The Diurnal Variation of Matrix Metalloproteinase-9 and Its Associated Factors in Human Tears

Maria Markoulli, Eric Papas, Nerida Cole, and Brien A. Holden

PURPOSE. Matrix metalloproteinases (MMPs) are degrading enzymes which maintain and remodel tissue architecture. Up-regulation of MMP-9 has been associated with corneal erosions and ulceration. As these conditions are often exacerbated on waking, suggesting that degrading activity is upregulated overnight, this study set out to determine the diurnal variation of MMP-9, Tissue Inhibitor of Metalloproteinase (TIMP)-1, and Neutrophil Gelatinase-Associated Lipocalin (NGAL).

METHODS. Flush tears were collected from 46 healthy, non-contact lens wearers at midday, before sleep, and immediately on waking. Total protein content (TPC) was measured using the bicinchoninic acid method, and MMP-9, TIMP-1, and NGAL concentrations were measured using sandwich enzyme-linked immunoassay. Statistical analysis was performed using repeated measures analysis of variance.

RESULTS. TPC was 3.4 ± 1.5 mg/mL, 5.0 ± 3.7 mg/mL, and 15.5 ± 8.4 mg/mL for midday, before sleep, and on waking respectively, the latter being significantly greater than the other two (P < 0.001). MMP-9 concentrations at the corresponding time points were 9.8 ± 14.3 ng/mL, 8.5 ± 11.7 ng/mL, and 2000.7 ± 1950.7 ng/mL. Again, the value on waking was significantly greater than the previous two visits (P < 0.001). TIMP-1 concentrations exceeded those of MMP-9 at midday but the ratio of the two reversed on awakening.

CONCLUSIONS. Concentrations of MMP-9 are negligible during the day and completely inhibited by TIMP-1. On awakening, MMP-9 increases 200-fold, an increase that is not completely inhibited by TIMP-1. This diurnal change, along with the presence of NGAL which protects MMP-9 from degradation, suggests that the closed eye is an environment conducive to extracellular matrix remodeling. (Invest Ophthalmol Vis Sci. 2012;53:1479–1484) DOI:10.1167/iovs.11-8365

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Tears were collected by instilling a 60-L drop of nonpreserved, unit-dose saline into each eye. Participants were asked to stay overnight in the clinic and tears were collected again before sleep and immediately on awakening. All samples were collected by the same investigator (MM, trained and experienced in the technique for several years at this institute).

**Day-To-Day Repeatability Study.** Tears were collected from both eyes of nine participants at midday and on awakening, on two separate days so that day-to-day repeatability could be assessed.

**Clinical Techniques**

Visual acuity using computer letter charts was measured and slit lamp biomicroscopy (Zeiss SL-120, Carl Zeiss Meditec, Jena, Germany) was performed at midday to exclude any pre-existing conditions. Bulbar and limbal redness as well as palpebral redness and roughness were assessed with white light and corneal and conjunctival staining in conjuction with fluorescein (Fluoresants ophthalmic strips, 1 mg, Chauvin Pharmaceuticals, Essex, England) and a filter (Wratten #12; Hauk & Lomb, Rochester, NY). The Brien Holden Vision Institute Grading scales were used. Bulbar and limbal redness were also assessed on awakening. The order of tests was such to minimize the impact on the ocular surface before flush tear collection. As such, visual acuity and evaluation of the cornea and conjunctiva without fluorescein were conducted before flush tear collection while fluorescein evaluation and lid eversion were conducted after flush tear collection.

**Flush Tear Collection.** Flush tears as described previously were collected from both eyes using a 10-L microcapillary tube (Blaubrand; intraMark, Wertheim, Germany) at each visit and stored separately for the first 37 participants to establish eye-to-eye variability. Right and left eyes were pooled for the final nine participants. Flush tear collection was randomized. This technique was previously validated for MMP-9 analysis.

**Sample Treatment**

After collection, samples were centrifuged at 11,450 g for 20 minutes at 4°C to remove cellular debris. The supernatants were collected and stored in siliconized polypropylene microcentrifuge tubes (Sigma-Aldrich) at −80°C in four aliquots, one for each analysis.

