

“Coffee Ring Effect” in Ophthalmology

“Anionic Dye Deposition” Hypothesis Explaining Normal Lid Margin Staining

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Abstract: The process of formation of Marx line is studied in this article. Various theories have been proposed previously, in order to explain the mechanisms which lead to the development of Marx line. These theories are based on the characteristics of stained area and do not pay attention to the behavior of dye solution itself on the surface. The aim of this study is to investigate the latter behavior and introduce a new theory based on it, in order to explain the process of the Marx line formation.

This study also introduces “Coffee Ring Effect” and its possible applications in explaining some ophthalmological phenomena.

The effect of dye solution’s behavior on the beneath surface is adopted in order to propose a novel theory. This new hypothesis is called “Anionic Dye Deposition” which was based on “Coffee Ring Effect” phenomenon. For evaluation of this theory, Evaporation pattern of Rose Bengal and fluorescein were analyzed on different surfaces. Furthermore, the effect of tear meniscus alteration on lid margin staining is studied.

During the evaporation process of dye solutions, it was observed that almost all of the solute was deposited at the edge of the drop on hydrophilic surfaces. Furthermore, in the study of lid margin staining, it is observed that tear meniscus alteration during gaze affects staining pattern. This observation invalidates former hypotheses which only focus on stained surface characteristics.

According to the observations in this study, it is proposed that Marx line staining occurs as a result of “anionic dye deposition” due to evaporation.

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Abbreviations: ADD = anionic dye deposition, LOM = line of Marx, LW = lid wiper, LWL = lid-wiper epitheliopathy, MCJ = mucocutaneous junction, ML = Marx line, SICS = Solution Induced Corneal Staining, ULFE = upper lid frictional epitheliopathy, ULIE = upper lid inner epitheliopathy, ULMS = upper lid margin staining.

INTRODUCTION

Millions of patients suffer annually from ocular symptoms which are related to lid margin pathologies. In 2010,

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Knop et al¹ introduced lid margin as an important and underestimated structure in the literature. During the past century since Marx analyzed normal lid margin staining,² accurate cause or causes of staining of Marx line (ML) has not been well understood in the literature.

The goal of this study is to propose a reasonable explanation for lid margin staining in healthy subjects. In this article, dye solution’s behavior on the beneath surface has been particularly investigated and it has been shown that it is a defining factor in the process of ML formation. Based on this behavior, a novel hypothesis is introduced. This theory has been named anionic dye deposition (ADD) by the authors.

In ADD hypothesis, when diagnostic ophthalmic dyes which are anionic, see Section 6, instilled in conjunctival sac, due to evaporation, dye molecules start to deposit in the apex of tear menisci, see Sections 4 and 5. As explained in Section 7, the authors believe that ADD at this bordering line causes cellular staining.

MATERIALS AND METHODS

Evaporation pattern of Rose Bengal and sodium fluorescein were analyzed on different surfaces.

Additionally, the effect of dye solution’s behavior on the beneath surface is adopted in order to propose the theory.

The study was implemented in accordance with the tenets of the declaration of Helsinki. The study protocol was approved by the local ethics review committee of Tehran University of Medical Sciences, and all participants provided us with written informed consents prior to inclusion.

ML AND EXISTING HYPOTHESES

Marx line, also called Marx’s line (ML) or line of Marx in the literature, is a narrow line in the margins of upper and lower lids which is demonstrated with anionic vital dyes such as fluorescein, Rose Bengal, and lissamine green. This line is located on mucocutaneous junction (MCJ) which is the junction between nonwetting cutaneous and wetting mucosal part of lid margin.³ ML in healthy young individuals is generally posterior to Meibomian (tarsal) glands’ orifices, on conjunctival side, but it may be located on these glands’ orifices or anterior to them (Figure 1).

