

Determination of Blood Age by Morphological Changes of Different White Blood Cells

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ABSTRACT

KEYWORDS

Age,
Blood,
White blood cells,
pH.

Determination of age of bloodstain is a crucial subject in forensic field. This study was done to demonstrate the morphological changes occurring in white blood cells with time pass and correlate them with changes of pH level of the in vitro sample. Blood film stained with Hematoxylin and Eosin and pH level determination was done at times (0, 6, 12, 24, 48 and 60 hours) for in vitro blood samples left at room temperature. Lymphocytes were the most resistant cells for morphological changes with time; they appeared normal after 12 hours. Changes were detected after 24 hours in the form of pyknosis and degeneration of nucleus. Morphological changes of other types of white blood cells appeared after 12 hours in the form of pyknotic swollen nucleus, degenerated nucleus and cytoplasmic and nuclear vacuolation. After 60 hours, the nature and type of the cells (except lymphocytes) could not be identified. There was change in pH level corresponding with the morphological changes of white blood cells. The pH level was significantly decreased with pass of time. At 60 hours, pH level was (6.167±0.03) compared to its level (7.273±0.021) at zero time. It can be concluded that: The morphology of white blood cells and pH level of in vitro blood sample could give an indicator for the age of the blood

Introduction

It is an important issue for forensic pathologist to determine the time passed since death either for the body as a whole or for some of its tissues and components. Blood has advantage from forensic perspective that it is unaffected by factors such as age, gender, diet, diurnal cycles and stress, so it is ideal for postmortem interval (PMI) determinations. Estimation of the age of a bloodstain can be particularly useful when bloodstains are the

only piece of evidence available (Donaldson and Lamont, 2013 a).

Many methods have been used for determination of the age of bloodstains. Physiological techniques, changes in enzyme activities and the surface absorption spectrum of bloodstains have been utilized (Fujita et al., 2005).

Blood is a fluid that normally consists of 45% erythrocytes, 54% plasma and 1% leukocytes and platelets. Numerous cells in blood shows varying degree of postmortem changes and these occurring cellular changes could be utilized to estimate death interval (Marieb et al., 1998).

White blood cells (WBCs) are divided into five main types, due to their difference in

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structure: neutrophils (60-70%), lymphocytes (20-25%), monocytes (5%), eosinophils (2-4%) and basophils (0.5%) (Kumar et al., 2014). Irreversible changes that occur in the WBCs in the internal environment may be due to non-availability of oxygen, accumulation of carbon dioxide, pH change and accumulation of toxic products (Bardale and Dixit, 2007).

Blood pH ranges from 7.35 to 7.45, and it is one of the most regulated systems in the body. Acid-base buffers such as carbonic acid and bicarbonate ion regulate blood pH through the respiratory system and the kidneys. After death, the body buffering system is not maintained and blood pH changes can occur (Cotran et al., 1994).

The aim of the study was to monitor in vitro morphological changes of white blood cells that occur with time from (0-60 hours), as indicators for age of the blood sample, and to correlate these changes with blood pH levels in relation to time pass.

Material And Methods

Morphological study

Ten milliliters (10 ml) blood was withdrawn from the cubital vein of 20 apparently healthy human volunteers after consent using wide bore plastic syringe. Samples were put immediately in glass tubes containing 0.2 ml of 10% ethylene diamine tetra acetic acid (EDTA) solution as anticoagulant (Bardale et al., 2010). Exclusion criteria's were: known blood diseases or general diseases (hypertension, diabetes or infection) and history of previous blood transfusion. The experiment took place in March with atmospheric temperature varied from 26°C at day to 12°C at night.

Samples were left in room temperature. At times (0, 6, 12, 24, 48 and 60 hours), a drop

of blood from each sample was spread on a glass slide and left to dry then stained with Hematoxylin and Eosin (H&E) for light microscope examination. The nuclear and cytoplasmic morphological changes in white blood cells i.e. neutrophils; lymphocytes, monocytes and eosinophils had been recorded in Histology Department, Faculty of Medicine, Assiut University.

pH level estimation

At times (0, 6, 12, 24, 48 and 60 hours), mixing of the sample was done and 1ml of the sample was taken and put into a clean tube, centrifuged at 2500 round per minute (rpm) for 15 minutes. Plasma pH level was measured by pH meter (Analyticon Biotechnologies AGD-35104 Lichtenfeis, made in Germany). pH meter calibrated each time of use with pH 4.0, 7.0, and 10.0 calibrators.

