

Postmortem corneal changes evaluated by fluorescent staining

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Abstract: Sodium fluorescein is commonly used in clinical ophthalmology to detect the presence and degree of corneal alterations of various etiologies. Postmortem processes also alters the integrity of the corneal epithelium, and based on these etiopathological analogies we used sodium fluorescein staining to assess postmortem characteristics of corneal damages (with clinical and medico-legal implications) and also to evaluate the correlation between the intensity and extent of these damages with the postmortem interval.

Key Words: sodium fluorescein, postmortem corneal alterations, postmortem interval.

The goals of this study were to assess the postmortem characteristics of corneal cells deterioration visualised with fluorescent staining and the possibility of this method as a fast, cheap and non-invasive means to evaluate the correlation between corneal damage and early postmortem interval, knowing that cellular death is a process which occurs gradually over time depending on cells ability to survive anoxia caused by cessation of blood circulation and respiration.

From this point of view, cornea is a special type of tissue, being avascular (to ensure its transparency) excepting the limbus irrigated by branches from anterior ciliary and the palpebral artery [1]. The oxygen is supplied through diffusion by the precorneal tear film, its base being the outer external membrane of the corneal

epithelial cells [2]. Other functions of the tear film in the living are: prevention of corneal dehydration, protection, removal of metabolites and cellular waste, nutrition and optical role. Tear film disappears after death through evaporation of the normal 6 µL volume [2] which is not replenished due to cessation of lacrimal glands secretion and blinking.

Damaging effects of the tear film disappearance on corneal epithelial cells integrity are caused by dehydration (especially when the eyes of the dead remain open), anoxia and acidosis from accumulation of lactic acid and pyruvate [3], all of which overlap and favour postmortem autolysis of the corneal cells, caused by proteolytic lysosomal enzymes [4].

These processes also cause a proportional

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alteration of the integrity of the tight junctions between adjacent epithelial cells which normally seal the intercellular space preventing the diffusion of tear film components into the corneal deeper layers [2, 5].

Alterations of corneal epithelial cells and their tight junctions (i.g. in dry eye syndrome) are routinely assessed in clinical ophthalmology using sodium fluorescein that stains damaged and devitalised cells, gaps between cells [3, 6] disrupted mucin layer [2] and does not stain the normal corneal epithelium.

Based on these etiopathological analogies, we used fluorescent staining to assess the characterises of the postmortal damage of corneal surface and the correlation between intensity and extent of damage and time of death.

Aspects of corneal anatomy applied to fluorescein staining assessment.

Cornea is a transparent anatomical structure with the central area steeper and thinner (0.5mm thick) than the periphery (0.7mm thick) [1, 2, 7, 8].

1. The outer epithelium has a constant thickness of approximately 50µm over the entire corneal surface [2] and consists of 5 to 7 layers of cells [1, 9, 10], from surface to depth:

- 2 to 3 layers of superficial nonkeratinized squamous epithelial cells connected by tight and adherens junctions [1, 2, 9] which impede the tear film and its components to penetrate intercellular space [2, 10]. Disruptions of the barrier function of the corneal epithelium are measured by fluorescein staining.

- 2 to 3 layers of suprabasal cells or wing-like epithelial cells, presenting lateral interdigitations with numerous desmosomes and gap junctions [1, 2, 9].

- single layer of columnar basal cells which posses mitotic activity [8, 11]. Adjacent cells are connected by desmosomes which also connect this layer to the overlying wing cells [11].

2. Bowman's membrane (Anterior Limiting Lamina) is 10-17µm thick [1], acellular, consisting of collagen fibrils [1, 11].

3. The stroma represents approximately 85-90% of the corneal thickness (0.42 – 0.54mm [1, 8] and consists of hydrated fibrils of collagen, glycoproteins and proteoglycans which regulate hydration, maintaining in this way corneal transparency [1, 8].

4. Descemet's membrane (Posterior Limiting Lamina) is approximately 10µm thick in adult cornea [1]. It is secreted by the endothelium, separating it from the stroma [1, 11].

