Research Articles

Quantitative evaluation of cellular intensity in cytologic staining over difference time period of post air-dried smear in canine mammary gland tumor

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Abstract

Diagnostic cytology is an initial laboratory testing to diagnose various types of cancer, infections and inflammatory diseases. The collection and processing of cytological specimens are very important to produce good quality specimens for accurate and reliable cytological interpretations. In this study, 208 cytologic specimens were obtained from 26 dogs that have diagnosed of canine mammary gland tumors. The quantitative of cellular intensity in staining quality was evaluated in varying interval time of post air-dried smear by usage of light microscope and computer software. The results showed that the intensity of cells, nucleus, cytoplasm, and the percentage of neoplastic cells with distinct cell boundaries, which were parameters indicating staining quality, were statistical significant at all period of post air-dried smear when compared to specimens immediately stained ($p<0.05$). In conclusion, the results were demonstrated that any delay in staining post air-dried smears affected the cellular intensity and consequence adversely affected the quality of cytologic specimens.

Keywords: Quantitative evaluation, cytologic staining, cellular intensity, canine mammary gland tumor

การประเมินเชิงปริมาณค่าความเข้มของเซลล์ในการย้อมสีทางเซลล์วิทยา ในระยะเวลาที่แตกต่างกัน หลังการสเมียร์และทำให้แห้ง ในเนื้องอกเต้านมสุนัข

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บทคัดย่อ

การวินิจฉัยทางเซลล์วิทยาเป็นการตรวจวินิจฉัยทางห้องปฏิบัติการขั้นต้น ซึ่งใช้ในการวินิจฉัยมะเร็ง โรคติดเชื้อ และการอักเสบ ขั้นตอนการเก็บและเตรียมตัวอย่างเพื่อส่งตรวจมีความสำคัญมาก เนื่องจากขั้นตอนดังกล่าวจะส่งผลต่อคุณภาพตัวอย่างและส่งผลต่อความถูกต้องแม่นยำในการวินิจฉัยทางเซลล์วิทยา ในการศึกษาครั้งนี้ตัวอย่างส่งตรวจทางเซลล์วิทยาจำนวน 208 ตัวอย่างที่เก็บจากก้อนเนื้อบริเวณเต้านมของสุนัขจำนวน 26 ตัวที่เข้ารับการผ่าตัดเนื้องอกเต้านม ณ โรงพยาบาลสัตว์ประศุอาทร คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล ได้ถูกนำมาศึกษาการประเมินคุณภาพเชิงปริมาณค่าความเข้มของเซลล์ในการติดสีหลังการสเมียร์และทำให้แห้งด้วยอากาศ ในระยะเวลาที่แตกต่างกันโดยใช้กล้องจุลทรรศน์ร่วมกับโปรแกรมคอมพิวเตอร์ในการตรวจวัดค่าความเข้มของเซลล์ นิวเคลียส ไซโตพลาสซึมและร้อยละของจำนวนเซลล์มะเร็งที่เห็นขอบเขตเซลล์ชัดเจน ซึ่งค่าดังกล่าวเป็นตัวบ่งชี้คุณภาพการย้อมสีของตัวอย่างที่ส่งตรวจทางเซลล์วิทยา โดยผลการทดลองพบว่าค่าความเข้มของเซลล์นิวเคลียส ไซโตพลาสซึมและร้อยละของจำนวนเซลล์มะเร็งที่เห็นขอบเขตเซลล์ได้ชัดเจนมีความแตกต่างอย่างมีนัยสำคัญทางสถิติในทุกช่วงเวลาหลังการสเมียร์และทำให้แห้งด้วยอากาศ เมื่อเทียบกับตัวอย่างที่ทำการเก็บและย้อมสีตรวจทันที ผลการศึกษาแสดงให้เห็นว่าการส่งตรวจตัวอย่างที่ล่าช้าหลังจากสเมียร์และทำให้แห้งด้วยอากาศส่งผลต่อค่าความเข้มของเซลล์และคุณภาพตัวอย่างในการย้อมสีทางเซลล์วิทยา

