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Estimation of Postmortem Interval from Cartilage Changes of Rabbit Auricle

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Abstract In forensic practice, estimating the time of death is one of the most important procedures but is often extremely difficult. There are many methods for estimating the time of death, which can be divided into two categories (rate methods and concurrence methods). Postmortem decompositional changes to auricular cartilage were analyzed to help establish a new methodology in determining the postmortem interval.
Methods: The ears were collected from rabbits buried in simulated shallow graves for different time periods (0, 5, 10, 15, 20, 25, 30 days). The auricles were examined both macroscopically and microscopically to detect changes occurring over a specified time.
Results: Numerous macroscopic changes including color and texture changes and gradual degradation of cartilage were observed. LM showed gradual structural changes in the tissue over time including: cartilage matrix density and nuclear material.
Conclusion: Postmortem degradation of ear cartilage may be useful for estimating a presumptive postmortem interval.

Keywords Time since death, ear cartilage, LM.

Introduction

In forensic practice, estimating the time of death is one of the most important procedures but is often extremely difficult (Kimura et al., 2011). There are many methods for estimating the time of death, which can be divided into two categories (rate methods and concurrence methods). In rate methods, which are mainly used in forensic practice, the time of death is estimated on the basis of postmortem changes, such as the amount and distribution of rigor mortis (Anders et al 2013, Bendall & Lawrie 1962), the change in body temperature (Smart & Kaliszan 2012, Al-Alousi et al., 2001) hypostasis (Honjyo et al., 2005), changes of potassium concentration in vitreous humor (Júnior et al., 2014), synovial fluid (Siddhamsetty et al 2014), pericardial fluid (Palmiere & Mangin, 2015) development and growth of insects in the corpse (Mohr & Tomberlin 2014) and the degree of putrefaction of the body (Buchan & Anderson, 2001).

Examples of concurrence methods include reading the time on a wristwatch stopped by a traffic accident. Another method is determining the extent of digestion of the last known meal, which may contribute to estimating the time of death (Horowitz & Pounder 1985).

It is stated that, the longer the time passed since death has occurred, the less accurate the PMI estimate would be (Hau et al., 2014). So there is need for more research on tissues which resist putrefaction to improve estimation of postmortem interval in advanced putrefaction; hence the cartilage role comes.

Cartilage is a special form of connective tissue that also develops from the mesenchyme. Similar to connective tissue, cartilage consists of cells and extracellular matrix composed of connective tissue fibers and ground substance. In contrast to connective tissue, cartilage is nonvascular (avascular) and receives its

nutrition via diffusion through the extracellular matrix (Eroschenko & Di Fiore 2013).

Cartilage exhibits tensile strength, provides firm structural support for soft tissues, allows flexibility without distortion, and is resilient to compression. Cartilage consists mainly of cells called chondrocytes and chondroblasts that synthesize the extensive extracellular matrix. There are three main types of cartilage in the body: hyaline, elastic, and fibrocartilage. Their classification is based on the amount and types of connective tissue fibers that are present in the extracellular matrix (Bradbury, 2014).

Elastic cartilage is similar in appearance to hyaline cartilage, except for the presence of numerous branching elastic fibers within its matrix. This type of cartilage is found where flexibility is required, together with ability to recover shape after deformation. Elastic cartilage is found in external ear, external auditory meatus, pharyngo-tympanic tube, epiglottis and some laryngeal cartilages (corniculate and cuneiform) (Zhang, 1999)

The objective of this paper was to find out whether macroscopic and microscopic changes in rabbit auricle could be useful in estimation of time passed since death or not.

Material & Methods

This work was carried out in the department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Minia University during January 2015. All aspects of animal care and treatment were carried out according to the local guide line of the ethical committee of Faculty of medicine, Minia University.

Seventy male rabbits weighting 2500-3500 grams were implemented in this study. All rabbits were sacrificed by decapitation after being deeply anesthetized with sodium phenobarbital 0.1ml/100 g body weight. Rabbit heads were interred in soil to a depth of 20 cm. The soil type at this site is sandy loam with an average pH of 5.5. The experiments occurred during winter (January). Excavation occurred at 5 days' intervals. During sample excavation soil was removed in small layers until the ears were exposed. The samples were lifted (sometimes block lifted in soil to prevent damage to the sample), placed in sealed plastic containers and stored at -20C to prevent further degradation.