5. Inner endothelial cell layer, represents the most posterior surface of the cornea consisting of a single layer of cuboidal cells [1,11] of approximately 5 µm thickness and 15-20µm width [1]. Adjacent cells are connected by interdigitations and leaky tight junctions [1] and their role is to keep cornea dehydrated removing excess fluid from corneal stroma to the aqueous humor [1, 12].

Limbus (corneoscleral junction) – represents a zone of 1.0 – 1.5mm width of gradual transition between the transparent cornea and opaque sclera [13]. The anterior border of the limbus is a line connecting the periphery of Bowman's and Descemet's membrane and the posterior border is a perpendicular line from the scleral spur to the surface of the eye [14]. It contains highly proliferative limbal epithelial stem cells (LECS) located in the limbal niche [9] which can divide into transient amplifying cells (early TAC) that migrate centripetally and superficially (from basal to superficial corneal layers). During migration, TAC progressively become more differentiated (late TAC, postmitotic cells) finally giving rise to fully differentiated corneal cells [15-17]. Studies of Miyashita [18] and Bath [19] showed that hypoxia enhances limbal cells proliferation and also induces immature limbal cells to migrate centripetally from the limbus [16].

MATERIAL AND METHODS

The study was carried out at the "Mina Minovici" National Institute of Legal Medicine and the Pathology Department, Bucharest Clinical Emergency Hospital. The corneal damage was evaluated by fluorescence staining in two study groups depending upon the known postmortem interval (PMI): for the first group consisting of 66 cases, PMI was less than or equal to 24 hours and for the second group consisting of 50 cases, PMI was greater than 24 hours.

We separately interpreted the results from this two groups because corpses from the first group were not refrigerated while those from the second group were stored in morgue refrigerator until the examination was performed, and the low temperature could have interfered with the postmortem development of corneal damage.

There were excluded from the study those cases with ocular traumatic lesions and those who had eyes open within the postmortem interval. Data collected included: age (ranging from 16 to 96 years old), sex (66 males and 50 females) and time of death (provided by clinical or police reports, ranging from 2 hours to 160 hours).

The corneal fluorescent staining examination was performed before autopsy. We used for each examined eye a filter paper strip impregnated with sodium fluorescein 1% wet with 2 drops of water, then gently applied on the entire surface of cornea and immediately afterwards 1- 2 drops of water were added on the corneal surface.

In order to detect the areas of fluorescein stained epithelial defects, cornea was illuminated with cobalt blue light and high resolution close up photographs of the entire eye were taken (for obvious reasons we could not use the slit lamp biomicroscope in our research). At the end of cornea examination and after picture taking, the eye was abundantly rinsed with water removing in this way all stain traces.

The close-up photographs were separately assessed by two examiners, with regard to:

-corneal degree of damage measured by corneal fluorescence intensity (CFI) - correlating Efron scale [20] with our own observations, the intensity of corneal fluorescence was numerically graded on a scale from 0 to 4: 0 = no fluorescence, 1 = trace (Fig. 1), 2 = mild (Fig. 2), 3 = moderate (Fig. 3), 4 = intense (Fig. 4).

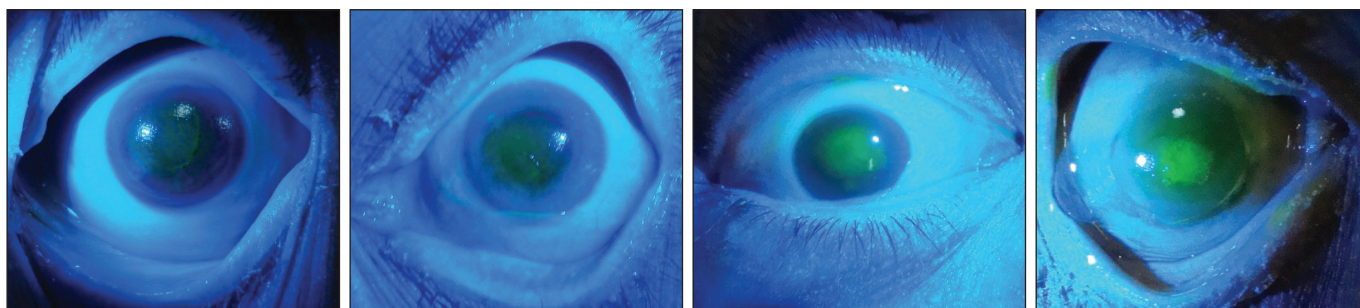


Figure 1. Trace and punctate staining (1).