Ethical considerations

This research was approved by The Research Ethics Committee of the Faculty of Medicine-Assiut University (Ref code: 17300062). Informed consent was taken from all individuals who participated in the study. Confidentiality of the data was guaranteed.

Statistical Analysis:

All analyses were performed with the IBM Statistical Package for Social Sciences (SPSS) program version 20.0 software. One way ANOVA test was used to determine whether there are any statistically significant differences between the means of pH levels at different time intervals. P value < 0.05 was considered significant.

Results

Morphological changes of white blood cells

Due to the low percent of basophils (0.5% of total white blood cells), their detection in blood film was rare so, the study of their morphological changes with time was difficult. After 60 hours the nature and type of the cells (except lymphocytes) could not be identified, so further estimation was meaningless.

Neutrophils: they constitute (60-70%) of the total white blood cells. At zero time, mature neutrophils had segmented nucleus with 2 to 5 lobes joined by a thin filament. The cytoplasm of a mature neutrophil was pink or nearly colorless and possesses moderate granules (Figure 1). At 6 hours, the cells appeared similar to zero time but with darker stained nucleus (Figure 2). At 12 hours and at 24 hours, the cells appeared with pyknotic nucleus (irreversible condensation of chromatin in the nucleus of a cell) (Figure 3) and pyknotic irregular shape nucleus (Figure 4), respectively. At 48 hours, the cells appeared with irregular degenerated nucleus (Figure 5). At 60 hours, the cells appeared with irregular swollen nucleus (Figure 6).

Lymphocytes: they constitute (20-25%) of total white blood cells. At zero time, mature lymphocytes had spherical nucleus, and scanty

pale blue cytoplasm (Figure 1). At 6 hours (Figure 2) and at 12 hours (Figure 3), the cells appeared normal. At 24 hours (Figure 4), at 48 hours (Figure 5) and at 60 hours (Figure 6), the cells appeared with pyknotic irregular shaped nucleus.

Monocytes: they constitute (5%) of total white blood cells. At zero time, mature monocytes had kidney-shaped nucleus (Figure 1). At 6 hours, the cells appeared similar to zero time but with darker stained nucleus (Figure 2). At 12 hours, the cells appeared with cytoplasmic and nuclear vacuolation (Figure 3). At 24 hours, the cells appeared with degenerated nucleus (Figure 4). At 48 hours, the cells appeared with irregular degenerated nucleus (Figure 5). At 60 hours, the cells appeared degenerated with pyknotic irregular shaped nucleus (Figure 6).

Eosinophils: they constitute (2-4%) of total white blood cells. At zero time, mature eosinophils appeared with bilobed nucleus and coarse cytoplasmic granules (Figure 1). At 6 hours, the cells appeared similar to zero time but with darker stained nucleus (Figure 2). At 12 hours, the cells appeared with eccentric nucleus (Figure 3). At 24 hours, the cells appeared with swollen vacuolated eccentric nucleus (Figure 4). At 48 hours, the cells appeared with nuclear degeneration (Figure 5). At 60 hours, the cells appeared degenerated with eccentric pyknotic nucleus (Figure 6).