The entire ear was subjected to macroscopic analysis to record any visually apparent changes in overall appearance. It was photographed using digital camera (Coolpix 4500, Nikon).

The ear cartilages were dissected, then they were divided into seven groups, 10 rabbits each:

Group I: the right ear cartilage (antihelix) removed immediately after death.

Group II: the right ear cartilage (antihelix) removed 5 days after death.

Group III: the right ear cartilage (antihelix) removed 10 days after death.

Group IV: the right ear cartilage (antihelix) removed 15 days after death.

Group V: the right ear cartilage (antihelix) removed 20 days after death.

Group VI: the right ear cartilage (antihelix) removed 25 days after death.

Group VII: the right ear cartilage (antihelix) removed 30 days after death.

Histopathological study

The cartilages were sectioned and stained with Haematoxylin and eosin, which stains cell nuclei dark pink to blue. After the stain had been applied, the samples were fixed in polystyrene and xylene (DPX) and covered with a standard microscope cover slip. Images of some slides were recorded using an Olympus computerized microscope in pathology department.

Statistical analysis

Fisher Exact Test was done to verify the significance between the grades of histopathological changes at the different postmortem intervals. $P< 0.05$ is significant.

Results

Macroscopic examination of rabbit auricle showed slightly progressive changes as regards its shape, hair covering, consistency and thickness. Figure (1) shows normal auricular shape at the time of death. Auricles examined 5 days postmortem showed slight changes in the form of slight loss of its consistency (figure 2). Ten days after death, auricles showed more loss of consistency and slight folding (figure 3). With longer postmortem interval (15 days), the rabbit auricles start to lose hair covering with complete loss of contour to become flat (figure 4). Twenty days after death, there was complete loss of hair covering. The auricle became folded and there was thinning of its thickness (figure 5). Twenty-five days after death, there were areas of tissue loss and marked thinning of cartilage thickness (figure 6). Figure (7) shows almost complete loss of articular tissues with small remnants attached to rabbit head.

As regards to microscopic examination of rabbit auricle using H&E stain, there was progressive degeneration of cartilage tissues in relation with time. After examination of all specimens, 5 grades of changes were noticed. This grading system followed the same principles used for other tissues examined for PM interval estimation (Kushwaha et al.,2010). The following grades were identifiable:

Go: No Change; preserved auricular tissue histology (Figure 8).

G1: Mild Change (architecture maintained, slight nuclear changes in the form of mild decrease in nuclear material, mild decrease in matrix density) (Figure 9).

G2: Moderate change (architecture maintained, more decrease in nuclear material with faint stain, moderate decrease in matrix density) (Figure 10).

G3: Severe change (architecture disturbed, loss of nuclear materials, marked decrease in matrix density, thinning of the tissues) (Figure 11).

G4: Very severe change (complete loss of architecture) (Figure 12).

With the application of the previous grading system, the following results were found (table 1):

Immediately after death: all cases showed normal tissue with no changes (G0). Five days PM, most cases (80%) showed no histopathological change (G0), and only 20% showed mild changes (G1). In cases examined 10 days PM, the predominant changes were G1 (70%), with 20% G0 and only 10% revealed G2.

Moderate changes (G2) were the predominant in both cases examined 15 and 20 days PM with percentage 80 and 60 respectively. Twenty-five days PM, about 70% of cases showed severe changes with only 20% moderate changes (G2). Thirty days PM, about 60% of cases showed complete loss of auricular cartilage architecture (G4) and 40% showed G3.

Fisher-exact test showed significant changes in different postmortem intervals (at death p=0.001; 5 days PM, p= 0.01; 10 days PM, p=0.01; 15 days PM, p= 0.03; 20 days PM, p= 0.03; 25 days PM, p= 0.01; and 30 days PM, p= 0.01.