Figure 2. Mild and coalescent staining (2).

Figure 3. Moderate staining (3).

Figure 4. Intense and patch staining (4).

- position of the fluorescent area: central (0), superior (1), inferior (2), nasal (3), temporal (4), supero-nasal (5), supero-temporal (6), infero-nasal (7), infero-temporal (8).

- the overall pattern of the fluorescent area: punctate (0) (Fig. 1), coalescent punctate (1)(Fig. 2), patch staining (2) (Figs. 3, 4),

- shape of the fluorescent area: linear (0), arcuate (1), round (2), oval (3), indefinite (4)

- surface of the fluorescent area (SFA) was visually estimated and numerically expressed depending on 10 percentage groups of surface involvement (1 = 1 – 10%; 2= 11 – 20%; 3 = 21 – 30%; 4 = 31 – 40%; 5 = 41 – 50%; 6 = 51 – 60%; 7 = 61 – 70%; 8 = 71 – 80%; 9 = 81 – 90%; 10 = 91 – 100%).

In order to prevent bias, the examiners did not know the postmortem interval for the assessed cases and the evaluation was repeated after one week. Statistical analysis was performed using IBM SPSS Statistics 22 and Analyse-it (Method Validation Edition) software.

Table 1. Number of radiographs by sex and age

	Minimum	Maximum	Mean	S.D.
CFI	1	4	2.61	0.857
SFA	2	7	3.91	1.344

Table 2. Numbers and percentages of CFI grades for PMI ≤ 24 hours

Grade of CFI	Frequency	Percent	Cumulative percent
1	7	10.6	10.6
2	21	31.8	42.4
3	29	43.9	86.4
4	9	13.6	100.0

Table 3. Location of the fluorescent area for PMI ≤ 24 hours

	Central	Superior	Inferior	Nasal
% of cases	86.36	10.60	1.51	1.51

RESULTS

The kappa statistic was used to assess interobserver agreement for two parameters: corneal fluorescence intensity and surface of the fluorescent area. Interobserver concordance was substantial (K = 0.677 > 0.6) for CFI parameter and poor (K = 0.187) for SFA parameter.

Intraobserver agreement was evaluated using Intraclass Correlation Coefficient (ICC) for the same parameters as above. ICC for CFI was 0.81 > 0.7 (range 0.73 - 0.87) indicating an excellent reproducibility. ICC for SFA was 0.89 (range 0.85 - 0.93) also indicating an excellent reproducibility, better than that for fluorescence intensity.

Statistical results for PMI ≤ 24 hours

Descriptive statistical data for CFI and SFA are shown in Table 1. CFI grades were ranging from 1 to 4 and SFA groups from 2 to 7.

Table 2 presents the numbers and percentages of cases from each group of CFI, resulting that most of the cases from this postmortem interval are of grade 2 (31.8%) and 3 (43.9%) intensity of corneal fluorescence. Table 3 shows that most (86.36%) fluorescent areas are centrally located, table 4 shows that coalescent and patch staining are found in almost 91% of the cases, and table 5 shows that the round shape of the fluorescent area is the most frequently found (68.18%) in our study group.

Table 4. Pattern of the fluorescent area for PMI ≤ 24 hours

	punctate	coalescent punctate	patch staining
% of cases	9.09	48.48	42.42

Table 5. Shape of the fluorescent area for PMI ≤ 24 hours

	linear	arcuate	round	oval	indefinite
% of the cases	3.03	3.03	68.18	13.63	12.12

Figure 5 presents the graph of CFI grades plotted against hourly intervals over the initial 24-hour postmortem period. Figure 6 presents the graph of SFA grades plotted against hourly intervals over the initial 24-hour postmortem period.

The test of linear correlation between PMI and CFI parameter gave the correlation coefficient $r = 0.33 > 0.24$ showing a moderate positive correlation between these two variables (Fig. 7). The relationship between these variables is expressed by a linear regression equation of the form $y = 8.14 + 2.42 \cdot x$ where $y = \text{PMI}$ and $x = \text{intensity of corneal fluorescence}$ (Fig. 8).