**Total Protein Content**

Total protein content (TPC) was determined using the bicinchoninic acid (BCA) method and using reagents (Pierce; Thermo Fisher Scientific) and flat-bottom 96-well microplates (Nunc-F Maxisorp; Thermo Fisher Scientific). Serial dilutions of bovine serum albumin (BSA) were used as standard. This was loaded in triplicate starting at 2 mg/mL down to 0.1 mg/mL in ultrapure laboratory grade water (MilliQ; Millipore, Billerica, MA) and 10 μL was added to each well. Tear samples were loaded at a 1:10 dilution. Tears were analyzed in duplicate and a 10 μL volume was added to each well. Solution A (BCA Protein Assay Reagent A; Pierce, Thermo Fisher Scientific) and solution B (BCA Protein Assay Reagent B; Pierce, Thermo Fisher Scientific) were combined in respective volumes of 20 mL and 0.4 mL. This mixture was then added at a volume of 200 μL per well. The optical density was read with a microplate reader (Spectra Fluor Plus, Tecan Multifunction Microplate reader using the X-Fluor 4 software; Tecan, Manneford, Switzerland) at 595 nm after 30 minutes and a standard linear curve generated using the BSA as a reference.

**MMP-9, TIMP-1, and NGAL Analysis**

Total MMP-9 concentration (92 kDa pro- and 82 kDa active forms), TIMP-1 and NGAL concentration were each determined using sandwich enzyme-linked immunosorbent assay (ELISA) with an ELISA development kit (Duoset kit; R&D Systems, Inc., Minneapolis, MN). These were performed according to the manufacturer’s directions.

**Gelatin Zymography**

Gelatinolytic activity of MMP-9 was determined using gelatin zymography. Ten micrograms of each tear sample was added to 5 μL of sodium dodecyl sulfate (SDS) sample buffer (2× Novex Tris-Glycine; Invitrogen, Carlsbad, CA) and the volume was made up to 12 μL with ultrapure laboratory grade water (MilliQ; Millipore). A concentration of 50 mg/mL of MMP-9 standard (R&D Systems) was activated by incubating with 1 mM 4-aminophenylmercuric acid21 (APMA; Sigma-Aldrich, Steinheim, Germany) and placed on ice until processing. A limit of 1 minute was imposed to avoid reflex tearing. All three visits were conducted consecutively. For on awakening, participants slept overnight in the clinic. In the evening before this visit, participants were informed that they would be woken up at an allocated time as agreed by both the Optometrist (MM) doing the tear collection and the participant. They were also advised that should they wake, not to open their eyes unless instructed to do so by the Optometrist. In the morning, they were awakened and instructed not to open their eyes as they were lead to the clinic chair. On instruction, the participants were asked to open their eyes and flush tear collection would commence immediately under dim lighting. All participants were questioned as to whether they had opened their eyes before instruction. In this case, the results were excluded from the analysis. The order of eyes for flush tear collection was randomized. This technique was previously validated for MMP-9 analysis.

Each sample was resolved using 10% zymogram gelatin gel (Novex; Invitrogen) under denaturing but nonreducing conditions. Gels were electrophoresed at a constant voltage of 120 V for 3.5 hours in running buffer (glycine, Tris base and 1% SDS) at 4°C. As proteolytic activity can be reversibly inhibited by SDS during electrophoresis and recovered by incubating the gel in aqueous Triton X-100, the gels were washed with 2.5% (vol/vol) Triton X-100 (Astral Scientific, Amresco, Solon, OH) for 1 hour. This was then decanted and the gels were equilibrated with developing buffer (Novex zymogram developing buffer; Invitrogen) for 30 minutes. The gels were then transferred to
Data Analysis

Normality was tested using the Shapiro-Wilk test and only the data for MMP-9 at midday and before sleep and TIMP-1 on awakening were not normally distributed when eyes were averaged. Repeated measures analysis of variance (ANOVA) was used to analyze the visits. Mauchly’s test was used to verify the sphericity assumption. Where main effects were significant, Student’s t-tests with Bonferroni correction were used post hoc. Software (PASW version 18.0 GP; IBM, Chicago, IL) was used for the analysis. In a small number of cases inadequate sample volume was obtained resulting in missing data. To avoid the need to delete all the data from affected subjects during repeated measures ANOVA, replacement was made using expectation maximization (EM), with any fresh developing buffer and incubated overnight at 37°C for 16 to 20 hours. Proteins were detected by staining with Coomassie Blue R-250 for a minimum of 2 hours and destaining in 50% (vol/vol) ethanol, 10% (vol/vol) acetic acid.

Disodium ethylene-diaminetetra-acetic acid (EDTA), which is known to inhibit MMPs, was added to a separate renaturing and developing buffer. After electrophoresis, the final well containing an identical sample to that in the third well, was cut off and renatured in buffer containing 20 mM EDTA, followed by incubation in developing buffer containing EDTA. These bands should have no proteolytic activity on staining and were used as controls (results not shown). Cancer serum samples were used as a positive control, courtesy of Michele Madigan, SaveSight Foundation.