In lid margin, upper and lower tear menisci form due to capillary pressure. The apex of tear meniscus is on the ML.³

Three general scenarios can be considered for the cause of ML staining based on its location. First, the focus is on the cells alone and staining is thought to be due to their innate characteristics. This class does not explain development of antroplacement of ML with aging.^{4,5} In the second scenario, anatomy of this region can be noted. It can be considered as marsh of the tear meniscus river which is suitable for dye deposition and is hard to be washed-out. Norn considered ML as the place for

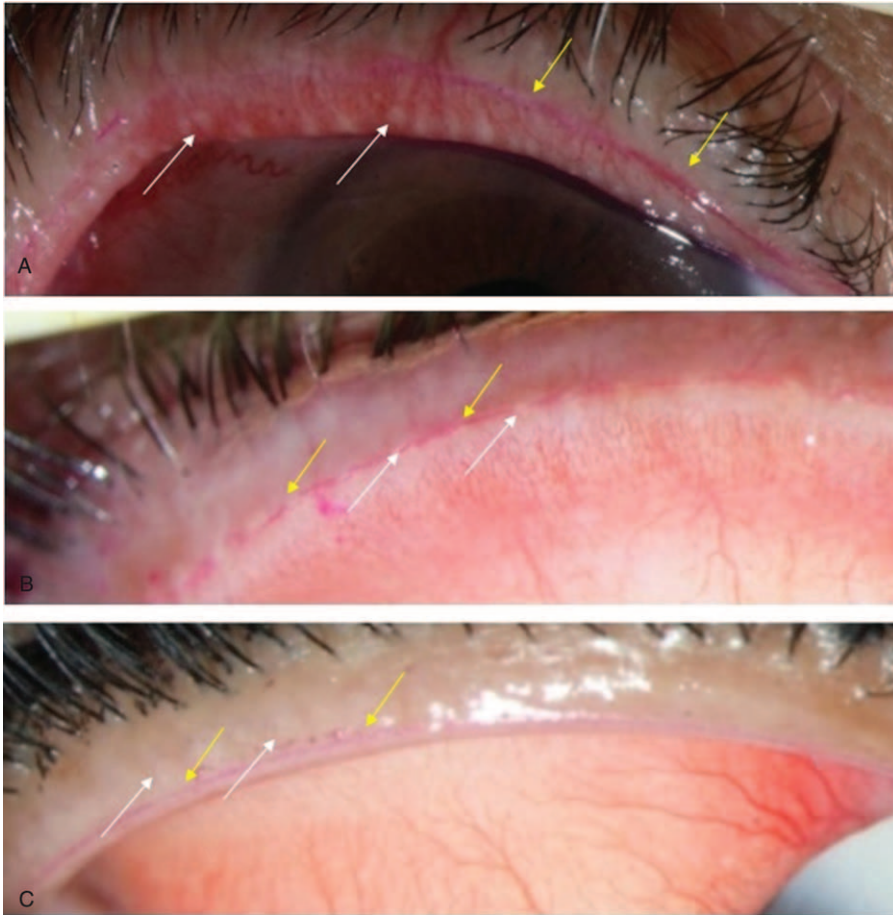


FIGURE 1. (A) Anterior migration of upper ML, (B) upper ML located on meibomian gland orifices, (C) ML located posterior to meibomian gland orifices. White arrows show meibomian gland orifices and yellow arrows indicate Marx line. ML = Marx's line.

debris and mucus deposition⁶ and the imprint of tear meniscus.⁷ Lastly, staining can be explained by environmental stress on cells such as desiccation or trauma while blinking. One such stress is hyperosmolarity. Bron et al³ suggested that high surface-area-to-volume ratio in the apex of tear meniscus located on ML, increases evaporation in this region and consequent increase of osmolarity of this region leads to immaturity of the cells beneath this region. This causes undifferentiation of mucins which in turn predisposes cells for staining.

“COFFEE RING EFFECT”

Desiccation Pattern of Drops Containing Rose Bengal and Sodium Fluorescein on Glass and Oily Surfaces

Sodium fluorescein (Merck millipore, Massachusetts, US, CAS Number: 518-47-8) and Rose Bengal solids (Sigma-Aldrich, Missouri, US, CAS Number: 632-69-9) were solved in distilled water in order to make solutions with concentrations of 1% and 0.1% w/w. One drop of each solution, approximately 30 μ L, was placed on 2 different surface types: glass surface and oily surface. These surfaces have been chosen due to their different characteristics. The glass surface is hydrophilic which is similar to conjunctivae and cornea in this feature. On the other hand, the oily surface is hydrophobic.

The study was performed at room temperature and relative humidity. After the completion of evaporation process, as shown in Figure 2, Rose Bengal and Sodium fluorescein drops left ring-shaped deposits on glass surface. This effect has not been seen on oily surface because of hydrophobicity of this surface. Also, it has been observed that in higher concentrations, the ring was thicker than lower concentrations (Figure 2).