The test of linear correlation between PMI and the surface of corneal damage visualised by fluorescence staining gave the correlation coefficient $-0.24 < r = -0.03$

> 0.24 showing a weak positive correlation between these two variables.

The test of linear correlation between:

-PMI and location of the stained area gave a correlation coefficient $r = 0.2$ showing no correlation.

-PMI and pattern of the stained area gave a correlation coefficient $r = 0.35$ showing a moderate positive correlation between these two variables.

-PMI and shape of the stained area gave a correlation coefficient $r = -0.11$ showing no correlation.

Statistical results for PMI > 24 hours

Descriptive statistical data for CFI and SFA is shown in Table 6. CFI grades were ranging from 2 to 4 (no trace stained cases) and SFA groups from 2 to 10

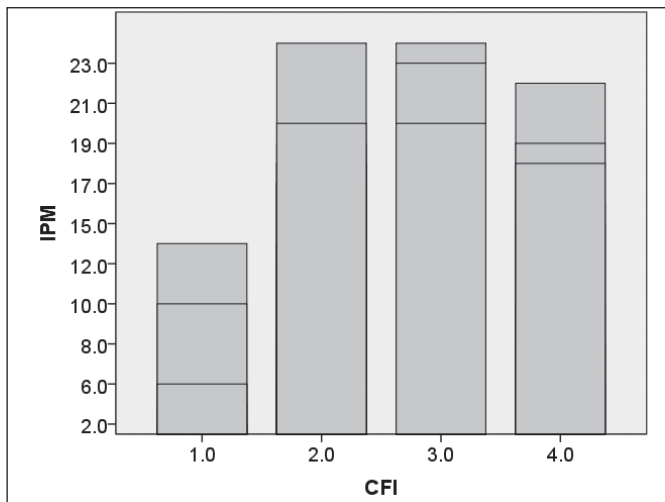


Figure 5. CFI grades plotted against time intervals.

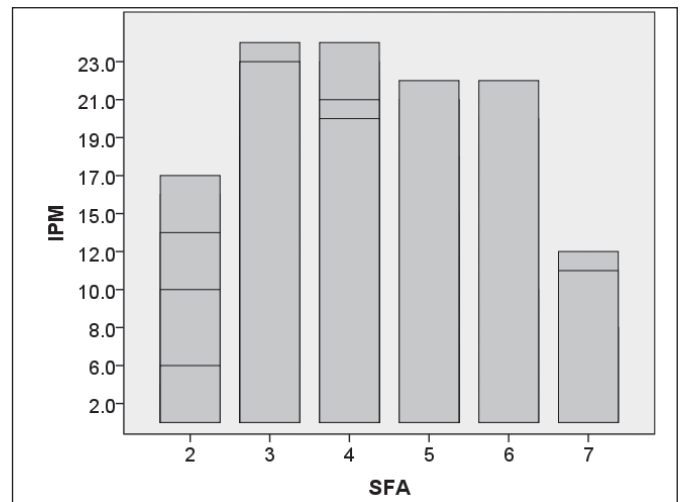


Figure 6. Surface of the fluorescent area grades plotted against time intervals.

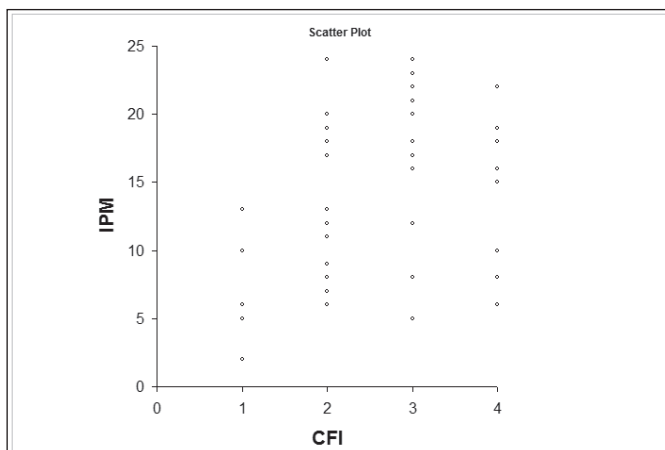


Figure 7. Linear correlation between PMI ≤ 24 hours and CFI.