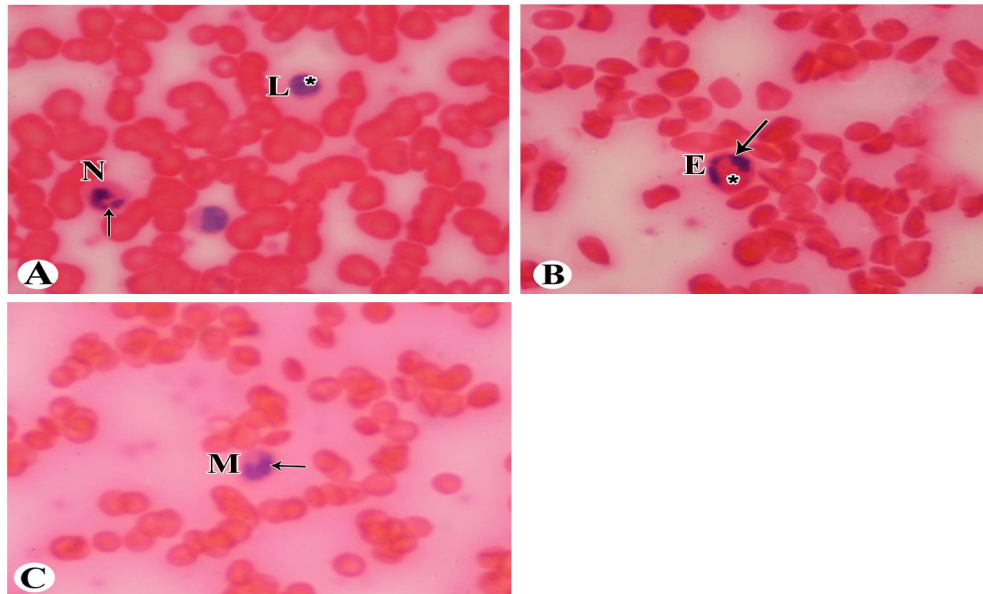


Fig. (1): A photomicrograph of peripheral blood films showing normal morphological appearance of white blood cells at 0 hour; (A) Neutrophil (N) appear with multilobal nucleus (arrow) and lymphocyte (L) with spherical nucleus (*) (B) Eosinophil (E) with bilobed nucleus (arrow) and coarse cytoplasmic granules (*) (C) Monocyte (M) with kidney-shaped nucleus (arrow) (H&E x1000).

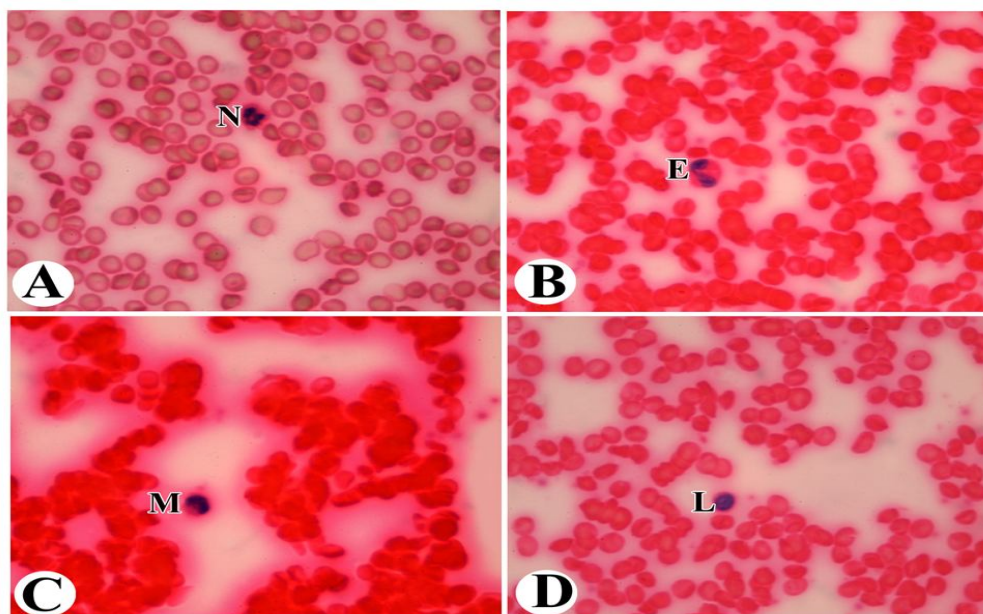


Fig. (2): A photomicrograph of peripheral blood films showing morphological changes of white blood cells at 6 hours; (A) Neutrophil (N) (B) Eosinophil (E) (C) Monocyte (M) (D) Lymphocyte (L). The cells appeared similar to zero time but with darker stained nucleus (H&E X 1000).

