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Original Article

Investigation of the Relationship Between Postmortem Interval and Pupil Response Following Pilocarpine Application in Rat Eyes: An Experimental Animal Study

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ABSTRACT

Objective: The aim of this study was to investigate the relationship between pupil diameter and postmortem interval (PMI) following topical pilocarpine application in rat eyes.

Methods: Thirty rats were grouped into 5 groups according to PMI (0-24 hours). One eye of each rat was used as the experimental eye and the other as the control eye. Topical pilocarpine (2%) was applied to the experimental eyes, while no application was made to the control eyes. Pupil diameter measurements were taken using a digital caliper with 0.01 mm precision. The difference between pre- and post-pilocarpine pupil diameters was recorded as the amount of pupil diameter change. Positive, negative, and paradoxical responses were classified.

Results: In the experimental group, pupil diameter after pilocarpine application was significantly reduced compared to the baseline pupil diameter at 0, 6, 9, and 18 hours postmortem (hpm). No significant difference in pupil diameter change was observed between the experimental and control groups at any PMI ($P > .05$). Additionally, no significant correlation was found between PMI and the amount of pupil diameter change in the experimental eyes ($P = .154$, $r = -0.201$). In the experimental group, positive responses were observed in all subjects at 0, 9, and 18 hpm, while paradoxical responses predominated at 3, 12, and 21 hpm.

Conclusion: This study found no correlation between PMI and the amount of pupil diameter change in rat eyes, and the distribution of positive, negative, and paradoxical responses after pilocarpine application was irregular. These results suggest that using topical pilocarpine for PMI estimation may be misleading.

Keywords: Pilocarpine, postmortem chemical iris excitability, postmortem interval, rat eye, supravital reactions

INTRODUCTION

Estimating the time elapsed since death using a reliable and reproducible method is one of the most crucial objectives in forensic medicine. Determining the time of death is particularly important in criminal investigations, as it helps to define the time frame during which the crime may have occurred. In routine practice, the most commonly used method for estimating the time of death is the temperature-based Henssge nomogram, which relies on the cooling rate of the body. The Henssge nomogram is a graphical method that illustrates how body temperature changes relative to ambient temperature and certain environmental factors during a specific period in the postmortem phase.[1] In addition to this gold-standard method, research continues to explore supplementary methods to reliably estimate the postmortem interval (PMI). One of these research areas focuses on

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the examination of supravital reactions, which refer to the reactions observed in tissues as a result of postmortem stimulation. The most significant of these reactions are the mechanical and electrical excitability of skeletal muscle and the pharmacologically induced excitability of the iris. Other supravital reactions include the response of sweat glands to drug injections, sperm motility, leukocyte viability, and postmortem blood clotting. However, these reactions generally lack practical significance in forensic applications.[2]

The eye has become one of the most prominent anatomical regions in PMI estimation research due to its ease of observation, resistance to decomposition and contamination, and accessibility through minimally invasive procedures. Research in this area encompasses a wide spectrum, ranging from the evaluation of macroscopic and microscopic changes in different anatomical regions of the eye to the postmortem biochemical analysis of aqueous humor and vitreous humor.[3,4] The iris tissue is also among the ocular tissues examined for postmortem changes. Previous studies have investigated changes in iris color and the excitability of iris tissue during the postmortem period.[5-8]

The iris tissue contains 2 muscle groups responsible for controlling pupil size: the sphincter pupillae, controlled by the parasympathetic nervous system, and the dilator pupillae, controlled by the sympathetic nervous system. The delicate balance between these 2 antagonistic muscle groups enables precise and rapid control of pupil diameter. Contraction of the sphincter pupillae and relaxation of the dilator pupillae result in pupil constriction (miosis), while contraction of the dilator pupillae and relaxation of the sphincter pupillae lead to pupil dilation (mydriasis). These muscles are innervated by postganglionic neurons originating from the ciliary ganglion and the superior cervical ganglion, which receive preganglionic inputs from the Edinger–Westphal nucleus and the intermediolateral nucleus, respectively.[9] The pupillary light reflex is the miosis response of the pupil to a light stimulus, and its disappearance during the postmortem period typically results in the pupils becoming moderately dilated.[3]

MAIN POINTS

- There is no correlation between PMI and the change in pupil diameter in rat eyes, and the pupil responses exhibit an irregular distribution.
- Using pupil response after topical pilocarpine application to estimate PMI may yield misleading results.
- More research is needed to explain the relationship between iris excitability and PMI.