คำสำคัญ : การประเมินเชิงปริมาณ การย้อมสีทางเซลล์วิทยา ความเข้มของเซลล์ เนื้องอกเต้านมสุนัข

Introduction

Mammary gland tumor is the most common tumor of reproductive system in female dogs (Zatloukal et al., 2005). Tumors can be classified into 2 types as benign or malignant and are generally recorded in dogs aged between 6-10 years. There are many causes of tumors, especially from imbalances of the hormones estrogen and progesterone produced by the ovaries (Toniti et al. 2009). The growth rate of tumors varies for different types and according to the level of hormonal changes (Allen et al. 1986), while neoplastic cells can metastasize through lymphatic vessels to superficial inguinal lymph nodes, axillary lymph nodes and even distant organs such as lungs. Presently, surgery is the common treatment method for mammary gland tumors through a cytological examination to screen for neoplasm and identify abnormalities (Clarke et al. 2001).

Cytology is an essential diagnostic test in veterinary practices; often used to diagnose cancer and identify abnormalities in any organs (Patel et al. 2012). Cytology can be performed immediately; it is inexpensive, offering less discomfort, minimal invasiveness and lowered risks to animals (Jain 2013). Cytological examination offers several advantages for disease diagnosis through clear identification of cellular morphology to differentiate between inflammatory lesions and tumors, while also revealing whether the etiologic agents that caused the lesions are bacteria, fungi or parasites. Lesions can be evaluated by cytology after collection from tumors, tumor-like lesions, inflammatory lesions and body fluids including sputum, urine, cerebrospinal fluid, pericardial, pleural and ascetic fluid by different methods involving fine needle aspiration, brushing and scraping. In veterinary practice, cytology is not only useful for screening of cancers and specific lesions but also provides essential data for precise diagnostic and prognostic evaluation and appropriate therapeutic planning (Latimer 2011).

Apart from these values, cytology has certain limitations because many factors affect specimen quality. Poor sample collection can result in inadequate amounts of specimen and poor specimen preparation, while inadequate smear and fixation methods are common problems which often cause adjustments of interpretation (Divani et al. 2009). Among these, fixation is an important step for cell preservation and should be performed immediately following sample collection to improve specimen quality. However, whether increased fixation time has any effect on the staining quality of cytologic specimens has not been prospectively evaluated (Jackson et al. 2013). Principle fixation methods include wet-fixed and air-dried. Air-dried smears are favored by clinicians before submitting specimens to the laboratory. Air drying is a simple, feasible and reliable fixation method, comparable to the wet-fixed conventional technique (Rupinder et al. 2013) and it is not necessary to fix the cytologic preparation after air drying (Rosenfeld and Dial 2011). Additionally, air-dried cells are flattened on the glass surface and thus appear larger; whereas, wet-fixed nuclei maintain their spherical form (Schulte 1986). One advantage of larger air-dried nuclei is the increase in anisokaryosis which improves sensitivity for cancer detection (Yang 1994). Romanowsky stains including Giemsa, Wright's and Wright's-Giemsa have long been championed in veterinary cytopathology staining (Jörundsson et al. 1999). For routine cytological diagnoses, air-dried slides submitted by clinicians are immediately stained and sent to the pathologist to make a diagnosis. However, delays in air-dried specimen submissions often occur, especially over long holidays when laboratory services are not operational.

No previous studies have been described about quantitative evaluation of cellular intensity in cytologic staining quality relevant the effect of time delay by varying post air-dried smear time points before staining on the quality of cytologic specimens collected from canine mammary gland tumors. Results will be advantageous for improvement of cytological staining techniques in the future.

Materials and Methods

Sample size

A total number of 208 cytologic specimens were collected from 26 female dogs of varying age, sex and breed diagnosed with mammary gland tumors at Prasu-Arthorn Animal Hospital, Faculty of Veterinary Science, Mahidol University. Specimens were obtained by surgical removal of mass from the mammary gland using fine needle aspiration cytology (FNAC). Statistical significance was defined at 95% confidence interval ($p<0.05$).