RESULTS

Flush Tear Collection

Flush tear collection rate was not different between right and left eyes ($P = 0.33$). We therefore averaged the eyes in Table 1. The flush tear collection rates were not significantly different between midday and on awakening ($P = 0.82$). The flush tear collection rate before sleep was significantly greater before sleep than that at both the midday ($P = 0.048$) and on awakening ($P = 0.02$).

### Table 1. Flush Tear Collection Rate (Mean ± SD)

<table>
<thead>
<tr>
<th>Visit</th>
<th>Flush Tear Collection Rate (µL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midday ($n = 46$)</td>
<td>22.8 ± 10.8</td>
</tr>
<tr>
<td>Before sleep ($n = 37$)</td>
<td>27.9 ± 17.1</td>
</tr>
<tr>
<td>Upon awakening ($n = 46$)</td>
<td>22.4 ± 12.6</td>
</tr>
</tbody>
</table>

Before sleep was significantly greater than that at both the midday ($P = 0.048$) and upon awakening ($P = 0.02$) visits.

Fresh developing buffer and incubated overnight at 37°C for 16 to 20 hours. Proteins were detected by staining with Coomassie Blue R-250 for a minimum of 2 hours and destaining in 50% (vol/vol) ethanol, 10% (vol/vol) acetic acid.

Repeatability between both days and eyes was evaluated by coefficients of repeatability defined as the SD of the mean difference between replicates multiplied by 1.96.$^{29}$

<table>
<thead>
<tr>
<th>Visit</th>
<th>Coefficient of Repeatability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyes ($n = 37$)</td>
<td></td>
</tr>
<tr>
<td>Midday</td>
<td>TPC (mg/mL) $P = 0.05$</td>
</tr>
<tr>
<td>MMP9 (ng/mL)</td>
<td>$P = 0.048$</td>
</tr>
<tr>
<td>TIMP-1 (ng/mL)</td>
<td>$P = 0.03$</td>
</tr>
<tr>
<td>NGAL (ng/mL)</td>
<td>$P = 0.03$</td>
</tr>
<tr>
<td>MMP9/NGAL ratio</td>
<td>$P = 0.03$</td>
</tr>
<tr>
<td>Days ($n = 9$)</td>
<td></td>
</tr>
<tr>
<td>Midday</td>
<td>TPC (mg/mL) $P = 0.001$</td>
</tr>
<tr>
<td>MMP9 (ng/mL)</td>
<td>$P = 0.01$</td>
</tr>
<tr>
<td>TIMP-1 (ng/mL)</td>
<td>$P = 0.01$</td>
</tr>
<tr>
<td>NGAL (ng/mL)</td>
<td>$P = 0.01$</td>
</tr>
<tr>
<td>MMP9/NGAL ratio</td>
<td>$P = 0.01$</td>
</tr>
</tbody>
</table>

### Table 2. The Coefficient of Repeatability for Eyes and Days for Each of the Proteins Measured

<table>
<thead>
<tr>
<th>Visit</th>
<th>Variable</th>
<th>Coefficient of Repeatability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midday</td>
<td>TPC (mg/mL) $P = 0.05$</td>
<td></td>
</tr>
<tr>
<td>MMP9 (ng/mL)</td>
<td>$P = 0.048$</td>
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</tr>
<tr>
<td>MMP9/NGAL ratio</td>
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<td></td>
</tr>
<tr>
<td>Before sleep</td>
<td>TPC (mg/mL) $P = 0.001$</td>
<td></td>
</tr>
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<td>MMP9 (ng/mL)</td>
<td>$P = 0.01$</td>
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<tr>
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<tr>
<td>MMP9/NGAL ratio</td>
<td>$P = 0.01$</td>
<td></td>
</tr>
<tr>
<td>Upon awakening</td>
<td>TPC (mg/mL) $P = 0.001$</td>
<td></td>
</tr>
<tr>
<td>MMP9 (ng/mL)</td>
<td>$P = 0.01$</td>
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</tr>
<tr>
<td>TIMP-1 (ng/mL)</td>
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</tr>
<tr>
<td>MMP9/NGAL ratio</td>
<td>$P = 0.01$</td>
<td></td>
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</tbody>
</table>

