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Tiger in the Veterinary Hospital of Ferdowsi University of Mashhad in 2024, photo by Dr. Ali Mirshahi, IRAN.

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Morphological and morphometrical study of the fore limb bone of common eland (*Taurotragus oryx*)

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ABSTRACT

This research paper presents a comprehensive morphologic and morphometric analysis of the forelimb bones (scapula, humerus, radius, and ulna) of the common eland (Taurotragus oryx) that are facing the threat of extinction. The study aims to contribute essential anatomical and clinical data, as well as facilitate biomedical research and educational endeavors. Bone specimens sourced from the Bangladesh National Zoo, Dhaka, were subjected to a standard hot water maceration technique, followed by sun-drying and treatment with 5% hydrogen peroxide. The scapula displayed a distinct triangular flat shape with a well-developed spine, and demarcating supraspinous and infraspinous fossae. Scapular weights were recorded at 698 gm (right) and 718 gm (left). The humerus, a robust and elongated bone measuring an average of 35.9 cm, exhibited a compressed cylindrical diaphysis and expanded epiphyses, with the right humerus weighing 944 gm and the left, 1016 gm. Notably, the humeral shaft displayed distinct regional compressions and expansions. The radius, slightly oblique and broader than the ulna, demonstrated cranial-caudal flattening. In contrast, the ulna, the longest bone at an average of 41.6 cm in the forearm, displayed distinct anatomical features. These findings offer valuable insights for comparative anatomical investigations within this taxonomic group and hold significance for clinical applications and educational purposes.

Keywords

Common eland; Scapula; Humerus; Radius; Ulna

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Abbreviations

No abbreviations.

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Introduction

The eland, a remarkable inhabitant of the African savannah, stands as the continent's second-largest antelope species, characterized by a size disparity between males and females. Within the eland genus, Taurotragus, the common eland (Taurotragus oryx), and its slightly smaller counterpart, the giant eland (Taurotragus derbianus), command attention [1]. Distributed across East and Southern Africa, the common eland, also known as the southern eland or eland antelope, assumes a prominent role among the savannah and plains antelopes [1]. This species falls within the Artiodactyla order, the Bovidae family, and the Taurotragus genus, a lineage initially outlined by Peter Simon Pallas, a distinguished German biologist and botanist, in 1766.

Eland's behavioral patterns have fascinated researchers, as it exhibits crepuscular tendencies—actively foraging during cooler periods, particularly in the early mornings and late evenings [2]. Remarkably, the common eland holds the potential for domestication, finding utility in meat and dairy production, coupled with its amenability to taming [3]. The eland's physiological efficiency, marked by low water requirements and substantial milk output, further supports its suitability for domestication [4]. While the physiological and behavioral dimensions of the common eland have attracted scholarly attention, a comprehensive elucidation of its skeletal composition remains notably absent. In the realm of anatomical education and research, the demand for accurate teaching aids remains pronounced, particularly within disciplines such as anatomy and biology. This need holds relevance in veterinary studies, facilitating a deeper comprehension of animal anatomy during the preclinical phase [4]. The skeleton, a structural cornerstone, furnishes critical support and protection to an organism's soft tissues [5]. Museums, research institutions, schools, and environmental organizations house vertebrate skeletons, pivotal in research and educational initiatives [6]. Among skeletal segments, the appendicular skeleton, comprising the limb bones, assumes significance in understanding animal locomotion and form [7].

This study embarks on the preparation and examination of the forelimb bones of the common eland, offering valuable insights for veterinary studies and facilitating an enhanced understanding of animal anatomy. Traditional skeletal preparation techniques, widely employed by anatomists, entail a considerable time investment, ranging from one to two months, contingent on the animal's size [8]. However, while extensive studies have explored the skeletal systems of diverse species—ranging from large ruminants such as cattle, small ruminants such as sheep and goats, [5], pet animals such as dogs [5, 9-10] and cats (1)], wild carnivores such as tiger [12], leopard and Asiatic cheetah [13], West African giraffe [14, 15], the African elephant [16], Guinea pig, rat and rabbit [17] have been documented. However, the morphology and morphometrics of the common eland's skeletal system have remained relatively uncharted territory.