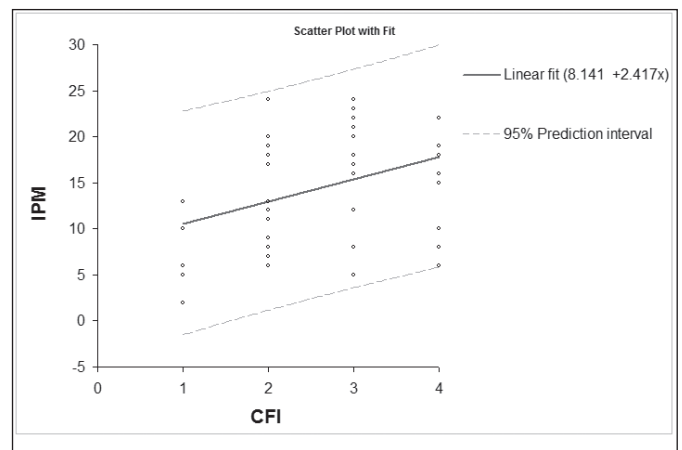


Figure 8. Linear regression between PMI ≤ 24 hours and CFI.

Table 6. CFI and SFA for PMI > 24 hours

	Minimum	Maximum	Mean	S.D.
CFI	2	4	3.38	0.567
SFA	2	10	5.20	1.938

Table 7. Percentage of CFI grades for PMI > 24 hours

Grade of CFI	Frequency	Percent	Cumulative percent
1	0	0	0
2	2	4.0	4.0
3	27	54.0	58.0
4	21	42.0	100.0

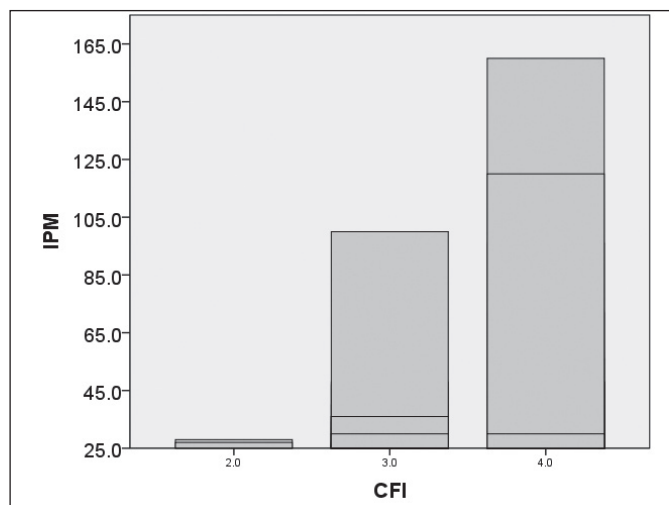


Figure 9. CFI grades plotted against time intervals.

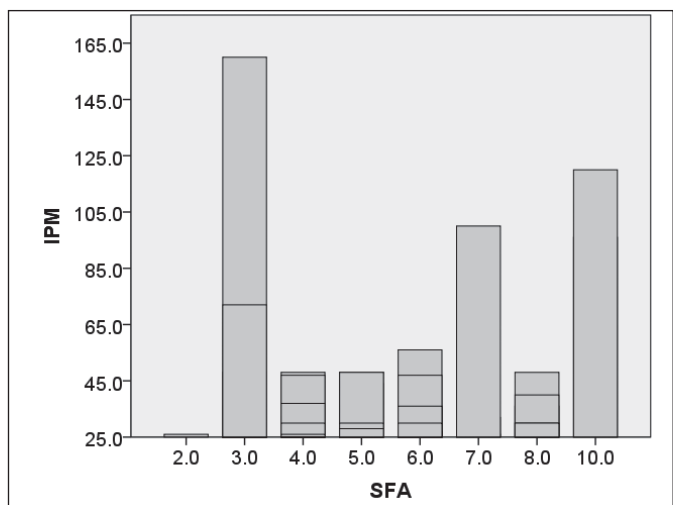


Figure 10. Surface of the fluorescent area grades plotted against time intervals.