Collection technique and specimen preparation

Areas for aspiration were chosen with no lesions of necrosis, hemorrhage or suppuration. A 21-gauge needle attached to a syringe was pierced into the surgical mass at 3-5 times, moving in fan-shape patterns radiating from its access site, and negative pressure inside the syringe was used to suck in the cells from the mass. After removing the needle from the tissue, specimens were spread and smeared on glass slides using the squash technique. The specimens were preserved by gently waving the slides in air until they were dried. Each specimen was kept in two separate slide boxes containing desiccants. For each tumor, cytologic specimens were collected for 8 slides; the first slide was immediately stained, regarded as day 0, and the remaining slides were stained on days 1, 2, 3, 4, 5, 6 and 7 following specimen collection and preservation.

Cytological staining technique

The modified Wright-Giemsa stain was used to stain the cytologic specimens. Optimal conditions were set to clarify the efficacy of the dye which was freshly prepared and filtered before use. After air drying, no other fixative was recommended since methanol was present in the dye to fix the cells. Optimal quality was determined for horizontal staining with modified Wright-Giemsa stain for 4 minutes. The slides were then dipped in phosphate buffer, pH 6.8-7.2 for 1 minute and washed gently with distilled water before drying and cover slip mounting in medium for microscopic examination.

Evaluation of staining quality

All the specimens were stained with modified Wright-Giemsa stain and 30 epithelial neoplastic cells per slide were examined under light microscope in areas of good cellular distribution, no cell distortion, and cells lying in a monolayer. The cells were observed at 1,000 magnification to increase cellular detail and accuracy of cellular intensity. Quantitative assessment of cellular staining was evaluated using the software 'NIS-Elements D (Documentation)' base on parameters including the intensity of whole cells, nuclei and cytoplasm, and the percentage of cells with distinct cell boundaries. For each specimen set, measurement were performed on days 0, 1, 2, 3, 4, 5, 6 and 7.

Computerized measurement of intensity

To determine the intensity of interested parameters, the software, 'NIS-Elements D (Documentation)' was desired to use. The intensity of nucleus, cytoplasm and the whole cell were examined. The areas of interested were selected, measured in triplicate and calculated to give average values.

Determination of distinct neoplastic cellular border

Neoplastic cells with distinct cellular borders were counted in five areas on each slide under light microscope at high magnification (100x).

Statistical analysis

SPSS version 21.0 statistical software was used with a 95% confidence level. Analysis of variance (ANOVA) was employed to compare differences in categorical variables between the groups.

Results

A quantitative evaluation of staining quality of the cytologic samples was conducted. Parameters were tested at varying time points prior to cytological staining on days 0, 1, 2, 3, 4, 5, 6 and 7 using computer software 'NIS-Elements D (Documentation)'. Parameters representing quantitative staining quality included the intensities of entire cells, nuclei and cytoplasm. Percentages of cells with distinct cell boundaries were calculated by Excel and expressed as geometric mean ± standard deviation (SD).

Mean values of cell intensity showed statistical significance ($p<0.05$) on days 1, 2, 3, 4, 5, 6 and 7 compared to mean intensity of cells on day 0. Means ± SD were 181.43 ± 0.53, 187.07 ± 0.73, 193.00 ± 0.61, 199.43 ± 0.78, 205.26 ± 0.89, 211.13 ± 0.79, 216.49 ± 0.84 and 220.72 ± 0.82 units, respectively and increased over time. Results are shown in Table 1 and Figure 1.

Significant differences of nucleus intensity ($p<0.05$) were found on days 1, 2, 3, 4, 5, 6 and 7 compared to day 0, with values showing an increasing trend over time from day 0 to day 7, respectively. Means ± SD of nucleus intensity were 179.77 ± 0.59, 185.45 ± 0.81, 191.52 ± 0.68, 198.06 ± 0.87, 203.96 ± 0.98, 209.95 ± 0.88, 215.39 ± 0.92 and 219.48 ± 0.93 units, respectively. Results are shown in Table 1 and Figure 1.