This research endeavors to address this gap, focusing on a comprehensive morphologic and morphometric analysis of the common eland's forelimb bones. By shedding light on the osteological intricacies of this species, the study not only aids in distinguishing it from other domestic ruminants but also presents insights applicable to surgical procedures and radiographic interpretations, thereby enriching the understanding of anatomical structures in practical contexts.

Results

The forelimb of the common eland was comprised of several key elements, including the scapula, humerus, radius-ulna, carpals, metacarpals, and digits (manus). This comprehensive study delves into the general morphological and morphometric characteristics of the scapula, humerus, and radius-ulna, comparing these features with those of other domestic and wild animals.

Scapula Morphology

The scapula of the common eland exhibited a distinctive, large, triangular-shaped flat bone structure, featuring lateral and medial surfaces, as well as three borders: cranial, caudal, and dorsal. These characteristics were found to be comparable to various species, including cattle [5], sheep, horses [5], lions [19], and dogs [9]. The scapula's weight was recorded as 698 gm for the right side and 718 gm for the left side (Table 1). It displayed an oblique and downward orientation, with a slight curvature and greater width dorsally compared to ventrally. Notably, the cranial border was convex, rough proximally, concave, and smooth distally, measuring an average length of 38.5 cm (Figure 1). The dorsal border, while slightly convex, proved to be the shortest (average 20.8 cm) among the borders (Table 1). In contrast, small ruminants tend to have a longer dorsal border and a narrower neck. Distinctive to the scapula of the common eland, the caudal border exhibited convexity along the proximal two-thirds and concavity along the distal third. This pattern of border differentiation was also observed in various domestic ruminants such as cattle [20], sheep [5], Black Bengal goats [21], and wild ruminants like

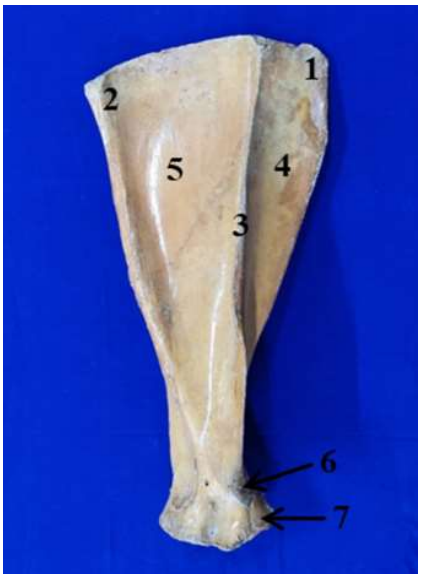

Figure 1.
Lateral aspect of Scapula. 1= Cranial angle, 2= Caudal Angle, 3= Spine of Scapula, 4= Supraspinous fossa, 5= Infraspinous fossa, 6 = Acromion process, 7= Supraglenoid Tubercle.

Table 1.
Morphometric data for various features of the scapula (N = 2)

Parameters		Measurements (cm)	
		Right	Left
Border:			
	Cranial	39	38
	Caudal	37.5	37.3
	Dorsal	20.7	20.9
Length of spine		36.8	36.7
Maximum length of dorsal border to the glenoid cavity		41.6	41.4
Height of supraspinous fossa to spine		5.0	4.8
Height of infraspinous fossa to spine		4.8	4.6
Glenoid cavity:			
	Height	6.5	6.5
	Width	6.9	6.8

giraffes [14, 15] and horses [20]. However, pigs [9] and certain carnivores displayed contrasting border configurations, with pigs appearing rhomboid and carnivores adopting a D-shaped structure. Moreover, the scapula's dorsal border measurements exhibited slight differences between the right (20.7 cm) and left (20.9 cm) sides, while the largest distance between the glenoid cavity and the dorsal border was 41.5 cm (Table 1).