(this means that for PMI > 24 hours, the surface of the fluorescent area ranged from 11 to 100% of the total corneal surface).

Table 7 presents the numbers and percentages of cases from each group of CFI, resulting that most of the cases from this postmortem interval are of grade 3 (54%) and 4 (42%) intensity of corneal fluorescence and just 4% of the case for grade 2 fluorescense intensity.

Figure 9 presents the graph of CFI grades plotted against hourly intervals over the PMI > 24 hours. Figure 10 presents the graph of SFA grades plotted against hourly intervals over the PMI > 24 hours.

Table 8 shows that most (80%) fluorescent areas are centrally located, table 9 shows that patch staining is found in 88% of the cases, and table 10 shows that the round shape of the fluorescent area is also the most

Table 8. Location of the fluorescent area for PMI > 24 hours

	(0) Central	(1) Superior	(2) Inferior	(3) Nasal
% of cases	80	2	4	14

Table 9. Pattern of the fluorescent area for PMI > 24 hours

	(0) punctate	(1) coalescent punctate	(2) patch staining
% of cases	0	12	88

Table 10. Shape of the fluorescent area for PMI > 24 hours

	(0) linear	(1) arcuate	(2) round	(3) oval	(4) indefinite
% of the cases	2	0	60	36	2

frequently found (60%) in this study group.

The test of linear correlation between PMI and the CFI gave the correlation coefficient $r = 0.36 > 0.28$ showing a moderate positive correlation between these two variables (Fig. 11). The relationship between these variables is expressed by a linear regression equation of the form $y = -15.4 + 16.65 \cdot x$ where $y = \text{PMI}$ and $x = \text{intensity of corneal fluorescence}$ (Fig. 12).

The test of linear correlation between PMI>24hours and SFA gave the correlation coefficient $r = 0.21 < 0.28$ showing a weak positive correlation between these two variables.

The test of linear correlation between:

- PMI and location of the fluorescent area gave a correlation coefficient $r = -0.1$ showing no correlation
- PMI and pattern of the fluorescent area gave

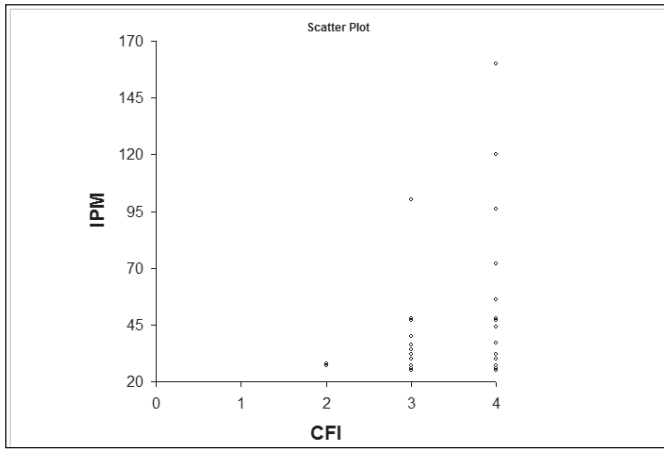


Figure 11. Linear correlation between PMI>24hours and CFI.

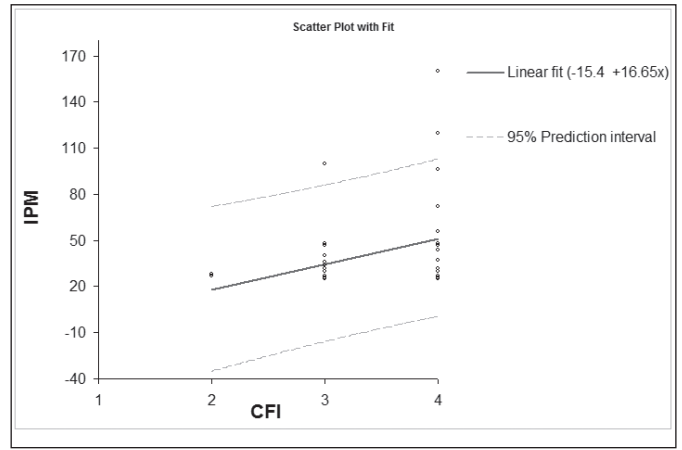


Figure 12. Linear regression between PMI>24hours and CFI.