Intensity of the cytoplasm was statistically significant on days 1, 2, 3, 4, 5, 6 and 7 as compared to day 0 ($p<0.05$). Values showed an increasing trend over time at 196.37 ± 0.15, 201.63 ? 0.30, 206.33 ± 0.21, 211.79 ± 0.34, 216.93 ± 0.22, 221.71 ± 0.34, 226.38 ± 0.17 and 231.83 ± 0.54 units, respectively. Results are shown in Table 1 and Figure 1.

Percentages of neoplastic cells with distinct cell boundaries, presented as mean ± SD, were 96.27 ± 1.35, 95.00 ± 1.26, 94.29 ± 1.62, 93.53 ± 1.86, 92.47 ± 2.00, 91.92 ± 2.18, 90.93 ± 2.45 and 89.31 ± 2.07 units, respectively. Statistically significant differences were observed on days 1, 2, 3, 4, 5, 6 and 7 and compared to day 0 ($p<0.05$). Values were found to be inversely proportional to time. Results are shown in Table 1 and Figure 2.

Over time, photographs of neoplastic cells showed continued color fade in the entire cell, nucleus and cytoplasm; similarly, contrast between the cellular border and background decreased. Comparisons of staining quality, captured and assessed by computer software over the period of 7 days, are shown in Figure 3.

Table 1. The intensity of cell, nucleus and cytoplasm and percentage of neoplastic cells with distinct cell boundaries, presented as mean ± SD are shown over the seven-day period

*Statistically significant at $P < 0.05$

Parameter (unit)	Post air-dried smear (Day)							
	0	1	2	3	4	5	6	7
Cellularity intensity	181.43 ± 0.53	187.07 ± 0.73*	193.00 ± 0.61*	199.43 ± 0.78*	205.26 ± 0.89*	211.13 ± 0.79*	216.49 ± 0.84*	220.72 ± 0.82*
Nuclear intensity	179.77 ± 0.59	185.45 ± 0.81*	191.52 ± 0.68*	198.06 ± 0.87*	203.96 ± 0.98*	209.95 ± 0.88*	215.39 ± 0.92*	219.48 ± 0.93*
Cytoplasmic intensity	196.37 ± 0.15	201.63 ± 0.30*	206.33 ± 0.21*	211.79 ± 0.34*	216.93 ± 0.22*	221.71 ± 0.34*	226.38 ± 0.17*	231.83 ± 0.54*
% Cell with distinct boundary	96.27 ± 1.35	95.00 ± 1.26*	94.29 ± 1.62*	93.53 ± 1.86*	92.47 ± 2.00*	91.92 ± 2.18*	90.93 ± 2.45*	89.31 ± 2.07*