Studies have shown that pupillary reactions to pharmacological stimuli persist for several hours after death, and that iris tissue can exhibit miosis or mydriasis in response to cholinergic or adrenergic drugs during the postmortem period.[3] It has been reported that the iris excitability induced by the subconjunctival injection of norepinephrine or acetylcholine can be maintained up to 46 hours postmortem (hpm) in some cases.[2] The relationship between changes in pupil diameter caused by the iris' supravital reaction and PMI estimation remains a topic of debate. While some studies in the literature suggest that pupil diameter changes are useful for estimating PMI, there are also cadaveric studies that suggest the results related to pupil diameter can be misleading.[6,7,10] However, to the best of knowledge, no animal studies have yet been conducted on this topic.

The size of the circular pupil in rats can change as a result of the iris expanding and contracting to regulate the amount of light entering the eye, similar to other mammals.[11] The aim of the study was to evaluate the relationship between pupil diameter changes and the PMI through the topical application of pilocarpine, an easily accessible agent used in the treatment of glaucoma, to rat eyes.

MATERIAL AND METHODS

The study was designed using excess ocular tissues from sacrificed animals in a study approved by the Kastamonu University Animal Experiments Local Ethics Committee (KU-AELEC) (Date: 12.11.2024 Number: 32) for animal experiments on March 11, 2024, with decision number 06. In addition, approval was received from (KU-AELEC) that no additional permission was required for the study. The study was designed to involve keeping rats under room climate conditions (23 ± 1°C and 35%-40% humidity) after sacrifice, followed by topical pilocarpine application at specific intervals and subsequent pupil diameter measurement. For this purpose, 30 male rats, aged 8 weeks, of the Sprague–Dawley strain, each weighing 250 ± 20 g, were included in the study.

Sacrifice

Rats were sedated with an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (5-10 mg/kg) to ensure adequate anesthesia and minimize pain and distress during the procedures. The depth was monitored by the absence of reflex responses, and animals were handled in accordance with ethical guidelines to ensure their welfare prior to sacrifice. Following sedation, rats were sacrificed using the cervical dislocation method, which is a rapid and humane technique when performed correctly.

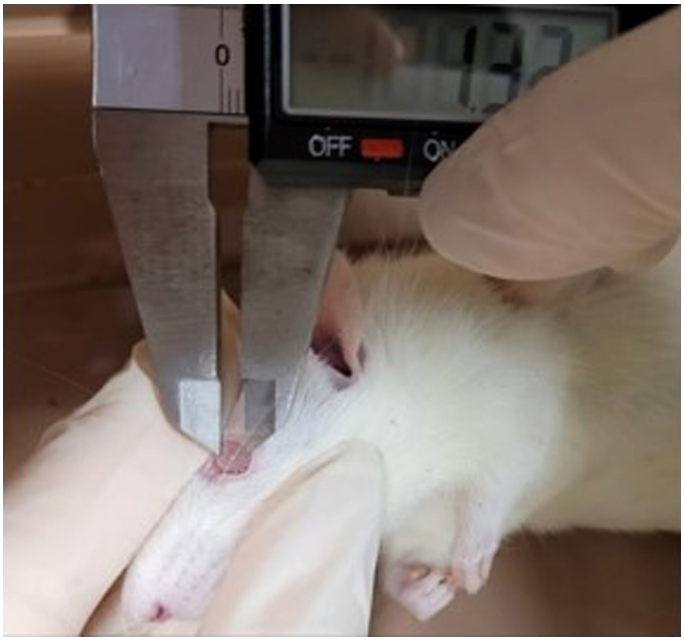

Figure 1. Pupil diameter measurement in the rat eye using a digital vernier caliper.

Experimental Design

The study included 5 rat groups, with 6 rats in each group. Initially, pilocarpine was applied to the right eyes of rats in each group, while the left eyes were used as control. Accordingly, pilocarpine was applied to the right eyes of group 1 at 0-hpm, group 2 at 3-hpm, group 3 at 6-hpm, group 4 at 9-hpm, and group 5 at 12-hpm. Subsequently, the left eyes of the rats in each group were used as the experimental group, and the right eyes were included as the control group. Accordingly, pilocarpine was applied to the left eyes of group 1 at 15-hpm, group 2 at 18-hpm, group 3 at 21-hpm, and group 4 at 24-hpm, and the study was concluded at the 24th hour. A digital vernier caliper, with a precision of 0.01 mm, was used for pupil diameter measurement (Figure 1).