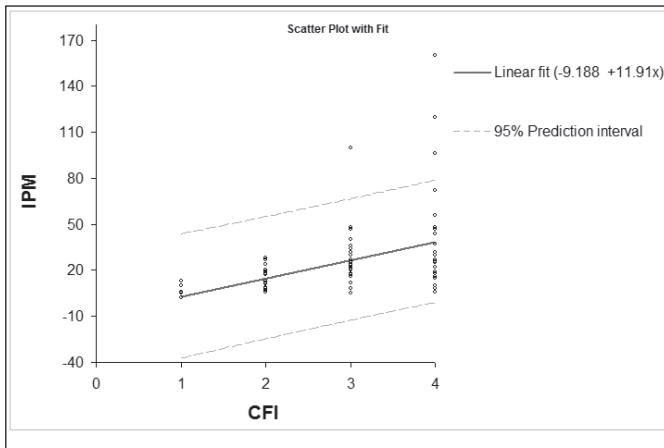


Figure 13. Linear regression between PMI and CFI for all 116 cases.

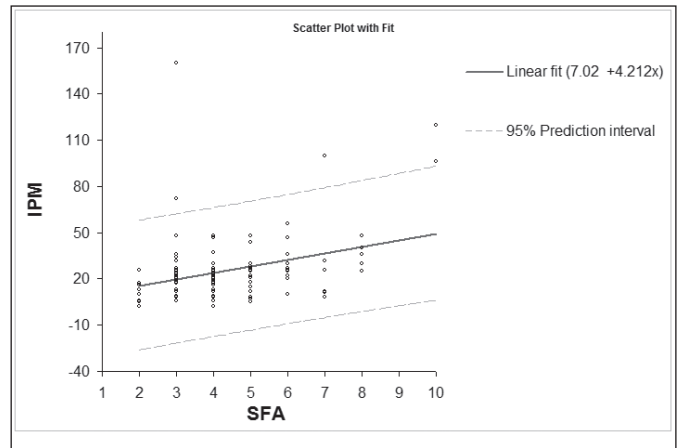


Figure 14. Linear regression for PMI and surface of the fluorescent area for all 116 cases.

a correlation coefficient $r = 0.18$ showing a moderate positive correlation between these two variables

-PMI and shape of the fluorescent area gave a correlation coefficient $r = 0.12$ showing no correlation.

If we take into consideration all 116 cases, the test of linear correlation between PMI and CFI gave the correlation coefficient $r = 0.45 > 18$ showing a tight positive correlation between these two variables. The relationship between PMI and CFI is expressed by a linear regression equation of the form $y = -9.19 + 11.91 * x$, where $y =$ PMI and $x =$ surface of corneal fluorescence (Fig. 13).

The test of linear correlation between PMI and SFA applied to all 116 cases gave the correlation coefficient $r = 0.33 > 0.18$ showing a tight positive correlation between these two variables but not so tight as in the case of PMI and intensity of staining. The relationship between PMI and surface of fluorescence is expressed by a linear regression equation of the form $y = 7.02 + 4.21 * x$, where $y =$ PMI and $x =$ surface of corneal fluorescence (Fig. 14).

DISCUSSION

Corneal fluorescent staining revealed interesting aspects of postmortem modifications of this avascular

tissue and from our knowledge no other studies of this type have been conducted.

I. In the first part of discussion, only the first study group (PMI ≤ 24 hours) was taken into consideration, because cases were exposed to normal room temperature and humidity.

1. All four grades of corneal fluorescence staining were found in this study group: most of the cases showed mild (31.8%) and moderate (43.92%) staining and the percentage of severe staining cases (13.63%) was greater than those with trace staining (10.59%). This finding shows that even though cornea is an avascular tissue, structural alterations develop and progress rapidly after death.