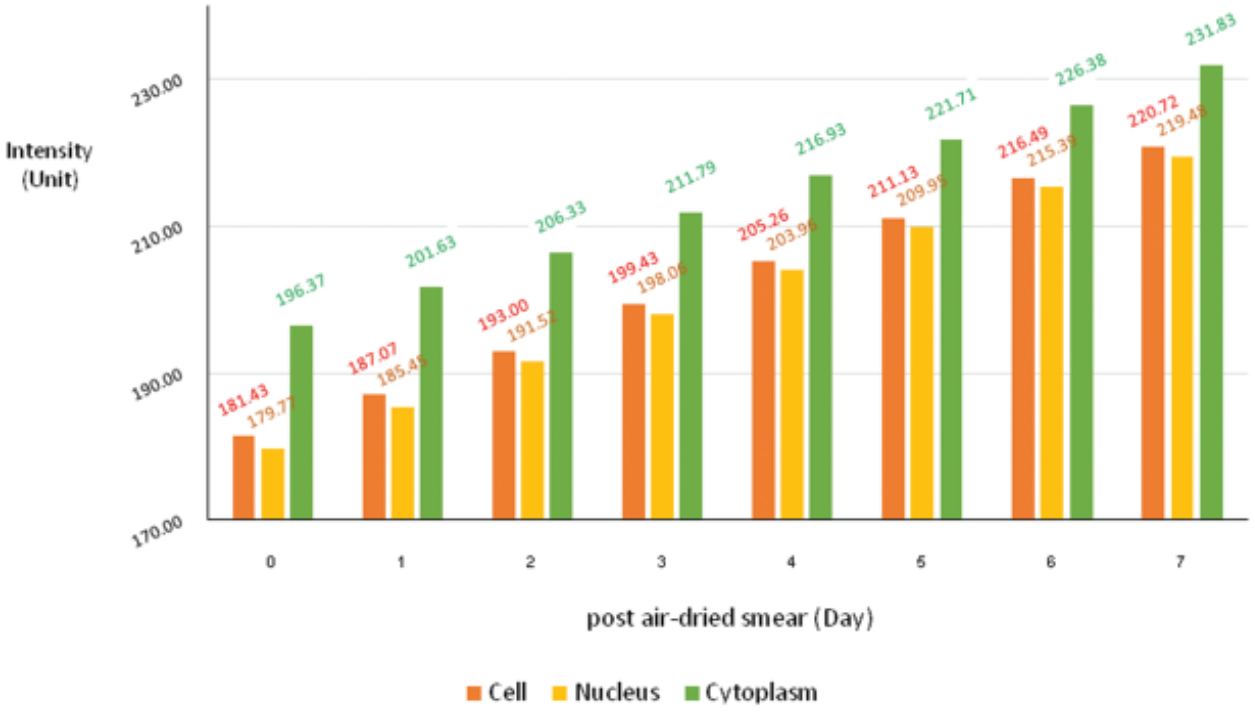

Figure 1. The mean value of intensity of cells, nucleus and cytoplasm of neoplastic cells over the seven-day period