To detect changes in pupil diameter after pilocarpine application, baseline pupil diameter measurements were taken before the drop application. For this, 3 consecutive pupil diameter measurements were made horizontally, and their average was calculated. Then, 0.05 mL of 2% pilocarpine (Pilosed®, 2%) was applied topically to the experimental group eyes, while no application was made to the control eyes. A 15-minute waiting period was allowed for the tissue concentration of pilocarpine to reach its maximum.[12] At the end of the waiting period, 3 consecutive pupil diameter measurements were taken horizontally for both the experimental and control eyes to assess secondary pupil diameter, and the average was calculated. The difference between the baseline and secondary measurements (baseline measurement—secondary measurement) was calculated for both the experimental and control eye groups and recorded as the amount of pupil diameter change. After pilocarpine application, the experimental eyes were categorized as follows: those with a decrease in pupil diameter were classified as a positive response, those with no change in pupil diameter as a negative response, and those with an increase in pupil diameter as a paradoxical response. The same procedure was repeated for each PMI (0, 3, 6, 9, 12, 15, 18, 21, and 24).

Statistical Analysis

Statistical analysis of the data was performed using IBM SPSS Statistics Version 26.0 software. The distribution of quantitative variables was assessed using the Shapiro–Wilk test. Descriptive statistics for the data included mean, SD, frequency, and percentage values. The Mann–Whitney *U* test was used for quantitative comparisons between groups. Baseline and secondary pupil diameters within the same group were compared using the Wilcoxon test. The relationship between the amount of pupil diameter changes and PMI was investigated using Spearman's correlation analysis. A *P*-value of <.05 was considered statistically significant for all analyses.

RESULTS

Pupil diameter measurements were started at 0 hpm after sacrifice. Each PMI group included 6 experimental eyes and 6 control eyes; however, one eye from each of the 18, 21, and 24 hpm groups was excluded from the study due to corneal opacity. The average baseline and secondary pupil diameters in the experimental eyes, based on PMI, are shown in Table 1. Accordingly, in the experimental group, at 0, 6, 9, and 18 hpm, the secondary pupil diameter was significantly smaller compared to the baseline pupil diameter ($P < .05$). In the control eyes,

Table 1. Comparison of Baseline and Secondary Pupil Diameter in the Experimental Group

Postmortem Interval	Baseline Pupil Diameter	Secondary Pupil Diameter	*P**
PMI—0 (n = 6)	1.54 ± 0.05	0.68 ± 0.41	**.027**
PMI—3 (n = 6)	1.55 ± 0.54	1.52 ± 0.69	.916
PMI—6 (n = 6)	1.78 ± 0.52	1.49 ± 0.60	**.046**
PMI—9 (n = 6)	2.19 ± 0.82	1.67 ± 0.59	**.028**
PMI—12 (n = 6)	2.37 ± 0.59	2.58 ± 0.52	.249
PMI—15 (n = 6)	2.33 ± 1.16	2.17 ± 1.32	.917
PMI—18 (n = 5)	2.11 ± 0.93	0.76 ± 0.04	**.043**
PMI—21 (n = 6)	1.37 ± 0.41	1.53 ± 0.42	.248
PMI—24 (n = 5)	1.26 ± 0.38	1.00 ± 0.33	.068

PMI, postmortem interval.
*Wilcoxon test.

Table 2. Comparison of Baseline and Secondary Pupil Diameter in the Control Group

Postmortem Interval	Baseline Pupil Diameter	Secondary Pupil Diameter	P*
PMI—0 (n = 6)	1.59 ± 0.17	1.29 ± 0.44	.116
PMI—3 (n = 6)	1.35 ± 0.69	1.66 ± 0.79	.249
PMI—6 (n = 6)	1.70 ± 0.58	1.61 ± 0.76	.500
PMI—9 (n = 6)	2.06 ± 0.66	1.90 ± 0.59	.345
PMI—12 (n = 6)	2.44 ± 0.56	1.84 ± 1.11	.249
PMI—15 (n = 6)	2.24 ± 0.68	2.70 ± 0.71	**.028**
PMI—18 (n = 6)	2.32 ± 0.79	2.42 ± 0.50	.833
PMI—21 (n = 5)	2.13 ± 0.81	2.41 ± 0.66	.686
PMI—24 (n = 6)	1.06 ± 0.38	1.04 ± 0.42	.753

PMI, postmortem interval
*Wilcoxon test.