Applications of “Coffee Ring Effect” in Ophthalmology

Different desiccated patterns occur when a colloidal drop evaporates. A common type of such patterns is the “coffee ring” pattern in colloidal depositions. In this pattern, solutes tend to deposit at the edge of the drop and form a ring shape footprint at the end of evaporation process.

It is known in physical chemistry that an aqueous liquid droplet spilled on a solid surface forms a meniscus at its edges due to capillary pressure. This meniscus forms an angle with the surface to overcome the capillary forces present at the interface. The value of this contact angle depends on the relative hydrophilicity of the surface. On the one hand, an aqueous solution with a hydrophilic surface forms an angle smaller than 90° , and on the other hand, the contact angle becomes $>90^\circ$ for hydrophobic surfaces. In fact, surface hydrophilicity itself is defined by the contact angle of the surface with a water droplet.

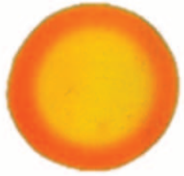
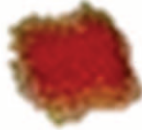

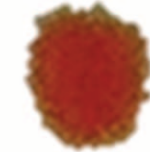
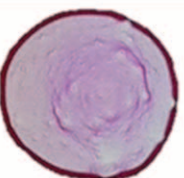



Dye percent (w/w)	Glass surface	Oil surface
Fluorescein 1%		
Fluorescein 0.1%		
Rose Bengal 1%		
Rose Bengal 0.1%		

FIGURE 2. Fluorescein and Rose Bengal drying patterns on glass and oil surface with different concentrations.

As explained above, an aqueous droplet of colloidal suspensions on a surface forms a meniscus at its interface with a hydrophilic surface as shown in Figure 3. Due to higher surface-to-volume ratio in the meniscus compared to the center, evaporation rate is higher at the edge which in turn reduces the aqueous solution volume at the edge. In this situation, one could think of two possible scenarios, either receding from the edge or compensating this volume loss by flowing more aqueous solution from the center as shown in Figure 3A and B, respectively. As deposition occurs at the apex of the meniscus, this point would be pinned to the edge; therefore, the latter scenario would occur. This results in a constant flow of solution from the center and deposition at the edge as shown in Figure 3C. This phenomenon was initially explained in 1997.⁸ This is also a familiar process, observed in everyday life after drinking a cup of coffee and is known as “coffee ring effect” in surface chemistry.

The mechanism of evaporation that causes “coffee ring effect” on hydrophilic surface is outward flow of solute from the interior. This flow leads to decrease of height of drop during evaporation while the area of drop is constant. This model of evaporation is known as “constant area mode”⁹ in which the contact line is stable (Figure 4). On the other hand, in evaporation of a hydrophilic drop on a hydrophobic surface, the height of drop reduces and the contact line slides toward the center but the contact angle is constant. This mechanism is known as

“constant angle” mode in which the final deposition pattern is a uniform dot with a dimple in the drop center.⁹

If solute particles size is very small, their diffusion coefficient is high (Table 1). Therefore, due to diffusion movements of particles, ring deposition does not occur. On the other hand, for very large particles, ring deposition does not occur either.¹⁰

Deposit pattern is also influenced by particles concentration. Higher initial concentration leads to a wider ring.⁸ High concentration also can inhibit ring formation and cause uniform deposition.^{11,12}

The “coffee ring effect” also highly depends on the shape of colloidal particles.¹³ Spherical particles deposit at the edge of the drop but ellipsoidal particle deposition is uniform.

“Coffee ring effect” has been applied in other disciplines of medicine in order to explain medical phenomena. As an example, this phenomenon was studied in drying blood plasma and synovial fluid.⁹ “Coffee ring effect” could also be used in order to explain phenomena in ophthalmology. For example, final pattern after desiccation of a drop of tear on a hydrophilic surface that is known as tear ferning test is also related to “coffee ring effect.” Pearce and Tomlinson¹⁴ observed that in tear ferning test, among tear solutes, Na, K, and Cl were deposited within the fern and macromolecules such as mucins and proteins were deposited at the very periphery of the dry tear drop. In previous studies, difference in solubility was thought to be the main cause of this pattern of solute segregation in tear