Sever alterations of corneal epithelium determining a grade 4 intensity of fluorescent staining was found in PMI ranging from 6 to 22 hours, grade 3 staining (moderate alteration) as early as 5 hours after death, grade 2 staining (mild alteration) after 6 hours and grade 1 intensity staining (incipient alteration) was found in a PMI ranging from 2 to 13 hours. The severity of the corneal damage in this early PMI is also demonstrated by the high frequency of confluent (48.48%) and patch staining (42.42%), revealing disruption of closely packed epithelial cells [21].

A moderate positive correlation between the intensity of fluorescence staining and PMI was found, showing an increase of the severity of the corneal epithelium and tear film alterations with the passage of postmortem time. This observation could be explained by the cumulative disruptive effects over time of the corneal desiccation, anoxia, acidosis and postmortem autolysis.

The heterogeneous development of postmortem corneal alterations could not only be explained by differences in environmental temperature or humidity since all cases were exposed to almost similar climatic conditions therefore future research is required to explain the mechanism underlying this phenomenon.

The fast and extensive development of corneal alterations after death, shown by our study, could also have implications in corneal transplant surgery: according to the Technical Guidelines For Ocular Tissue [22] the average delay from death to corneal retrieval should be 13.05 hours (min. 2 hours, max. 35 hours). Our study found that in this period of time important destructive postmortem processes occur, causing mild to severe structural alterations of the corneal epithelium that might not be considered suitable for transplantation.

2. Fluorescent stained area was centrally located in 86.36% of the cases and had a regular shape: round in more than half of the cases (68.18%) and oval in 13.63% of the cases. These two characteristics suggest that the postmortem damage of the corneal epithelium starts in the centre of cornea and expands centripetally and symmetrically over time, affecting in our study group up to 70% of the corneal surface in the first 24 hours after death. Another possible conclusion resulting from this observation is that the resistance over time to anoxia of corneal cells, increases progressively from central areas to periphery where paralimbal and limbal areas are the last to be affected by postmortem anoxia. This could be explained by the resistance to anoxia of the less differentiated cells, their number decreasing progressively from periphery to the central areas, as they migrate from the limbus and progressively become more differentiated and in this way less resistant to anoxia.

3. In the second group where PMI > 24 hours and the corpses were stored at low temperature, the overall severity of corneal damage was higher than that found in the first group: grade 1 staining intensity was not found, grade 2 in just 4% of the cases and the rest of the cases were of grade 3 (54%) and 4(42%) intensity of corneal

staining. Patch staining was seen in 88% of the case and coalescent punctate in 12%. The stained area was centrally located in 80% of the cases and shape was round in 60% of the cases and oval in 30%, having a surface ranging from 20 -100%.

II. Regarding the correlation between the degree of corneal alteration and PMI we can make the following observations:

- the interobserver reliability was substantial for the assessment of CFI and discrepancy was observed in the assessment of SFA, most likely caused by the subjectivity of the visual method employed in absence of uniform surface landmarks.

- a moderate correlation was observed between PMI and CFI in both groups (the same correlation was noticed between PMI and pattern of the stained area parameter) and no correlation between PMI and SFA for the ≤ 24 -hour group and poor correlation between PMI and SFA for the >24 -hour group.

- when we applied the test of linear correlation between PMI and CFI for all 116 cases a tight positive correlation was obtained similar to that between PMI and SFA for the same number of cases. However, in case of the second study group where PMI > 24 hours and corpses were refrigerated, we have to consider the fact that low temperature could have altered the development of postmortem corneal damage.

- grade 1 intensity of corneal fluorescence (trace staining) was not observed in cases from PMI >24 hours and also in this study group grade 2 intensity (mild staining) was seen in just 4% of the cases. The application of this finding in current forensic practice is that their presence would most likely indicate a PMI interval of less than 24 hours (grade 1 would indicate a PMI interval less than 13 hours).

- the surface of stained area for PMI ≤ 24 hours ranges from 11% to 70% of the total corneal area, therefore in practical forensic applications stained areas greater than 70% would most likely indicate a PMI of more than 24 hours. The same PMI could be suggested by the absence of punctate pattern of staining, seen only in early hours postmortem.

Regarding the applicability in forensic estimation of PMI, corneal fluorescence staining is a cheap, fast, easy to use and non –invasive method that used in conjunction with other postmortem time indicators improves the accuracy of the PMI estimation.

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