Table 3. Comparison of Pupil Diameter Changes Between the Experimental and Control Groups

Postmortem Interval	Pupil Diameter Change		P¥
	Experimental Group	Control Group	
PMI—0	0.85 ± 0.41	0.31 ± 0.49	.065
PMI—3	−0.13 ± 0.49	−0.31 ± 1.20	.336
PMI—6	0.29 ± 0.36	0.09 ± 0.27	.423
PMI—9	0.52 ± 0.31	0.17 ± 0.43	.173
PMI—12	−0.21 ± 0.50	0.60 ± 1.10	.150
PMI—15	0.16 ± 0.86	−0.45 ± 0.34	.078
PMI—18	1.35 ± 1.95	0.23 ± 0.99	.068
PMI—21	−0.16 ± 0.33	−0.29 ± 1.04	.583
PMI—24	0.06 ± 0.41	0.02 ± 0.11	.361

PMI, postmortem interval.
¥Mann–Whitney *U* test.

a significant increase in the secondary pupil diameter was observed only at 15 hpm ($P = .028$) (Table 2). In the analysis of pupil diameter changes, no significant differences were found between the experimental and control groups at any PMI ($P > .05$) (Table 3). Additionally, no significant correlation was observed between the PMI and the amount of pupil diameter change in the experimental eyes ($P = .154$, $r = -0.201$).

The distribution of responses in the experimental eyes after pilocarpine application at different PMIs is shown in Figure 2. A positive response (Figure 3) was observed in all subjects at 0, 9, and 18 hpm, while paradoxical responses predominated at 3, 12, and 21 hpm. Among the 52 experimental eyes evaluated, 33 (63.5%) showed a positive response, 1 (1.9%) showed a negative response, and 18 (34.6%) exhibited a paradoxical response. Among the 53 control eyes evaluated, 25 (47.2%) exhibited miosis, 27 (50.9%) exhibited mydriasis, and no change in pupil diameter was observed in 1 (1.9%).

DISCUSSION

In this study, iris excitability, a supravital reaction, was evaluated at different PMIs using pilocarpine, a parasympathomimetic agent. Pilocarpine was selected due to its use in glaucoma treatment and its easy accessibility. For the sake of application ease, topical administration was preferred over anterior chamber injection. To the best of knowledge, this study is the first animal experiment to examine the relationship between iris excitability and PMI during the postmortem period.

There are conflicting results in the literature regarding the evaluation of iris excitability in postmortem cases using

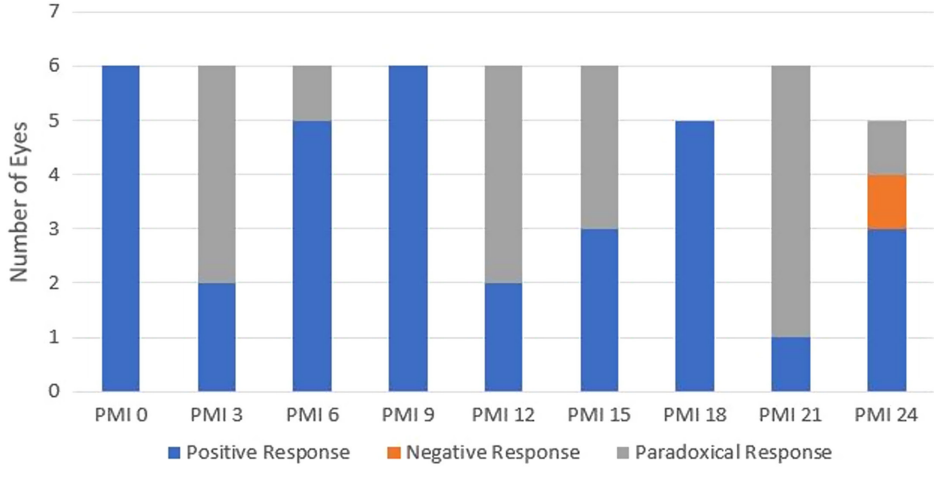

Figure 2. Pupil response types at different postmortem intervals (PMI) after pilocarpine application.