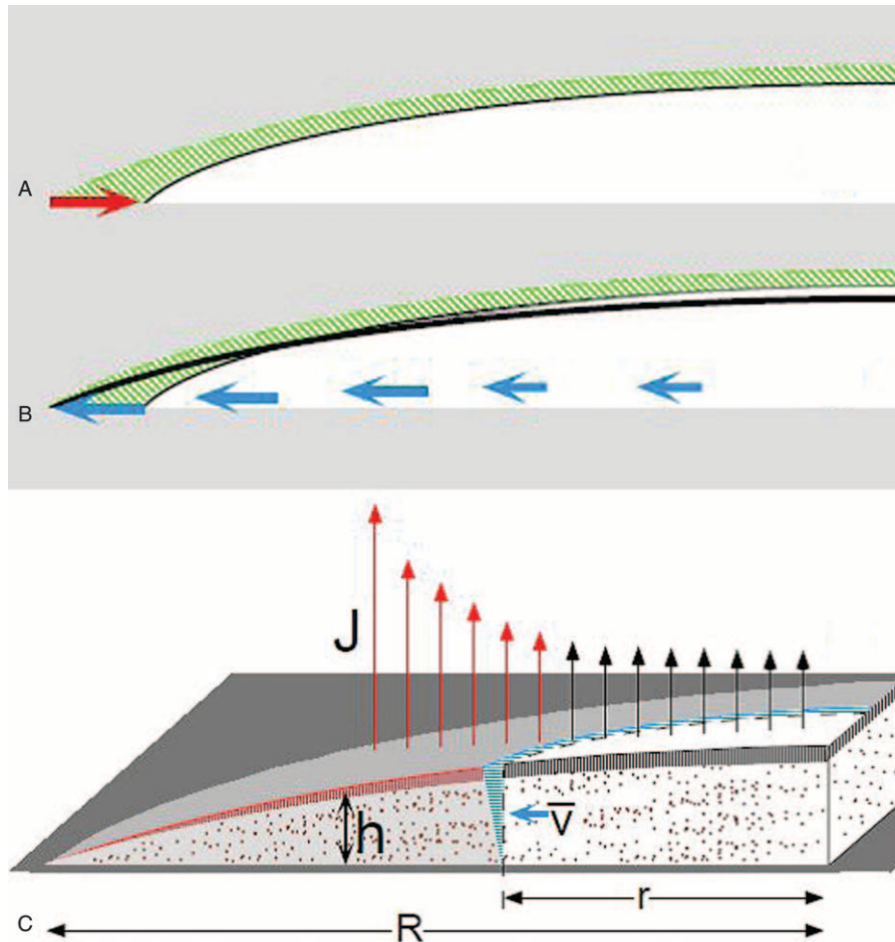


FIGURE 3. Mechanism of outward flow during evaporation, (A) receding from the edge, (B) outward flow of solution from the center to the edge, (C) faster evaporation at the apex of meniscus and deposition at the edge, reprinted with permission from Deegan et al.⁸

ferning test.^{15–17} It had been suggested that insoluble solutes deposit at the ring and highly soluble solutes tend to deposit somewhere inside the region encircled by the outer ring.¹⁸ However, in this study, Rose Bengal and Sodium fluorescein deposited at the ring, are both highly soluble. On the contrary, it should be concluded that the “size”,¹⁰ of molecules, which affect their diffusion coefficient, and “shape”,¹³ of molecules, along with ionic strength of the solution and other factors,¹⁹ play more important role in drying pattern and segregation.

Based on Pearce and Tomlinson¹⁴ observation, it could be predicted that if some amount of tear solute deposit or gelate at the MCJ, this deposition contains higher amount of mucins and proteins and lower amount of Na, K, and Cl. It is also predicted that deposition of this macromolecules can easily be washed to puncta in healthy subjects.

In this study, we observed that when a solution containing Rose Bengal and sodium fluorescein evaporates on a hydrophilic surface, almost all the dye deposits at the edge of the drop. It is known that this process of deposition starts early and its progress is a function of time.¹⁰ Deposition of these ophthalmic dyes occurs on all hydrophilic surfaces such as conjunctivae. According to this, it is predicted that following instillation of the dye solution in conjunctival sac, dye molecules start to deposit

at the MCJ line. As explained in Section 7, this process leads to cellular staining at the bordering lines of tear menisci.

TEAR MENISCUS DYNAMIC

During interblink time, immediately, capillary pressure makes a liquid flow from tear film to upper and lower tear menisci.²⁰ Although it seems that gravity generates a flow downward from upper tear meniscus to tear film and from tear film to lower tear meniscus, at these micro-dimensions, gravity’s effect becomes negligible in comparison to molecular forces leading to capillary effect.