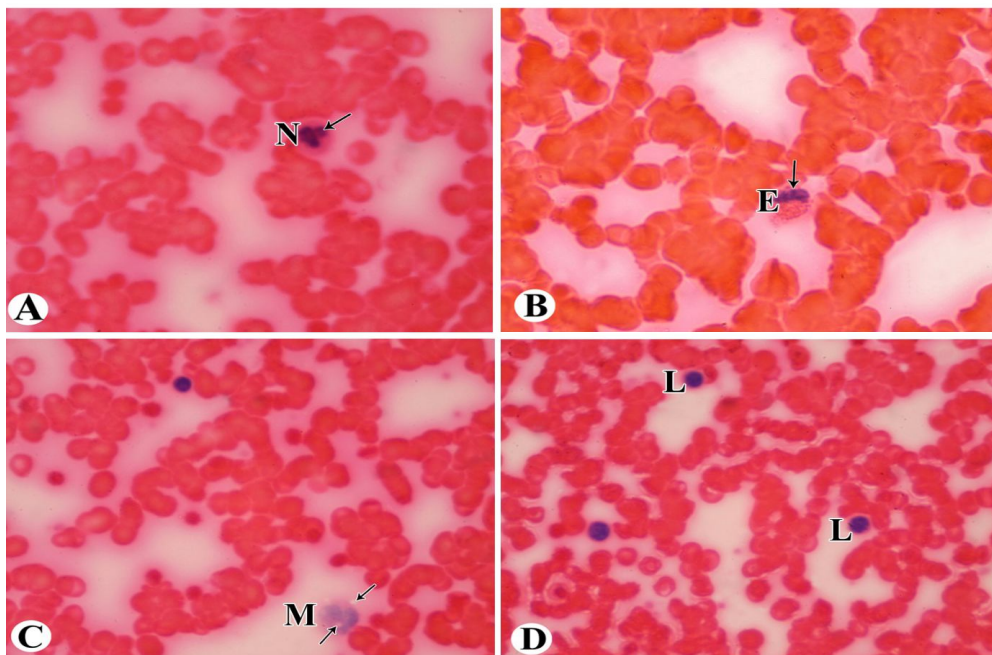


Fig. (3): A photomicrograph of peripheral blood films showing morphological changes of white blood cells at 12 hours; (A) Neutrophil (N) with pyknotic nucleus (arrow) (B) Eosinophil (E) with eccentric nucleus (arrow) (C) Monocyte (M) with cytoplasmic and nuclear vacuolation (arrow) (D) Lymphocytes (L) with normal appearance (H&E x1000) .

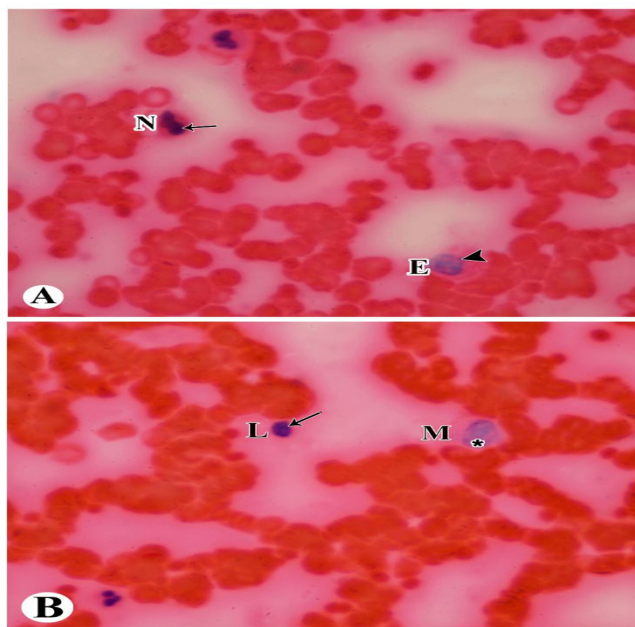


Fig. (4): A photomicrograph of peripheral blood films showing morphological changes of white blood cells at 24 hours; (A) Neutrophil (N) with pyknotic irregular shaped nucleus (arrow) and Eosinophil (E) with swollen vacuolated eccentric nucleus (▲) (B) Monocyte (M) with degenerated nucleus (*) and lymphocyte (L) with irregular pyknotic nucleus (arrow) (H&E X 1000).