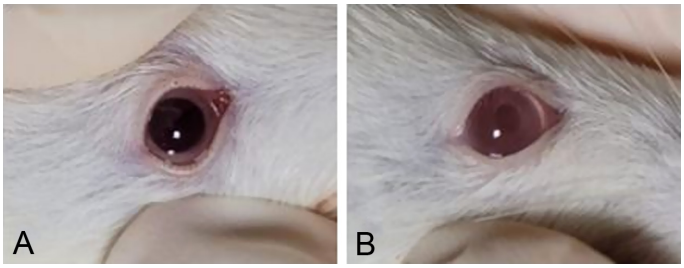

Figure 3. A positive response at 6 hpm following pilocarpine application in the rat eye. A. Pupil appearance before pilocarpine application. B. Pupil appearance after pilocarpine application.

pilocarpine. In the study by Larpkrajang et al.,[10] a correlation between pupil response and PMI was found in postmortem cases where pilocarpine was applied. They reported that the pupil changes observed after pilocarpine application could be used to estimate PMI through a regression equation. In contrast, Orrico et al.[6] reported observing a positive response to pilocarpine in 16.2% of 309 eyes and found no significant relationship between miosis and PMI. They emphasized that using pupil diameter measurement to estimate PMI could be misleading. In their study, Koehler et al.[7] investigated iris excitability in 137 individuals with a known time of death, using a control group. They reported that, similar to the experimental eyes, the eyes in the control group could exhibit positive, negative, or paradoxical responses. In this study, no correlation was found between PMI and the amount of pupil diameter change in standardized subjects. The observation of both increases and decreases in pupil diameter at different PMIs in both the experimental and control group eyes suggests that these changes in pupil diameter may be due to postmortem changes rather than the pharmacological effects of pilocarpine.

After pilocarpine application, a positive response was observed in 63.5% and a paradoxical response in 34.6% of the 52 experimental eyes. In the control group, the miosis response was close to 50%. The presence of PMIs with predominantly paradoxical responses starting from 3 hpm in the experimental group suggests that the distribution of responses was irregular. In contrast to the findings, some studies in the literature report a high positive response to pilocarpine during early postmortem hours.[10,13] The differing results in the studies may be related to differences in measurement methods or systemic variables in the included cases.

In this study, iris excitability was evaluated using pilocarpine, a parasympathomimetic agent. There are also studies in the literature that assess iris excitability using sympathomimetic agents. In a study by Englisch et al.[8] phenylephrine, a mydriatic agent, was applied to the eyes of 16 cadavers, and they reported a significant difference between baseline and post-medication pupil diameters. A limitation of the mentioned study is that the cadavers were stored at 13°C. The fact that the cadavers were not kept under room temperature conditions may make it challenging to generalize the study's findings to broader crime-related situations. In this study, an environment closest to a real crime scene was attempted to be created by keeping the experimental animals under room climate conditions (23 ± 1°C and 35%-40% humidity).

This study has some limitations. One of these limitations is the use of one eye as the experimental eye and the other as the control in the same subject. However, we chose this approach because, during the postmortem period, systemic circulation and the nervous system are not functional, and the pupil response is a local supravital muscle reaction. Therefore, the theoretical possibility of medication applied to one eye affecting the other is questionable. Additionally, the use of eyes initially treated with pilocarpine as controls after 15 hours, as well as the small sample size, are also limitations of the study. Future studies with larger sample sizes are needed. Another limitation of this study is that it was conducted under room conditions, which restricts the generalizability of the findings to different environmental conditions.

In conclusion, no correlation was found between PMI and the amount of pupil diameter change in rat eyes, and the distribution of positive, negative, and paradoxical responses after pilocarpine application was irregular. The results suggest that using topical pilocarpine for PMI estimation may be misleading. However, further research on this topic is needed to strengthen the evidence.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

Ethics Committee Approval: Ethics committee approval was received for this study from the Kastamonu University Animal Experiments Local Ethics Committee (Date: 12.11.2024; Number:32).

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

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N.S.; Materials – B.Ş.D.E., P.G., S.K., U.A., İ.Ç., N.S.; Data Collection and/or Processing – B.Ş.D.E., P.G.; Analysis and/or Interpretation – B.Ş.D.E., P.G., İ.Ç., N.S.; Literature Search – B.Ş.D.E., S.K.; Writing Manuscript – B.Ş.D.E., S.K.; Critical Review – B.Ş.D.E., S.K., U.A.

Declaration of Interests: The authors have no conflict of interest to declare.

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