Table (1): Microscopic degenerative changes according to PMI in rabbit auricular cartilage

PMI	G0		G1		G2		G3		G4	
	No	%	No	%	No	%	No	%	No	%
0 d	10	100	0	0	0	0	0	0	0	0
5 d	8	80	2	20	0	0	0	0	0	0
10 d	2	20	7	70	1	10	0	0	0	0
15 d	0	0	2	20	8	80	0	0	0	0
20 d	0	0	2	20	6	60	2	20	0	0
25 d	0	0	0	0	2	20	7	70	1	10
30 d	0	0	0	0	0	0	4	40	6	60

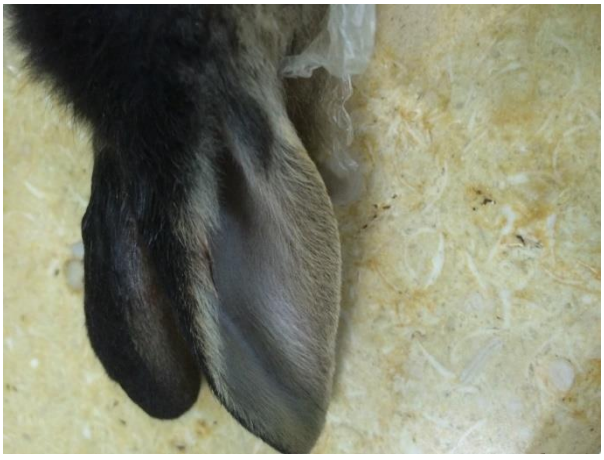


Figure 1: shows the rabbit auricle at death with normal shape and hair covering

Figure 2: Five days' postmortem, the auricle still has its shape as at the moment of death but there is slight loss of consistency

Fig 3: Ten days postmortem it became softer, losing its shape, but still having hair covering.

Fig 4: 15 days PM complete loss of contour and start of loss of hair covering.

Fig 5: Twenty days after death, complete loss of hair becoming folded and very thin.

Fig 6: 25 days after death with much more thinning in thickness and start of loss of tissues (the arrow).

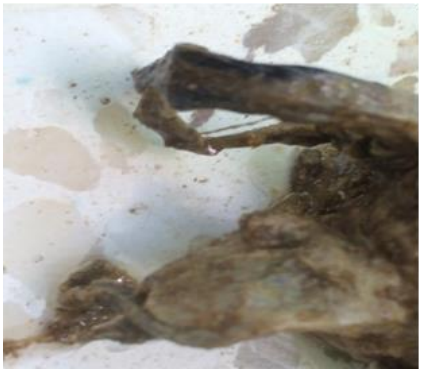


Fig 7: 30 days after death, almost complete loss of cartilage tissue with small remnants attached to head

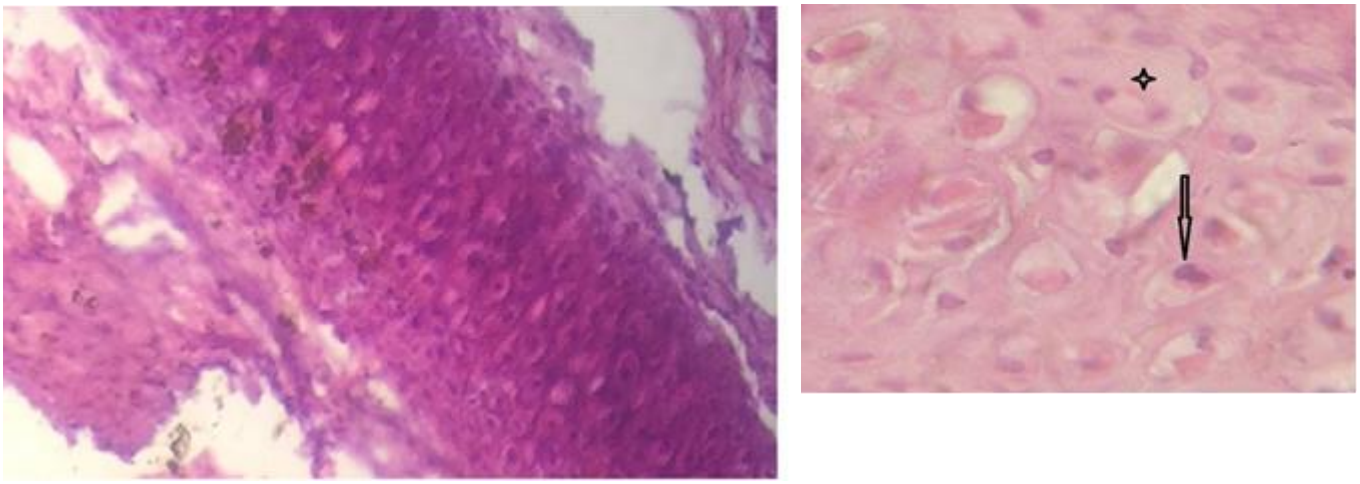


Figure (8): photomicrograph of normal auricular cartilage showing normal matrix tissue (star) and nuclear material (arrow) (H&E, right: X100 and left: X400).