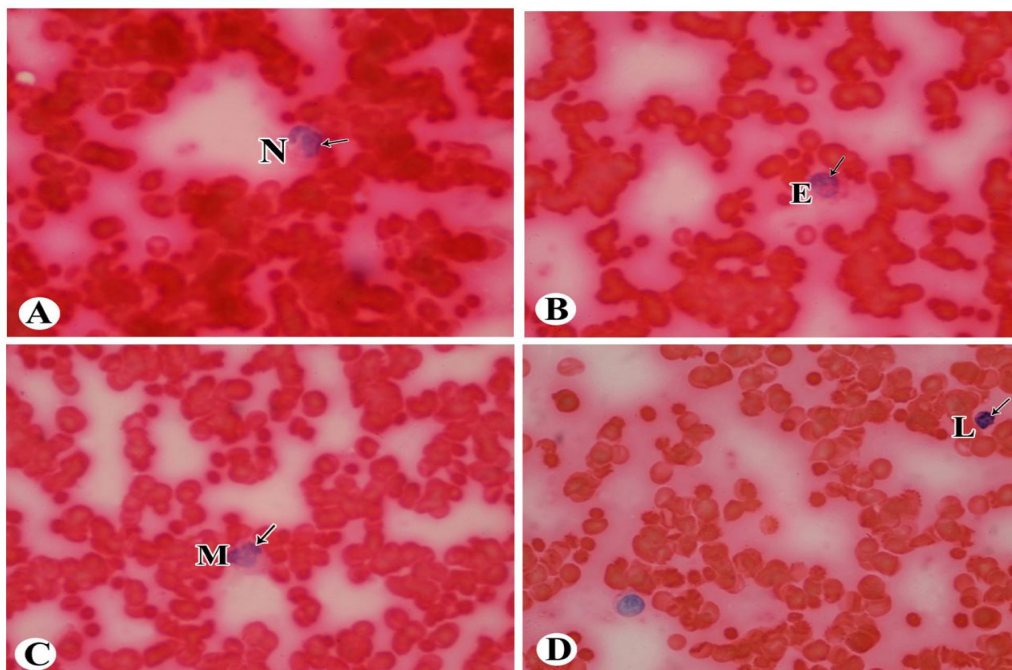


Fig. (5): A photomicrograph of peripheral blood films showing morphological changes of white blood cells at 48 hours; (A) Neutrophil (N) with irregular degenerated nucleus (arrow) (B) Eosinophil (E) with nuclear degeneration (arrow) (C) Monocyte (M) with irregular degenerated nucleus (arrow) (D) Lymphocyte (L) with irregular pyknotic nucleus (arrow) (H&E X 1000).