### Table 3. The Diurnal Variation of Each of the Proteins Measured in the Tear Film

<table>
<thead>
<tr>
<th>Visit</th>
<th>TPC (mg/mL)</th>
<th>MMP9 (ng/mL)</th>
<th>TIMP-1 (ng/mL)</th>
<th>MMP9/TIMP-1 Ratio</th>
<th>NGAL (ng/mL)</th>
<th>MMP9/NGAL Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midday</td>
<td>3.4 ± 1.5</td>
<td>9.8 ± 14.3</td>
<td>74.5 ± 39.7</td>
<td>0.3 ± 0.4</td>
<td>680.8 ± 523.3</td>
<td>0.01 ± 0.04</td>
</tr>
<tr>
<td>Before sleep</td>
<td>5.0 ± 3.7</td>
<td>8.5 ± 11.7</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Upon awakening</td>
<td>15.5 ± 8.4</td>
<td>2000.7 ± 1950.7</td>
<td>277.8 ± 282.2</td>
<td>13.9 ± 15.8</td>
<td>3620.3 ± 1832.1</td>
<td>0.16 ± 0.14</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD; $n = 46$ except for NGAL and MMP9/NGAL ratio, where $n = 9$, and before sleep where $n = 37$. Brackets denote statistical significance ($P < 0.05$). Exact $P$ values are reported in the text.

Repeatability

Coefficients of repeatability$^{29}$ were calculated at each time point and for each protein between replicates taken from the two eyes of each subject when randomized (Table 2). There were no significant differences between eyes.

Day-to-day repeatability was determined from the pooled tears of nine participants. Of the proteins measured only NGAL at midday was significantly different ($P = 0.03$). Table 2 lists the coefficients of repeatability.

As the eyes and days were not significantly different, except for NGAL, these were averaged and repeated measures was performed on this to determine the diurnal variation.

### Diurnal Variation

Table 3 lists the concentrations of the proteins measured at each time point. TPC was significantly greater on awakening than at midday and before sleep ($P < 0.001$) and midday and before sleep were also significantly different from each other ($P = 0.03$).

The concentration of MMP-9 was significantly greater on awakening compared with both midday and on awakening ($P < 0.001$) and midday and before sleep were not significantly different ($P = 0.6$).

TIMP-1 was measured as it is the main inhibitor of MMP-9. Concentrations were significantly greater on awakening compared with midday ($P < 0.001$). The MMP-9/TIMP-1 ratio provides an indication of the potential for MMP-9 to cause tissue damage. The MMP-9/TIMP-1 ratio was significantly greater on awakening than at midday ($P < 0.001$). These ratios indicate that at midday, the concentration of TIMP-1 exceeded that of MMP-9, hence completely inhibiting MMP-9. In contrast, on awakening the ratio indicates that this balance is reversed, with an excess of MMP-9 in the tear film, indicating a shift from TIMP-1 dominance during the day to MMP-9 dominance on awakening. NGAL was significantly elevated on awakening compared with midday ($P = 0.002$). When considered as an MMP-9/NGAL ratio, of the MMP-9/NGAL ratio, where $n = 9$, and before sleep where $n = 37$. Brackets denote statistical significance ($P < 0.05$). Exact $P$ values are reported in the text.
NGAL ratio, the ratio on awakening was significantly greater than that at midday ($P = 0.006$). At both the midday and on awakening visits, NGAL exceeded the concentration of MMP-9 present in the tear film.

Sex was not significantly different for TPC, MMP-9, NGAL or TIMP-1 and its ratio with MMP-9 ($P = 0.86, 0.13, 0.39$, and 0.23 respectively). The ratio of MMP-9 to NGAL showed a significant difference between the sexes, with males having a significantly greater ratio on awakening than the females and the reverse at midday ($P = 0.048$).

**Zymography**

Figure 1 shows a Coomassie Blue-stained gel of tear samples of two non-contact lens wearing participants at midday and on awakening. The clear bands against the stained gelatin background are areas of gelatinolytic activity.\(^{27}\) Overnight closure resulted in prominent bands at 92, 135, and $>200$ kDa (Fig. 1, lanes 2 to 5). These bands are consistent with those previously identified by others and correspond to pro-MMP-9, NGAL, or $\alpha_2$-macroglobulin complexes not dissociated by SDS, and a dimer of MMP-9, respectively.\(^{30-33}\) At midday (Fig. 1, lanes 6 to 9), faint bands were detected at 135 kDa and 92 kDa, corresponding to the MMP-9 complex and pro-MMP-9 respectively. The difference between the on awakening lanes and the midday lanes is consistent with the diurnal variation of MMP-9 measured with ELISA.

**Clinical Correlation**

Correlation analysis did not show any association between changes in clinical signs and TPC or MMP-9. A significant, though weak, positive correlation was found between TIMP-1 and limbal redness ($R = 0.44; P = 0.04$). A significant positive correlation was also found between age and the diurnal change in NGAL ($R = 0.83; P = 0.01$).