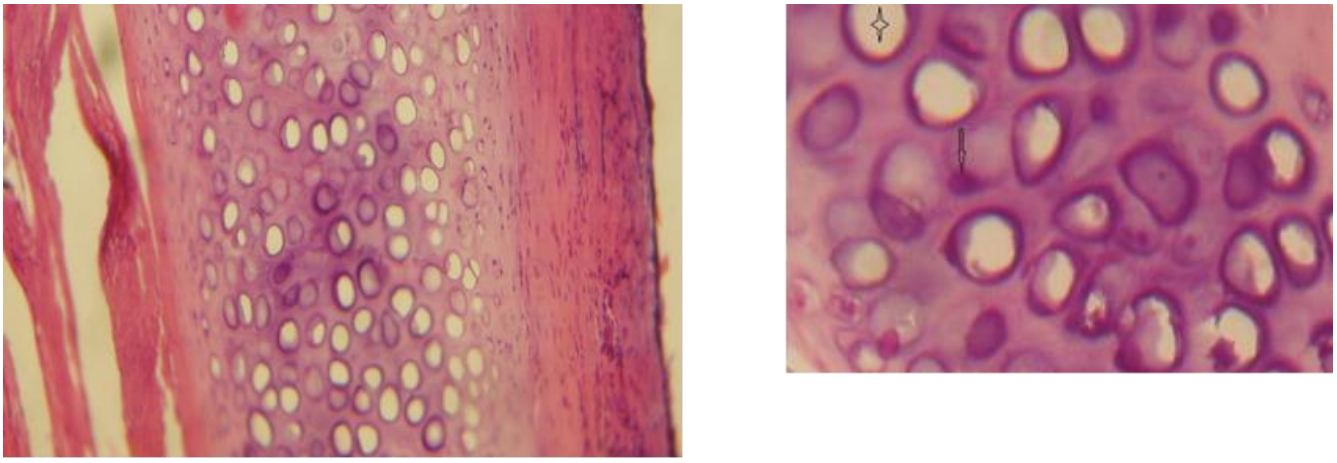


Figure (9): G1: mild decrease in matrix, slight decrease in nuclear material (arrow) and empty lacunae (star) (H&E, right: X100 and left: X400).

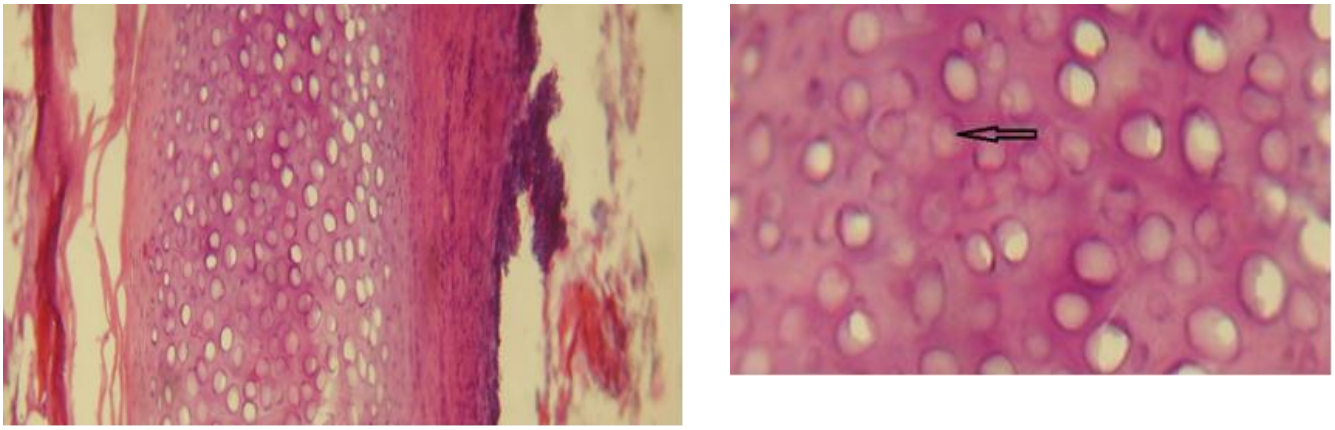


Figure (10): G2: more decrease in the nuclear material (arrow) with faint stain, moderate decrease in matrix density (H&E, right: X100 and left: X400).