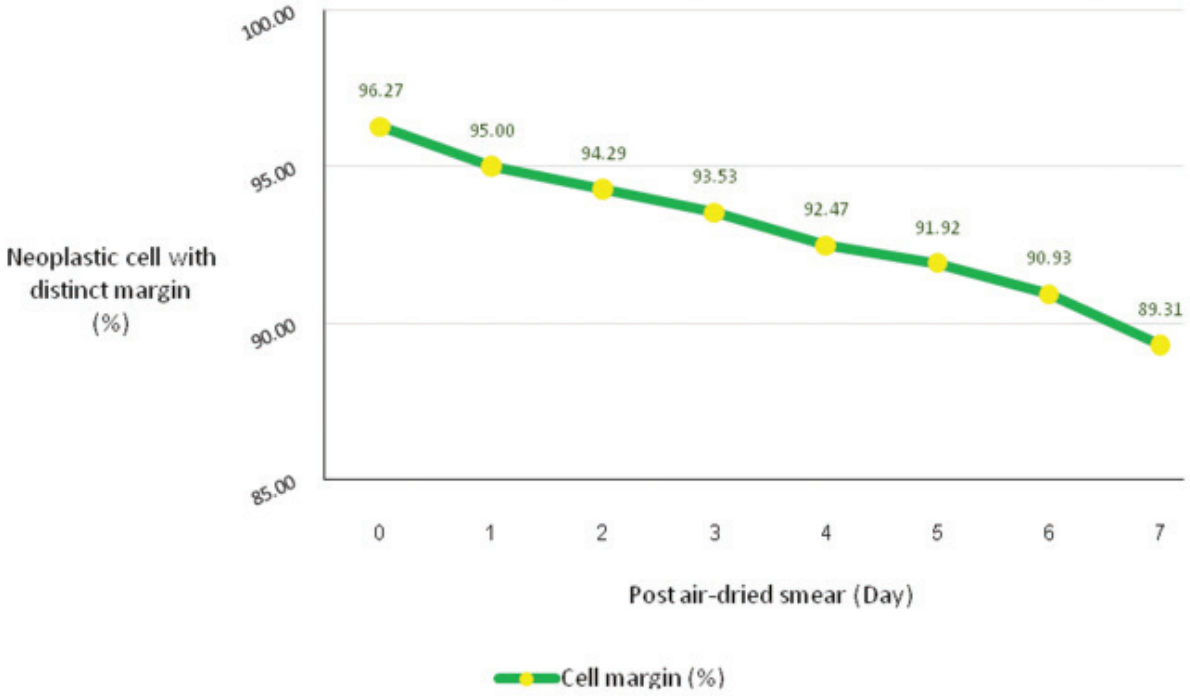

Figure 2. The mean percentage of neoplastic cells with distinct boundaries over the seven-day period.

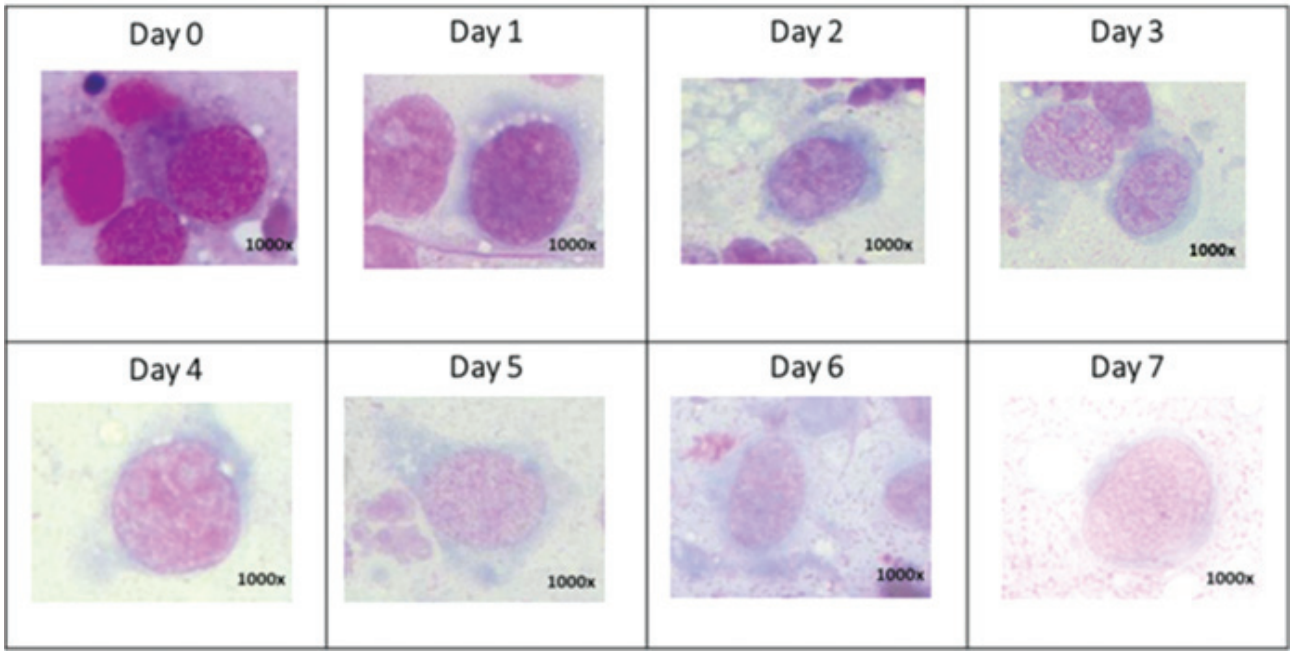

Figure 3. The figure shows the comparison of staining quality over the period of 7 days, the photography of neoplastic cells showed continue fade color by more time.