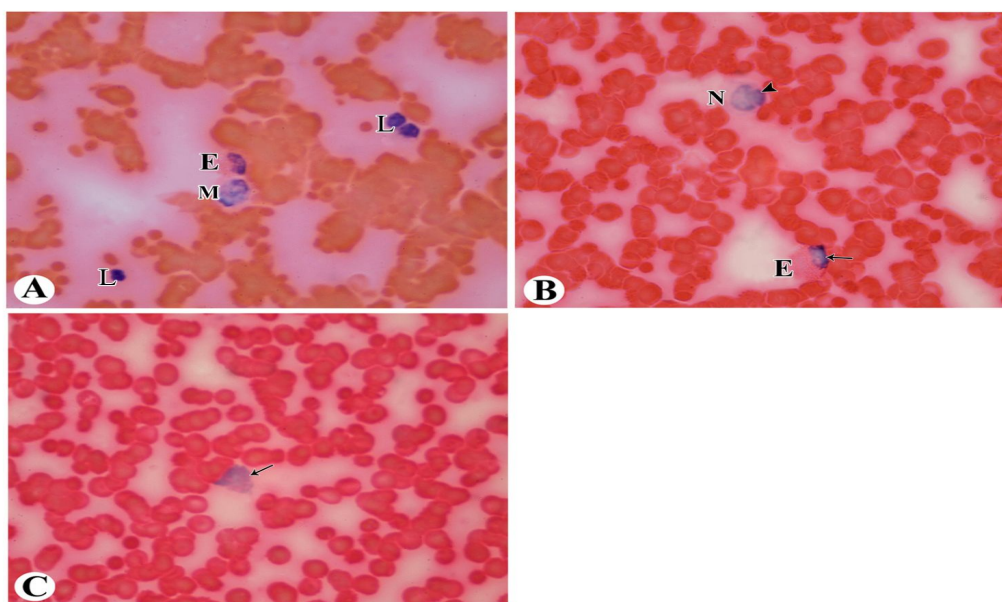


Fig. (6): A photomicrograph of peripheral blood films showing morphological changes of white blood cells at 60 hours; (A) Degenerated monocyte (M), eosinophil (E) and lymphocytes (L) with irregular pyknotic nuclei (B) eosinophil (E) with eccentric pyknotic nucleus (arrow) and neutrophil (N) with irregular swollen nucleus (▲) (C) Degenerated leukocyte (arrow) (H&E X 1000).

pH level estimation:

The pH level for samples at different time intervals was measured and results were expressed in mean ± SD as shown in table (1) and figure (7). There was significant difference ($p < 0.05$) between pH level at zero time (7.273 ± 0.021) and pH level at 6 hours (7 ± 0.057). There was significant difference

($p < 0.01$) between pH at zero time and pH at 12 hours (6.9 ± 1). There was highly significant difference ($p < 0.001$) between pH level at zero time and its level at 24 hours (6.85 ± 0.03), at 48 hours (6.35 ± 0.02) and at 60 hours (6.167 ± 0.03) respectively as shown in table (2).

Table (1): pH levels (presented as mean ± SD) at different time intervals

Time (Hours)	At zero time	At 6 h	At 12 h	At 24 h	At 48 h	At 60 h
pH	7.273 ± 0.021	7 ± 0.057	6.9 ± 1	6.85 ± 0.03	6.35 ± 0.02	6.167 ± 0.03

SD: Standard Deviation

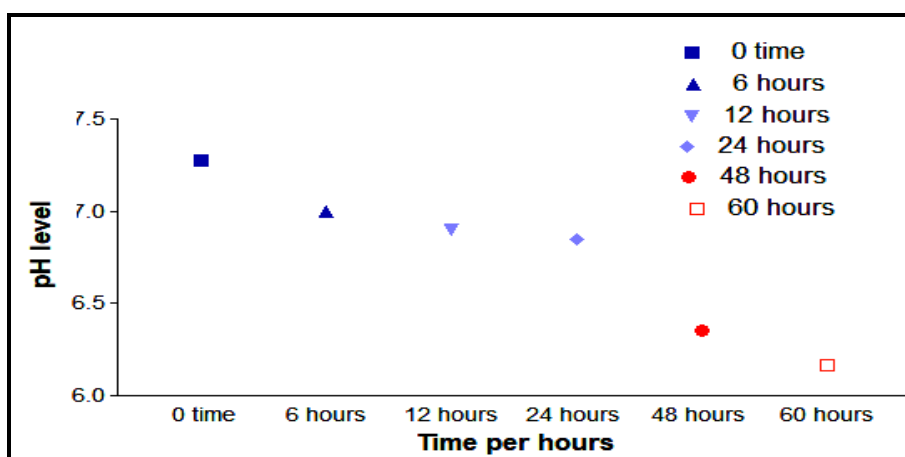


Fig. (7): pH levels (presented as mean ± SD) at different time intervals

Table (2): Comparison between the means of pH levels at different time intervals.

	At zero time	At 6 h	At 12 h	At 24 h	At 48 h
At 6 h	$p = 0.0114^*$				
At 12 h	$p = 0.0038^*$	$p = 0.2879$			
At 24 h	$p = 0.0003^*$	$p = 0.0808$	$p = 0.4818$		
At 48 h	$p < 0.0001^*$	$p = 0.0005^*$	$p = 0.001^*$	$p = 0.0003^*$	
At 60 h	$p < 0.0001^*$	$p = 0.0002^*$	$p = 0.0004^*$	$p = 0.0001^*$	$p = 0.0142^*$

* Significant.