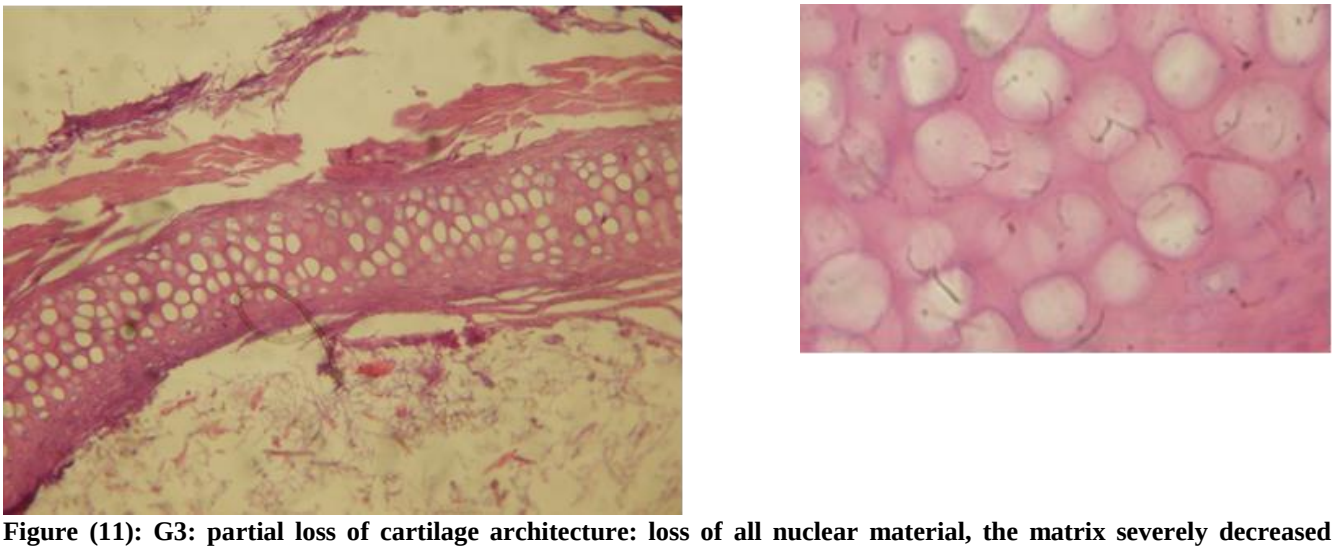


Figure (11): G3: partial loss of cartilage architecture: loss of all nuclear material, the matrix severely decreased (H&E, right: X100 and left: X400).

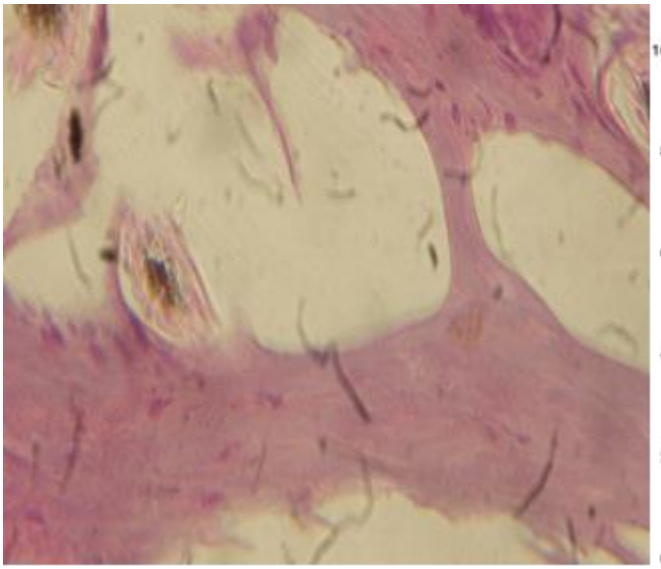
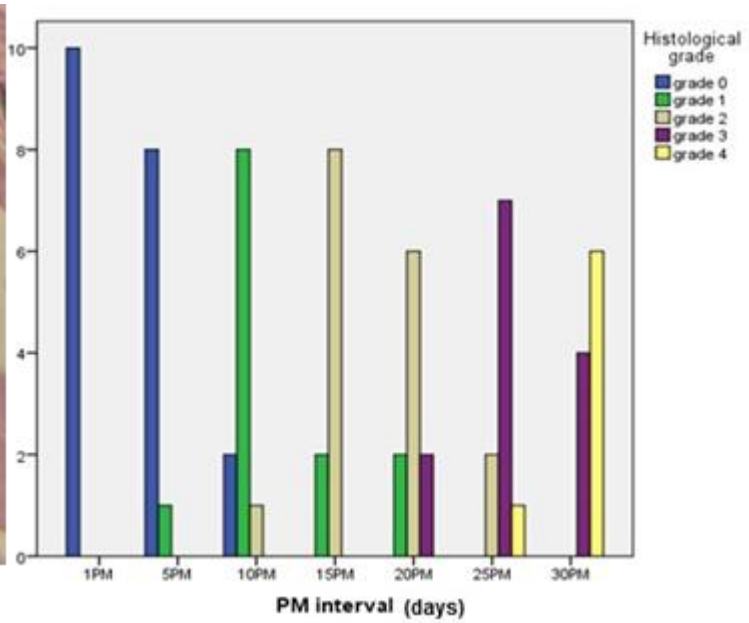