Discussion

Cytopathologists are concerned regarding cytological quality as this is crucial for correct diagnostic assessment. As mentioned above, many factors affect specimen quality including collection, handing and sample staining (Wiener et al. 2007). Poor specimen quality subsequently leads to false cytological interpretation and misdiagnosis of diseases. Previously, studies investigated the parameters affecting the quality of cytological diagnosis including cytoplasmic content, nuclear features, nuclear size, nuclear chromatin, cell size, cell uniformity, and cellular pleomorphism (Wani et al. 2010; Saha et al. 2013). Staining quality is an important aspect for the accurate interpretation of these parameters. Staining systems stain cells to allow high specificity and visibility influenced by microscopic resolution, contrast and operator skill (McGavin 2014). However, staining quality is sensitive to pH, temperature, buffer, dye concentration and fixation. Smears must be rapidly air-dried and should not be heated above 37°C in Romanowsky stain (Ng et al. 1994). Moreover, staining solutions and procedures differ in various stain modifications, resulting in variable shades of color (Stanley and Löwhagen 1993). The best staining provides clarity of cytoplasmic and nuclear cell morphological characteristics. To determine the diagnosis, cytopathologists need to evaluate whole cells, especially the nucleus and cytoplasm through good staining quality. Tumors, nuclear features, and cytoplasmic content are essential in evaluating malignancy. Accordingly, stains that optimize nuclear detail are preferably used in combination with those that emphasize cytoplasmic and extracellular information to differentiate cell origin (Grundsson et al. 1999) and increase confidence in the diagnosis.

In this experiment, parameters of cytological staining quality were evaluated by quantitative assessment of the intensity of cells, nuclei, cytoplasm, and percentage of neoplastic cells with distinct cell boundaries, by varying the interval time of post air-dried smear in canine mammary gland tumors. Results indicated that mean values of intensity of cells, cytoplasm and nuclei increased over time. Cell color continuously faded over time. A pale cell color requires higher intensity to produce a prime picture in the eye (Wisslar 2013). Thus, a higher light intensity was required to view cells which reduced in color over time.

Alternatively, the percentage of neoplastic cells with distinct cell margins was found to be inversely proportional to time. This suggested that contrast between cell color and background decreased over time and significantly reduced image clarity. Therefore, cellular margins became difficult to distinguish and, consequently, the percentage of cells with distinct margins decreased. Thus, delay in staining the specimen after air drying the smear resulted in color loss and low contrast of cells, nuclei, cytoplasm and cellular borders.

Our results showed that specimens which were stained immediately, or on day 0, had the best staining quality and cell preservation, with lowest intensity and highest percentage of distinct cell borders. These findings were consistent with previous authors who reported that delayed fixation and staining of cytologic urine specimens negatively impacted on cellular preservation (Ahmed et al. 2011). Findings also demonstrated that the quality of staining decreased from day 1 to day 7. Photographs of the neoplastic cells displayed continuous fading of color intensity and decline in the number of distinct cell borders from day 0 to day 7. Consequently, increased time of post air-dried

smears prior to staining had a detrimental effect on slide quality.

Other influencing factors that can affect the quality of cells in smear samples before staining are moisture and formalin. Formalin can alter cell staining quality. In addition to dampness, moisture condensation on the slide can cause cell lysis (Rosenfeld and Dial 2011). Moreover, if the specimen dries slowly, the liquid can become progressively hypertonic. This may cause poor cell preservation and result in artifacts due to insufficient dye uptake, lack of cell spreading (Ng et al. 1994) and consequent rupture of cell and nuclear membranes (Stanley and Löwhagen 1993). Furthermore, incompletely dried smears cultivate blue nuclear artifacts that may cause erroneous enlarged, dark, blue nuclei (Schulte 1986). Therefore, in our experiment, samples were kept in slide boxes containing desiccants to protect confounding variables from light, humidity and formalin.

Our results indicated that delays in staining post air-dried smear samples affected the cellular intensity and consequence adversely affected the quality of cytologic specimens. Therefore, specimens should be submitted immediately to the pathology laboratory to achieve optimal quality and promote accurate cytological diagnosis.

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Declaration of Conflicting Interests

The author (s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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