As shown by Kimball et al,²¹ in interblink period, after immediate thinning of tear film due to capillary pressure, later thinning starting 2 s after blinking is caused by evaporation. The authors predict that at this point, evaporation causes dye deposition at tear meniscus apex and a liquid flow from interior to apex of tear meniscus occurs.

Considering tear meniscus volume of about 0.5 μL ,²² tear meniscus height of 0.3 mm, tear meniscus length of 30 mm, and evaporation rate of 13.57×10^{-7} g/cm² per s,²³ only about 1.5% of whole tear meniscus volume evaporates during a minute. But this evaporation rate gets higher toward the edge of tear meniscus and it becomes very large at the edge line. Due to “coffee ring effect,” dye molecules start to deposit at this line.

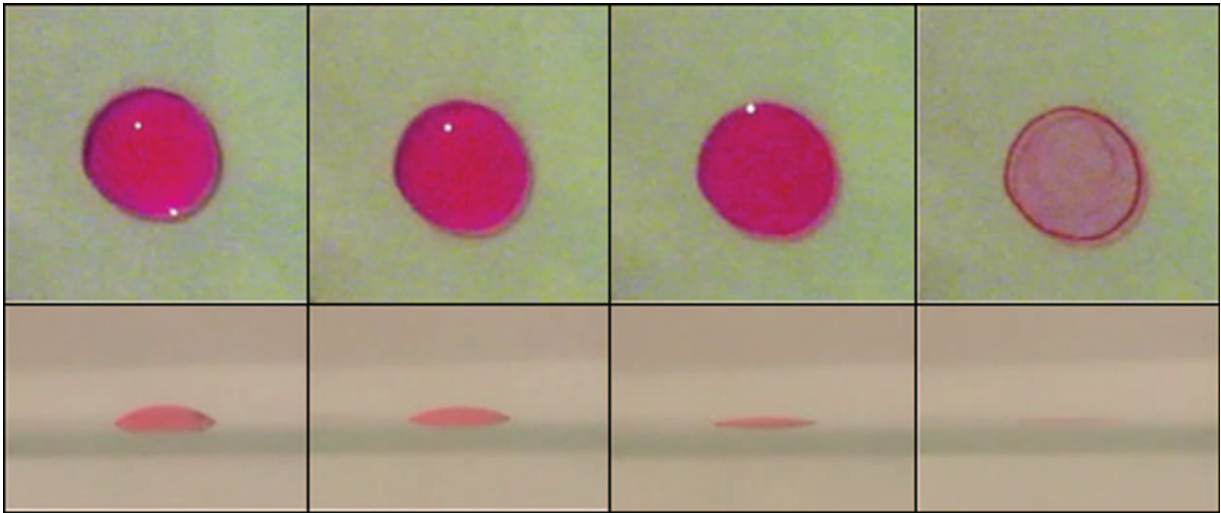


FIGURE 4. Constant area mode in evaporation of 0.1% Rose Bengal solution on glass surface. From top left to bottom right: 0, 10, 20, and 33 min after the drop spills on glass. Top view of the drop showing the drop area and its side view showing the drop height at the same time point.

It should be mentioned that coffee ring effect (evaporation induced capillary flow) occurs after the edge of drop pins to the hydrophilic surface. During interblink time, this effect due to the flow of fluid to the edge of tear meniscus helps to prevent apex of tear meniscus from receding avoiding desiccation stress on conjunctivae.

If blinking is prohibited for longer than normal periods, reflex tearing happens and tear meniscus swell quickly.²⁴ The authors believe that lid margin staining may be decreased as a result of staining being washed out in prohibited blinking.

During blinking, thickness of upper and lower lid margins changes.²⁵ Furthermore, during lid closure phase of blinking, upper and lower tear menisci are combined together.²⁵ In the opening phase of blinking, lid margins return to their previous locations and tear menisci start to form again due to capillary pressure. In addition, during opening phase of blinking punctal drainage happens.²⁶ It is predicted that the change of tear meniscus during blinking may increase surface to volume ratio at its apex which leads to higher evaporation rates.