Figure (12): G4: Complete loss of cartilage architecture (H&E, X400).



Histogram (1): the distribution of different histological grades among different groups.

Discussion

Estimation of PMI is very important in crime investigations, event reconstructions and case solving. An accurate estimation of time of death can help the courtroom in accepting or refusing of suspects and witnesses (Bohra et al., 2014). Hence, its determination is one of the most studied points in forensic science. Most of researchers focused on estimation of time since death from early postmortem changes such as body cooling (Hubig et al., 2015), lividity (Sterzik et al., 2014), rigor mortis (Anders et al., 2013), muscle excitability (Elmas et al., 2002), and chemical changes in the fluid compartments (Yahia & El-Hakiem,2013). These changes are applicable in the early postmortem period, but their reliability and accuracy progressively decrease over time. Generally, the longer the time passed since death has occurred, the less accurate the PM interval estimate would be (Metcalf et al., 2013). So this study investigates a tissue which could be used in late postmortem interval namely, cartilage.

Cartilage is particularly useful for late postmortem analysis because the tissue possesses a low cell density. Also, the chondrocytes depend predominantly on the nutrient diffusion from the abundant matrix, which makes their metabolism anaerobic and relatively resistant to oxygen starvation and acidosis. All these factors may delay autolytic processes (Buckwalter & Mankins, 1997, Temenoff & Mikos, 2000).

Research on the chondrocytes long-term survival was done mainly with the purpose of the chondrocytes' cultivation or their conservation in osteochondral samples for clinical use, especially for articular cartilage transplantation. In these studies, the difference in the proportion of chondrocytes that survived during a specific time and at different temperatures was observed. Studies on human allografts showed that approximately 70% of the chondrocytes survived for one month, and that approximately 35% of the chondrocytes survived for two months if the samples were kept in the tissue banks at 4 C, and under optimal conditions (Csönge et al., 2002, Williams, et al., 2003, Drobnic et al., 2005, Hicks et al., 2006,).

Postmortem changes of cartilage have received very little research. It should be noted that most of studies used cartilage as a tool for estimation of postmortem interval focused only on the viability of isolated chondrocytes (Alibegović et al., 2011, Alibegović et al 2012, Alibegović et al 2014), or within cartilage (Drobnic et al.,2005). Also, several methods were used for examination of viability of chondrocytes some of which were H&E (Rogers et al 2011), manual counting under a microscope, viability cell analyzer and the flow cytometer of isolated chondrocyte in cell culture media (Alibegović et al., 2011), and confocal laser microscope (Alibegović et al 2014). Very few work was used cartilage as whole tissue (Rogers et al., 2011) and knee joint was the main source of chondrocytes for most of the studies.

This work focus on auricular cartilage. It showed that there are both macroscopic and microscopic changes of this neglected tissue which may help us in estimation of PM interval especially in late postmortem changes. Changes in the auricle did not start before 5 days PM (where many methods of early PM changes could be used). With increase time after death; the auricle

started to loss its consistency and thickness until it disintegrated completely after 30 days. Also, histologically, changes began to appear 5 days PM in few cases (20 %) to complete loss of cartilage architecture 30 days after burial.

