinou et al. (13), was used to determine the recovery of s-IgA from the various eye spears, relative to s-IgA recovery from the untreated reference samples. The plates (96-well, Immulon 4HBX; Fisher Scientific, Pittsburgh, PA, USA) were left overnight at 4°C with 100 μL of an α-chain specific goat F(ab’)2 anti-human IgA (InvivoGen, San Diego, CA, USA) antibody (primary antibody), at 2 μg/mL, in 0.05 M carbonate-bicarbonate buffer (pH 9.6). After three washes with phosphate-buffered saline containing 0.05% Tween-20 (PBST), the plates were blocked with 200 μL/well of 2% bovine serum albumin in PBST (blocking buffer) for 1 h at 31°C. An 8-point standard curve was constructed using dimeric IgA (Sigma-Aldrich, St. Louis, MO, USA) isolated from human colostrum, and by diluting this standard two-fold in blocking buffer to create an operating range of 0.391-50 ng/mL. The reconstituted salivary fluid samples were plated in duplicates, diluted two-fold starting from 1:10,000, and incubated at 31°C for 2 h. After washing as before, the goat anti-human IgA-HRP (Fc-specific) (Antibodies Online, Atlanta, GA, USA) was diluted 1:2,000 in blocking buffer and applied at 100 μL/well for 1 h at 31°C. The plates were then washed (6×) and ABTS (KPL, Inc, Gaithersburg, MD, USA) was added and allowed to develop for 1 h at 31°C. Absorbance values were measured at 405 nm using SoftMax Pro system.

Statistical analysis
To measure the efficacy of each spear in the recovery of the salivary s-IgA concentrations, the concentration of s-IgA in each sample of saliva incubated with an eye spear was compared to the concentration of s-IgA in a sample of the untreated saliva. This untreated sample was a standard of reference, representing an s-IgA concentration of 100%. If the concentration of s-IgA in the fluid recovered from a spear was equal to the s-IgA concentration in the reference sample, this spear would be considered to show 100% efficacy. This percentage recovery value was used to evaluate the performance of the various eye spears at various dilutions. The Wilcoxon rank-sum test was used to compare each IgA concentration with that in the corresponding untreated, standard, saliva sample. All reported P values were based on 2-sided tests and compared with a significance level of 5%. Stata 9.1 for Windows (Stata Corp LP, College Station, TX) was used for all statistical calculations and plots.

Results
Comparing the different eye spears
Salivary IgA concentrations in samples recovered from the eye spears centrifuged at 10,000 rpm differed significantly from the IgA concentration detected in the original reference sample (Fig. 2a). The average absorbance values obtained by ELISA at each dilution approximated a concentration of 126.652 μg/mL in the reference saliva
sample. This value was set as 100% s-IgA recovery, and the results obtained for samples incubated with eye spears were expressed relative to this standard. The Visispear, Keraspear, Weck-Cel, and Merocel on average recovered s-IgA at the concentrations of 168.203 μg/mL, 315.391 μg/mL, 226.612 μg/mL, and 205.022 μg/mL, respectively. These correspond to 34.2% (P = 0.002), 150.4% (P < 0.001), 79.6% (P < 0.001), and 62.9% (P < 0.001) more salivary IgA than in the untreated reference sample. However, when the eye spears were centrifuged at 2,000 rpm, the measured s-IgA yield of the Weck-Cel did not differ significantly from the 148.013 μg/mL s-IgA detected in the reference sample (155.558 μg/mL, 7.8%, P = 0.07). The samples incubated with the Visispear, Keraspear, and Merocel spears yielded the s-IgA concentration of 122.529 μg/mL (−18.4%), 262.573 μg/mL (80.7%), and 375.534 μg/mL (163.8%), respectively (P < 0.05) (Fig 2b).

**The effect of centrifugation speed**

Weck-Cel was the eye spear used in all the subsequent experiments since it was the only one yielding s-IgA concentrations comparable to the standard. No significant differences were found between the average s-IgA concentration in an untreated whole saliva sample (256.659 μg/mL), and the concentrations in untreated saliva samples (no eye spear contact) centrifuged at 2,000 rpm (233.649 μg/mL, P = 0.393), and 4,000 rpm (211.088 μg/mL, P = 0.069) (Fig. 3a). However, the untreated saliva sample centrifuged at 8,000 rpm showed a significant difference in s-IgA yield in comparison with the reference sample (182.729 μg/mL, P = 0.007). The average IgA concentration in the sample incubated with a Weck-Cel eye spear centrifuged at 2,000 rpm did not differ significantly (263.937 μg/mL, P = 0.622) from the reference sample (256.659 μg/mL) (Fig. 3b). However, Weck-Cel samples centrifuged at 4,000 and 8,000 rpm

**Fig. 3** Percentage of salivary IgA recovery in comparison with baseline at various centrifugation speeds, relative to the original (reference) saliva sample. (a) Untreated samples were not kept in contact with eye spears. (b) Treated samples were incubated with Weck-Cel eye spears for 1 h. Error bars refer to duplicate measurements for each sample. *P = 0.05-0.001; **P = 0.01-0.001; NS, no significant difference.

**Fig. 4** Percentage of salivary IgA recovery in comparison with the baseline from samples kept with the spears for 1, 2, and 4 h, relative to the original (reference) saliva sample. (a) Uncovered vials of salivary fluid remained open to air throughout each period. (b) Covered vials were sealed off during each period of storage. Error bars refer to duplicate measurements for each sample. ***P < 0.001; NS, no significant difference.
yielded significantly different s-IgA concentrations in comparison with the reference sample (327.971 μg/mL and 200.491 μg/mL, \( P < 0.05 \)) (Fig. 3b).