**DISCUSSION**

MMP-9 is a vital but potentially destructive enzyme by virtue of its ability to degrade corneal collagen. Control of this activity is thus critical to maintaining corneal health. When the eyes are open, there is little cause for concern as, in agreement with previous studies,\(^{6,8-10,15}\) these data suggest that MMP-9 concentrations are negligible and significantly exceeded by those of the inhibitor molecule TIMP-1. The potential for proteolytic activity is thus inhibited and hence the corneal epithelium and basement membrane are protected from degradation.

During sleep however, this situation changes dramatically as MMP-9 concentration increases roughly 200-fold, while TIMP-1 barely triples. The shift in the balance from TIMP-1 to MMP-9 dominance, which may be a downstream effect of increased neutrophil activity,\(^{7,24}\) suggests that there may be an increased propensity for basement membrane degradation overnight. This is further supported by the excess NGAL in the tear film compared with MMP-9, both at midday and on awakening, a finding that is in agreement with previous studies.\(^{7}\) While this diurnal variation may be necessary to facilitate increased cell desquamation during sleep, or it may be that this increase in MMP-9 is a byproduct of epithelial cell breakdown during eye closure, it also apparently sets the scene for tissue destruction by virtue of the excess MMP-9 present. In reality however, such tissue damage was not evident among the participants in this study, nor does it typically occur overnight in the general population. Clearly then, our understanding of the regulatory mechanisms of MMP-9 in the tear film is incomplete.

Looking at the zymography results indicates that the MMP-9 in the closed-eye tear film is in thezymogen form rather than the active form (Fig. 1), suggesting that this may be one contributory factor. Additionally, some MMP-9 was detected at molecular weights in excess of 220 kDa indicating it to be in the dimeric state which does not differ in enzymatic activity from the inactive form at 92 kDa.\(^{30}\) It may also be that MMP-9 is being regulated by nonspecific inhibitors such as $\alpha_2$-macroglobulin which traps activated MMP so that the complex can be removed by the receptor.\(^{34}\)

Many conditions such as recurrent corneal erosions and corneal ulcers are known to be exacerbated by eye closure.\(^{35}\) Due to its action on collagen and hence the anchoring complexes of the corneal epithelium, MMP-9 could contribute to the increased incidence of recurrent erosions by virtue of its diurnal variation, particularly when the regulatory mechanisms are not controlled.\(^{11,36}\) The diurnal profile of MMP-9 and its associated factors could also indicate why the epithelium is particularly prone to erosion and infection with overnight contact lens wear,\(^{37,38}\) as well as corneal ulceration\(^{10,12,13,39}\) and corneal desiccation on awakening.

MMP-9 concentration in the tears varies considerably within the population at any given time as demonstrated by the coefficients of repeatability presented in Table 2. This may be

![Figure 1. Coomassie Blue-stained zymogram of on awakening tears in either 5 or 10 µg and midday tears in either 5 or 10 µg. Lanes 2 and 3 are on awakening tears of one participant in 5 and 10 µg, respectively, while lanes 4 and 5 are those of another participant, also on awakening tears. Lanes 6 and 7 are the midday tears of one participant in 5 and 10 µg, respectively, while lanes 8 and 9 are those of another participant.](image-url)
in part attributable to the variability in the flush tear collection rate as shown in Table 1. The lack of correlation with clinical signs of redness suggests that this ubiquitous sign of ocular distress is not particularly helpful as a marker for raised MMP-9 levels however. It may also be that this presence of elevated levels of the inactive form of MMP-9 does not produce redness because it does not constitute an insult to the ocular surface.

Finally, it may be that these results also have implications for certain topical therapies, in particular the use of prostanoid analogues. These compounds are used for the treatment of glaucoma and work by increasing the concentration of MMP-940 as a means of degrading ciliary muscle extracellular matrix and increasing uveoscleral outflow.41 Concurrent increased MMP-9 concentrations have also been found in the tear film,42 increasing the potential for corneal damage as has been found in both animal43 and human studies.44 It may then be advisable to adjust the instillation timing for prostaglandin drops to avoid the periods before sleep and immediately on awakening, particularly for those already prone to corneal erosions.

In conclusion, this study has shown that there is a substantial diurnal variation of MMP-9 and its associated factors. Concentrations of MMP-9 are negligible during the day and completely inhibited by TIMP-1. On awakening MMP-9 increases 200-fold, an increase that is not completely inhibited by TIMP-1. This diurnal change, along with the presence of NGAL, suggests that the closed eye is an environment conducive to extracellular matrix remodeling. The fact that we typically do not see corneal damage on awakening suggests that other regulatory mechanisms are active to prevent excess extracellular matrix degradation.

Acknowledgments
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References