To the knowledge of the authors, there is no other study in the literature using auricular cartilage as a whole for estimation of PM interval. Instead, two studies researched postmortem changes in articular cartilage of knee joint (Rogers et al., 2011, and Bolton et al., 2015). In comparing with auricular cartilage with articular cartilage; it was found that articular cartilage resisted putrefactive changes for much longer periods than auricular cartilage. Rogers et al., (2011) found that; cartilaginous tissue of the knee started to show macroscopic and microscopic changes after 4 weeks and loss of cartilage after 13 weeks. Bolton et al., (2015) found that there is a gradual change in the color of porcine trotter cartilage. It changed from pearly white to pink and evidence of resorption over a period of 6 weeks.

Although study of articular cartilage has an advantage over auricular cartilage as it is isolated and protected within the joint, auricular cartilage is more easily accessible. Also, changes of auricular cartilage are more rapid in comparison with articular cartilage which allow determination of PM interval in a range of days while articular cartilage gives a range of weeks (Rogers et al., 2011).

This work offers preliminary promising data suggesting that auricular cartilage can be simply used for estimation of PM interval. Both macroscopic and microscopic changes can be used. Changes start to appears 5 days PM and progressively increase over the period of 1 month. This method can be used with other methods such as forensic entomology (Byrd & Castner, 2009).

There are several methods available for determining PMI between a few days and several months especially in burial environments. Using auricular cartilage may help narrow down the PMI to a range 5 days with techniques employed by most forensic services.

There are limitations to the application of this data such as environmental influences, namely temperature and soil type. This study was done in winter and in sandy soil. Further study is recommended in different temperatures and soil. As it is difficult to obtain human tissues in Egypt, this study was conducted on rabbits, but it is also important to apply this method to human remains to see if the same processes occur. If so, then the results from this research can be used by forensic pathologists and anthropologists who routinely analyze remains in various stages of decomposition.

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الملخص العربى

تحديد فترة ما بعد الوفاة من تغيرات غضروف الأذن للأرانب

ميلاد جاد بولس و إيمان إسماعيل حسن[1] و نسرين عبد التواب عبد الجابر[2]

في مجال ممارسة الطب الشرعي، تقدير وقت الوفاة هي واحدة من أهم الإجراءات المطلوبة. ولكن غالبا ما يكون ذلك صعبا للغاية. فهناك العديد من الطرق لتقدير وقت الوفاة، والتي يمكن تقسيمها إلى فئتين (معدل وتزامن). وقد ذكر سلفا أنه كلما زادت فترة ما بعد الوفاة، كلما كان تحديدها أقل دقة وهذا بسبب التعفن. لذلك هناك حاجة للاعتماد على أنسجة مقاومة للتعفن لتحسين تقدير هذه الفترة وخاصة في حالات التعفن المتقدم، ومن هنا يأتي دور الغضروف.

وقد تم سابقا فحص التغيرات التحليلية للغضروف المفصلي بعد الوفاة للمساعدة في تأسيس منهجية جديدة في تحديد فترة ما بعد الوفاة. اما في هذا البحث فقد أقمنا باختيار اذان الارانب لفحصها بعد الوفاة وتقييم ما إذا كانت مفيدة في تحديد فترة ما بعد الوفاة ام لا.

فقد تم جمع آذان الأرانب بعد دفنها في مقابر ضحلة لفترات زمنية مختلفة (٠، ٥، ١٠، ١٥، ٢٠، ٢٥، ٣٠ يوما) وفحصها بالعين المجردة، ثم تم تشريح غضاريف الاذان وفحصها بالميكروسكوب الضوئي.

ومن الفحص العيني تم ملاحظة العديد من التغيرات بما في ذلك تغيير اللون والملمس، كما لوحظ تدهور تدريجي في الغضروف والأنسجة الرخوة المجاورة. كما أظهر الفحص بالميكروسكوب الضوئي التغيرات الشكلية والتكوينية للأنسجة تدريجيا مع مرور الوقت وتشمل: سمك الغضروف، كثافة المصفوفة، والتغيرات النووية.

استنادا الى هذه النتائج، يمكننا القول بان التغيرات التي تحدث للغضروف الأذني بعد الوفاة قد تكون مفيدة لتقدير الفترة الزمنية التالية للوفاة.

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