**Assessing the time effect**
When the tubes containing the saliva and eye spears were covered and stored (for 1, 2, or 4 h), there were no significant differences between the recovery of s-IgA at each time point (133.522 μg/mL, 99.052 μg/mL, and 131.183 μg/mL, respectively) in comparison with the s-IgA recovery from the reference sample (129.265 μg/mL). In contrast, when the samples were left with the spears in uncovered tubes for similar periods, the concentrations of recovered s-IgA were significantly higher (208.539 μg/mL, 238.559 μg/mL, and 238.098 μg/mL after 1, 2, and 4 h, respectively) than in the untreated reference saliva sample (126.336 μg/mL) (Fig. 4a, b).

**Assessing inter-individual differences**
To rule out inter-individual differences in saliva composition that might affect s-IgA measurements, saliva samples from 11 individuals (median age 32 years; range, 23-36 years) were collected. Each sample was collected using simple expectoration or the Weck-Cel eye spears. Both sets of samples were initially centrifuged at 2,000 rpm for 5 min. The final s-IgA concentrations were all similar (\( P > 0.250 \)) in the pairwise comparison (data not shown).

**Discussion**
Our study extended the work of Strazdins et al. by examining the variations of salivary IgA concentrations in samples obtained using different collection devices (10). Our results showed that s-IgA recovery results vary for different types of eye spears, depending on the absorbent material used in these devices. Other factors also affected the concentration of recovered s-IgA. The recovery yield depended on the centrifugation speed and on the manner of storage (covered or uncovered tubes). The recovery did not seem to be affected by the length of storage of the spears with the saliva (1, 2, or 4 h).

Of the four types of eye spears tested here, only the Weck-Cel eye spear centrifuged at 2,000 rpm yielded s-IgA concentration not significantly different from that in the reference sample. With the other spears, the s-IgA recovery generally exceeded the reference concentration, even at higher centrifugation speeds. The concentrations of s-IgA recovered from Weck-Cel samples at higher centrifugation speeds differed significantly from that in the reference sample.

Our initial findings showed a gradual increase in the total IgA concentration in the reconstituted samples with the increasing incubation time. These extended periods of spear submersion (storage) were chosen to mimic the process of sample collection and processing in a realistic clinical setting, where the sample processing is unlikely to be immediate. When the centrifuge tubes were covered with their caps during such storage periods, the subsequent s-IgA recoveries did not differ significantly from the standard regardless of the storage duration (up to 4 h spear submersion time).

Most of the reconstituted samples showed s-IgA recovery slightly above 100%. This phenomenon could be attributed to the evaporation of salivary fluid in the uncovered tubes, which would concentrate all salivary proteins, producing assay results that implied more than 100% s-IgA recovery. A similar result has been reported by Chang et al., who have suggested that the absorbent device may concentrate the immunoglobulins in a dilution-dependent manner (5). According to Chang et al., the percentage of IgA extracted from the absorbent device may be affected by the initial antibody concentration of the sample (5). In our study, however, this parameter could not be examined because all experiments were based on a single sample, and consequently from a constant initial antibody concentration.

The mechanical forces in the sponge might also affect the recovered concentration. Samples not kept with the eye spears did not yield s-IgA concentrations greater than the one detected in the reference sample. Storing the samples with the eye spears, however, resulted in s-IgA recoveries >100% in two out of three cases, suggesting that the sponges were involved in concentrating the immunoglobulins. This increase in IgA concentration might have been caused by cleavage of the dimeric IgA to the monomeric form or by an impaired fluid absorption and subsequent reconstitution by the absorbent material. We observed that the Keraspear and Merocel spears did not release all of the salivary fluid upon centrifugation, which might be related to their PVA composition. Incidentally, on average more s-IgA was recovered from these two spear types than from the two cellulose-based spears. It is not clear why only one of the two cellulose-based spears performed reliably, although a different manufacturing process might account for this observation.

To our knowledge, there is no study examining a comprehensive range of absorbent devices and their corresponding salivary IgA recoveries. The effects of centrifugation speed, storage time, and exposure of saliva samples to air (i.e., capped tubes vs. uncapped tubes) have not been examined, although the impact of these factors on s-IgA recovery is evident. Our ELISA results
suggested that the use of eye spears for saliva collection might achieve even more accurate salivary IgA level determination than the assays of the whole, untreated saliva. The samples obtained with Weck-Cel eye spears centrifuged at 2,000 rpm had s-IgA concentrations closer to 100% s-IgA recovery values, as opposed to the saliva samples not kept with such spears.

We sought to close the gap in the existing literature regarding the efficacy of various absorbent materials in the collection and recovery of salivary IgA. It seems reasonable to state that eye spears can be effective tools for biomarker collection once their appropriate composition and conditions for collection are determined. Thus, they might present numerous advantages to patients, caregivers, and health care professionals. In particular, our findings indicate that the salivary IgA assays can be reliably performed on samples collected using Weck-Cel eye spears. To maximize the accuracy, we would suggest that the spears be placed in covered tubes and centrifuged at 2,000 rpm for 5 min. The length of spear storage did not significantly affect the assay readings for the covered samples. Thus, it might not be necessary to perform centrifugations immediately after collection. To corroborate these findings we need further studies with larger sample sizes, analyzing fluid samples obtained directly from the oral cavity.

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Conflict of interest
The authors declare no conflicts of interest.

References