ANIONIC DYES VERSUS CATIONIC DYES

In 1924, Marx used many dyes in order to assess this line of staining. He reported that this line is stained by Rose Bengal, eosin, water blue, and nigrosin. He also reported that methylene blue, gentian violet, toluidine blue, and dahöia strongly bind to conjunctivae and MCJ remains almost unstained.²

TABLE 1. Factors Influencing Coffee Ring Formation in Evaporation of an Aqueous Drop

Factors Amplifying Coffee Ring Effect	Factors Inhibiting Coffee Ring Effect
Solute particles being not very small or very large	Solute particles being very small or very large
Hydrophilic surface	Hydrophobic surface
Lower concentration	Higher concentration
Spherical shape of particle	Ellipsoidal shape of particle

As ocular barrier function is not yet fully understood,²⁷ we assume that similar to endothelial glycocalyx barrier, conjunctival barrier function also depends on the size and charge of the particles. Since endothelial glycocalyx has negative charge, it repels negative particles and binds to cationic dyes.^{28,29} Similarly, ocular glycocalyx contains polyanions³⁰ such as sialylated O-glycans^{31,32} that have negative charge. The authors hypothesize that cationic dyes bind to conjunctival glycocalyx while anionic dyes are repelled by glycocalyx. Tables 2 and 3 list the chemical structure of some anionic and cationic dyes studied in the previous researches.

ADD AT TEAR MENISCUS EDGE CAUSING CELLULAR STAINING AND ML FORMATION

It is predicted that when anionic dyes deposition occurs, their cations neutralize either anionic dye itself or glycocalyx. This results in the increase of transmembrane diffusion of dye molecules which leads to cellular staining. The same process has also been observed in the physiological corneal staining phenomenon which is known as Solution Induced Corneal Staining (SICS). SICS occurs due to the uptake of lens preservative solution’s cationic particles by glycocalyx on cornea surface.³³ Furthermore, we should remember that dye deposition at the deposition site caused by coffee ring effect, increases dye concentration to about 100%, in fact, the term “purity” is more suitable than “concentration.”

As shown in Figure 5, it is observed that contact of anionic dye strips to conjunctivae leads to mucins and cellular staining. In Figure 5, nonwetted Rose Bengal strips were inserted in conjunctival sac. After removal of strip, mucins and cellular staining was observed. It should be mentioned that in addition to the causes discussed above, contact pressure between lid and globe can also amplify conjunctival staining. Similar to this observation, Norm³⁴ observed that 10% Rose Bengal solution can stain the site of drop instillation. However, no similar phenomenon was detected after instillation of the weaker 1% Rose Bengal solution.³⁴

We predict that ADD at the edge of tear meniscus due to coffee ring effect, leads to staining of cells beneath the line of deposition.

TABLE 2. Anionic Dyes That Stain Marx Line

Name of dye	Chemical structure	Name of dye	Chemical structure
Sodium fluorescein		Rose Bengal	
Lissamine green		water blue	
Eosin		Nigrosin	
Trypan blue			

TABLE 3. Cationic Dyes That Bind to Conjunctivae

Name of dye	Chemical structure	Name of dye	Chemical structure
methylene blue		gentian violet	
toluidine blue		Alcian blue	

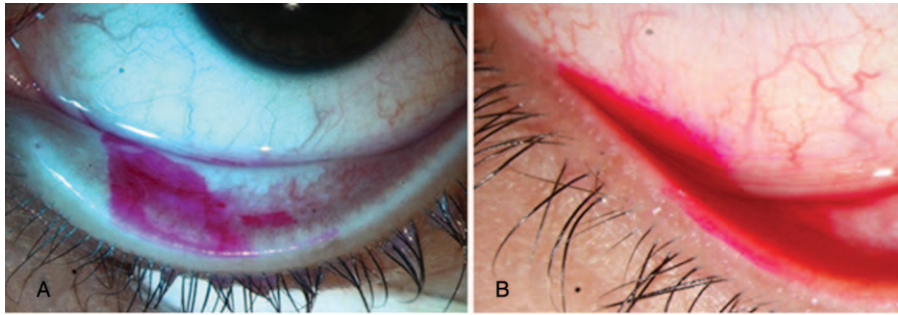


FIGURE 5. Inserting nonwetted Rose Bengal strips in conjunctival sac of (A) dry eye patient, (B) normal subject.

EFFECT OF TEAR MENISCUS ALTERATION ON LID MARGIN STAINING

Korb and Blackie³⁵ reported that in upward gaze, ML of upper lid is visible. According to this observation, in upward gaze, upper tear meniscus is located posterior to the line of staining from its previous location.