Discussion

As other tissue cells, blood cells lose their normal morphology as a result of postmortem autolysis and putrefaction until they are unidentifiable. The changing process through normal morphology to unidentification period can be a useful criterion for estimating postmortem interval (Henssge and Madea, 2004).

It is recommended to use untreated whole blood to study blood samples, *in vitro*, for simulation of blood at crime scenes as realistic as possible. But, without adding anticoagulant blood will clot within 30 seconds, so anticoagulants are used to prevent early clotting. Many anticoagulants exist for preventing *in vitro* blood coagulation, including heparin, citrate, and EDTA. However, no evidence is reported in the literature for the influencing of the anticoagulants on the processes associated with ageing of bloodstains (Bremmer et al., 2012).

In the current study, the morphological changes of different white blood cells that occur with time pass from (0-60 hours), as indicators for age of *in vitro* blood sample, were studied using light microscope after staining with H&E. The results showed that lymphocytes were the most resistant cell for morphological changes with time. They appeared normal after 12 hours and changes began to be obvious after 24 hours in the form of pyknotic degenerated irregular shaped nucleus.

In agreement of the present study the results of (Penttilä and Laiho, 1981) who reported that lymphocytes were more resistant to autolysis than the other types of white blood cells. But they stated that normal stained lymphocytes were seen in up to 270 hours postmortem.

Also, in agreement with this, Dokgöz et al. (2001) detected similar morphological changes of white blood cells, on their examination of blood smears from blood samples taken from non-refrigerated cadavers (for 21 hours postmortem) and hospital patients (for 120 hours total storage time). They reported that the degenerative changes of lymphocytes started at 24 hours in both groups. Also, their results confirmed that anticoagulants have no influence on the processes associated with ageing of white blood cells of bloodstains. In the current study, morphological changes of neutrophils, monocytes and eosinophils appeared after 12 hours in the form of pyknotic nucleus, irregular swollen nucleus, degenerated nucleus, as well as, cytoplasmic and nuclear vacuolation.

Also Dokgöz et al. (2001) noticed the same changes in the form of pyknosis, cytoplasmic and nuclear vacuolation, and degeneration of nucleus but started at 6 hours.

In the present study, the nature and type of the cells (except lymphocytes) could not be identified after 60 hours. In controversy to these results, Dokgöz et al. (2001) reported that degenerated neutrophils were unidentifiable beyond 96 h *in vitro*, while eosinophils and monocytes were unidentifiable beyond 72 h after *in vitro* storage. Lymphocytes were still identifiable beyond 120 h and later. This longer period than the present study may be explained by other factors mainly the room temperature (24 °C) and type of stain (May-Grunwald stain, followed by Giemsa stain) used by them.

Also, in disagreement with the present results, Bardale and Dixit (2007) reported that degenerative changes were early identified in monocytes and eosinophils (4-6 hours), intermediate in neutrophils (5-15 hours) and late in lymphocytes (25-27 hours). The morphology of any cell beyond 30-hour PMI was not identifiable.

As well as, Babapulle and Jayasudera (1993), noticed that identifiable eosinophils and monocytes were first to 'disappear' (by 60 hours), followed by neutrophils (by 66 hours), and finally identifiable lymphocytes disappeared completely at or around 84 hours from the time of death.

Laiho and Penttilä (1981) reported that well-preserved neutrophils and monocytes were seen in the peripheral blood after over 200 hours, from cadavers with known PMI.

The use of post-mortem biochemistry is now a standard practice along with conventional, physical methods to determine the PMI (Vass et al., 2002). The ante-mortem blood pH is regulated to stay within the narrow range of 7.35 to 7.45 (Sawyer et al., 1988).

This morphological change could be explained by the change of the surrounding media of WBCs which, on the current study, was the change of pH of blood from normal to acidic (less than 7) with pass of time. The results of the current study showed that pH level of the in vitro blood sample was significantly decreased with pass of time.

Poloz and O'Day (2011) explained this by the fact that death results in global anoxia, after blood circulation ceases, and thereby cells cannot produce energy in the form of ATP. As energy production stops, cells die and cell membrane permeability is changed in. Once membrane permeability is compromised, breakdown of cellular homeostasis occur and ions and other molecules flow in and out uncontrollably.