We dissolved Rose Bengal strips (OptiRos, opthechnics unlimited, Haryana, India, 1.5 mg) in distilled water with the ratio of 1 strip per 30 µL of water. Each drop of this solution, which is equivalent to 1 strip, contains about 30 µL of 5% Rose Bengal. Two drops of this solution were instilled in lower lacrimal sac of a healthy subject. Then, the subject was asked to look at a constant point forward. After 30 s, the subject was asked to look at another constant point that was higher than the previous point for 1 min. Then the lid margin staining was assessed by slitlamp photography. During this procedure, we made sure that subject felt comfortable and could blink normally at any time that needed.

As shown in Figure 6, another cellular staining happens posterior to the upper ML. This cellular staining is related to the new location of tear meniscus. This observation confirms that the dye characteristics and behavior play the main role in lid margin staining and contradicts the previous theory³ which focused on cellular characteristics neglecting the dye effect.

Also, from this observation and Norm study,³⁴ it can be concluded that in case of using high concentration of dye, the width of Marx line will be greater than 0.1 mm that was previously reported for Marx line width.³⁶ As a result, behavior of dye itself should be considered in clinical studies as an important influential factor.

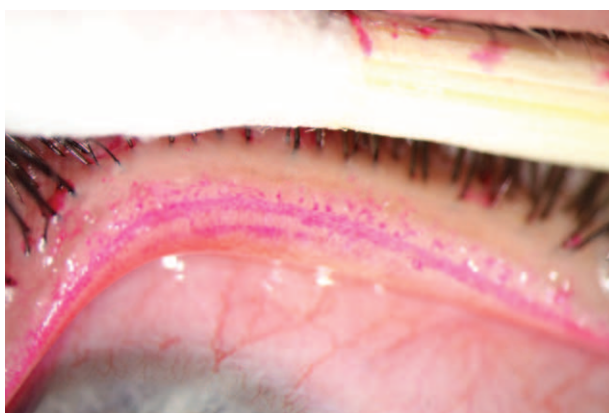


FIGURE 6. Cellular staining located posterior to initial Marx line, due to altered tear meniscus location.

PATHOLOGICAL LID MARGIN STAINING, LID-WIPER EPITHELIOPATHY

Lid wiper (LW) is the contact surface of upper and lower lid with globe (or lense) while blinking. This surface does not have an even and constant shape and alters under the effect of different factors like gaze³⁵ or blinking.²⁵ LW is posterior to ML and is wider than it (approximately 2 mm vs nearly 0.1 mm).³⁶ In contrast to ML, LW shows no staining under normal circumstances. In patients with dry eye symptoms, LW gets wider and shows staining. Korb et al³⁷⁻³⁹ reported and called this condition lid-wiper epitheliopathy (LWE). Inadequate lubrication between LW and globe can increase frictional coefficient and damage to LW or globe.³⁸ LWE was first evaluated in upper lid and it is also known as upper eyelid inner epitheliopathy, upper lid margin staining, and upper lid frictional epitheliopathy.⁴⁰ However, lower LWE is also currently investigated in research studies.⁴¹ Nevertheless, some authors still refer to all types of stainings in the margin of the lower lid as ML and believe LWE solely belongs to the upper lid.

LWE has 3 different patterns: feather-like pattern (stainings as lines perpendicular to ML); band pattern, the most common type of staining in the shape of a band whose width varying from nasal to temporal sides of the inner surface of lid margin; and irregular pattern (staining with unrecognizable shape).⁴⁰ Also other authors have demonstrated further types for LWE.⁴² A band pattern of LWE in a dry eye syndrome case is shown in Figure 7.

ADD SUPPORTED BY OBSERVATIONS IN PREVIOUS LITERATURE

Previous studies have been done in order to assess ML and LWE. In this section, those observations that could be explained by ‘‘ADD’’ hypothesis are discussed.

ML in Entropion and Ectropion Cases

Norm⁶ wrote: ‘‘The Marx’ line always touches the punctum. There are, however, 2 exceptions to the rule, these are in



FIGURE 7. Upper lid-wiper epitheliopathy in a dry eye patient.

TABLE 4. Previous Studies and Methods for Lid Margin Staining Supporting Anionic Dye Deposition Process

Year of Study	Title of Study	Method of Lid Margin Staining
2012	Lid margins: sensitivity, staining, meibomian gland dysfunction, and symptoms ⁴³	A 25- μ L drop of 2% fluorescein was instilled into the lower conjunctival sac of the right eye, followed by a 25- μ L drop of lissamine green dye preparation 1 min after. After 4 min, a second drop of 25 μ L of 2% fluorescein was instilled, followed by a second 25 μ L of lissamine green dye 1 min after. One minute after the last instillation, the eye was examined for lid margin staining
2010	Marx's line of the upper lid is visible in upgaze without lid eversion ³⁵	A 20- μ L drop of 2% unpreserved fluorescein was instilled into the conjunctival sac of the right eye after gentle depression of the lower eyelid. The 20- μ L drop was chosen to ensure adequate fluorescein residence time for both the evaluation without eversion and after eversion. The patient was instructed to blink 3 times, and 1 min after the instillation of the fluorescein, the patient was instructed to fixate superiorly on a fixation target, approximately 45° upward and to abstain from blinking. The examiner refrained from manipulation of either lid If Marx's line was not visible on examination 1 min after the instillation of fluorescein, the examination was deferred for additional 1 to 3 min to allow the tear volume in the meniscus to retreat posteriorly on the upper lid margin, exposing the stained line
2010	Prevalence of lid wiper epitheliopathy in subjects with dry eye signs and symptoms ³⁹	A drop of a mixture of 2% FL and 1% LG solution were instilled, at time 0 and at 5 min. One minute later the upper eyelids were everted and the lid wipers of all subjects were examined

ectropion where the Marx' line is found a certain distance behind the everted punctum, and in pronounced entropion where the Marx' line is either found in front of the punctum or is missing.” To explain Norm's observation, it can be mentioned that in lid malposition, ML position changes due to alteration of tear meniscus location. However, in entropion cases, if the new surface beneath tear meniscus, beyond MCJ, remains hydrophobic, coffee ring effect does not occur. As it was explained in Section 4, coffee ring deposition happens only on a hydrophilic surface since the contact angle is smaller than 90°.

Interval Between Staining and Lid Margin Assessment

From ADD hypothesis, it is predicted that low amount of dye, reflex tearing, and other factors can cause ML not to get stained well. Unlike this prediction, in previous studies for all normal subjects, ML was reported to be stained well. In many previous works, authors used multiple dyes and waited for a time period between staining and lid margin assessment. Some previous methods for lid margin staining are listed in Table 4.^{35,39,43} We predict that during the interval between staining and assessment, dye concentration decreases in tear while deposition occurs at the apex of tear meniscus.

CONCLUSION

This study proposed a novel theory for explaining ML formation. This hypothesis called ADD explains deposition of vital dyes as the cause of ML staining.

In the first step, we examined if the ophthalmic dye molecules have the required characteristics to deposit at the edge of a drop on hydrophilic surfaces. It is observed that during evaporation, dye molecules deposit at the edge of the drop. From this observation, it is predicted that these molecules would start to deposit at the edge of tear meniscus.

The mechanism of this observation is similar to the “coffee ring effect” phenomenon. “Coffee ring effect” is known and fully studied in surface chemistry. This phenomenon was

explained in detail in Section 4. The authors predict that this phenomenon will find other applications in ophthalmological phenomena.

In the second step, based on previous studies, ophthalmic dyes were divided in 2 major categories: anionic and cationic dyes. While cationic dye adheres to mucins, anionic dyes do not have this characteristic. Based on anionic dye characteristics, they are utilized as diagnostic dyes in ophthalmology. We described that due to ADD process occurring at the edge of tear meniscus, a line of cellular staining occurs.

In third step, effect of tear meniscus location alteration on lid margin staining is studied. As tear meniscus location changes, new area of staining is observed. This observation confirms that dye solution behavior on conjunctivae plays an important role in lid margin staining.

Lastly, observations in previous studies supporting this new hypothesis are discussed. ADD process well explains ML changes during lid malposition. Also, it is expected that during interval between staining and lid margin assessment reported in previous methods, dye concentration decreases in tear while deposition occurs at the apex of tear meniscus.

These results support the theory that ADD process plays an important role in explaining ML formation. More studies could be done to further evaluate the effect of this process in lid margin staining. Finding out cause or causes of physiological lid margin staining is important to propose standard methods for lid margin staining. This would improve lid margin staining methods which in turn also helps us better compare the results of different studies using the same method. Finally, understanding ML formation mechanism would enhance our knowledge about lid margin which significantly affects several ophthalmic diseases.

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