While, Donaldson and Lamont (2013 b) reported that the pH of human blood stored in a tube decreased very slightly compared to ante mortem levels going from 7.4 to 7.1 over 96 hours. They stated that, in vitro blood in a tube has no glucose store to supply fuel for anaerobic metabolism; consequently no

significant lactate accumulation occurs to help lower blood pH. But, in vivo, the rapid decrease in blood pH is most likely due to the accumulation of metabolites and ions such as bicarbonate, carbon dioxide, hydrogen ions, dihydrogen phosphate ions, and lactic acid building up in a corpse due to autolysis.

Coe (1993) previously found that pH level changed from 7.0 to 5.5 by 20 hours post-mortem in cardiac blood taken directly from 11 individual human remains. This change in in vivo blood pH may occur due to accumulation of acidic metabolites. Lactic acid is produced by lactate dehydrogenase from pyruvate via anaerobic glycolysis in skeletal muscle, liver and red blood cells when oxygen is insufficient.

However, Thibodeau and Patton (2007) reported that autolysis does not occur in erythrocytes because they do not have lysosomes so, acidic cellular metabolites such as carbon dioxide, hydrogen ions, formic acid and lactic acid generated inside cells are not released rapidly from lysing cells to significantly lower pH as would occur in a corpse.

Conclusion

The results showed that lymphocytes were the most resistant cell for morphological changes with time. Changes in lymphocytes were detected after 24 hours in the form of pyknosis and degeneration of the nucleus. Morphological changes of neutrophils, monocytes and eosinophils were detected at 12 hours in the form of pyknotic nucleus, irregular swollen nucleus, degenerated nucleus and cytoplasmic and nuclear vacuolation. After 60 hours the nature and type of the cells (except lymphocytes) could not be identified. The morphology of white blood cells and pH level of in vitro blood sample could be used as indicators for the age of the blood.

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تحديد عمر الدم بواسطة التغيرات الشكلية لخلايا الدم البيضاء المختلفة

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تحديد عمر الدم هو موضوع مهم في مجال الطب الشرعي. وقد أجريت هذه الدراسة لتوضيح التغيرات الشكلية التي تحدث في خلايا الدم البيضاء مع مرور الوقت وربطها مع تغيير مستوى الرقم الهيدروجيني للدم المخزن خارجياً. تم عمل فيلم للدم مصبوغ بالهيماتوكسولين والأيسوسين وتحديد مستوى الرقم الهيدروجيني في أوقات (٠، ٦، ١٢، ٢٤، ٤٨، و ٦٠ ساعة) ليقع الدم المخزنة في المختبر التي تركت في درجة حرارة الغرفة. النتائج: كانت الخلايا الليمفاوية الأكثر مقاومة للتغيرات الشكلية مع مرور الوقت و بدت طبيعية بعد ١٢ ساعة والتغيرات المكتشفة بعد ٢٤ ساعة على شكل تغلظ وتحلل في النواة. ظهرت التغيرات المورفولوجية لأنواع الأخرى من خلايا الدم البيضاء بعد ١٢ ساعة على شكل تغلظ وانتفاخ في النواة، تحلل للنواة و فجوات في السيتوبلازم والنواة. لم نستطع تحديد طبيعة ونوع الخلايا (باستثناء الخلايا الليمفاوية) بعد ٦٠ ساعة. وكذلك كان هناك تغير في مستوى الرقم الهيدروجيني متوافق مع التغيرات الشكلية لخلايا الدم البيضاء. كان هناك إنخفاض له دلالة إحصائية في مستوى الرقم الهيدروجيني مع مرور الوقت. بعد ٦٠ ساعة حيث كان مستوى الرقم الهيدروجيني (٠,٠٣ ± ٦,١٦٧) مقارنة بمستواه في وقت الصفر (٠,٠٢١ ± ٧,٢٧٣). لذا فإن مورفولوجيا خلايا الدم البيضاء ومستوى الرقم الهيدروجيني لبقع الدم المخزنة خارجياً يمكن أن يعطي مؤشراً لعمر الدم.