Scapular Fossae and Spine

The lateral surface of the scapula was characterized by the presence of two fossae: a cranio-dorsal supraspinous fossa and a caudo-ventral infraspinous fossa, divided by a well-developed scapular spine (Figure 1). This spinal feature, more prominent and less sinuous in the common eland, displayed distinct bending characteristics. Unlike the horse [5], where a small spinal tuber was observed centrally, the scapular spine of the common eland lacked this feature. Additionally, the spine's projection in the pig [22] significantly differed from the findings of this study. Furthermore, the acromion process of the scapula extended prominently from the spine like cattle, sheep, Black Bengal goats, and dogs. Comparable to lions [19] and African elephants [23], the acromion process in the common eland presented an overhanging structure toward the glenoid notch. The average length of the scapular spine in the studied individual was 36.75 cm, contributing to its unique morphology (Table 1). Notably, the infraspinous fossa was larger than the supraspinous fossa, maintaining a 3:1 ratio (Figure 1), consistent with patterns observed in various ruminant and non-ruminant species [5]. The supraspinous fossa housed the supraspinatus muscle, while the infraspinous fossa accommodated the infraspinatus muscle, resembling muscle distribution in other domesticated and wild animals.

Subscapular Fossa and Glenoid Cavity

The medial surface of the scapula revealed a shallow middle subscapular fossa, hosting the subscapularis muscle (Figure 2). Notably, the areas designated

Figure 2.
Medial aspect of the scapula. 1= Cranial angle, 2= Caudal angle, 3= Caudal Ridge, 4= Cranial Ridge, 5= Dorsal Margin, 6= Cranial margin, 7= Caudal Margin, 8= Scapular notch.

for the attachment of serratus ventralis muscles lacked distinct boundaries from the subscapularis fossa. This finding contrasted with cattle [24], sheep, and goats [24], where distinct boundaries were evident. The distal extremity of the scapula, connected to the bone's body by the neck, featured the glenoid cavity for articulation with the humerus. This glenoid cavity displayed circular characteristics akin to cows, Black Bengal goats, sheep, horses, and giraffes. In comparison, variations were observed in the shape of the glenoid cavity among other species, such as the elongated cavity in Asian elephants [25], the pear-shaped cavity in cats [11], and the shallow cavity in dogs [9]. Moreover, the edge of the glenoid cavity exhibited a slight cranial indentation. The presence and distinctness of the glenoid notch, however, varied across species, with more distinct notches observed in horses [5], dogs [9], and cats [11], but there is no distinct notch in Black Bengal goats [21], sheep, cattle [5]. The presence of the small and rounded supraglenoid tubercle, located close to the glenoid cavity on the cranial aspect of the scapula in the common eland, aligned with cattle features, is different from those seen in horses [5], dogs [9], and cats [11]. On the medial aspect of the glenoid angle, the tiny, rounded coracoid process was projected caudally to the supraglenoid tubercle, like that of cattle, horses [5], giraffes [14, 15], and other wild animals.

Humerus Morphology

The humerus, forming the arm of the common eland, constituted a prominent bone within the appendicular skeleton. It extended proximally from the scapula to the elbow distally, serving as a crucial link between the shoulder and the radius-ulna complex. The humerus displayed distinctive characteristics, both in terms of its length and various structural features, as discussed below. The average length of the humerus in the common eland was measured at 35.9 cm. Notably, variations were observed in weight between the right humerus (944 gm) and the left humerus (1016 gm), suggesting subtle asymmetry between the limbs (Table 2). The humerus comprises three main components: the body or shaft, the proximal extremity, and the distal extremity. The shaft exhibited an irregular cylindrical shape and displayed a noticeable twist, contributing to its unique appearance (Figure 4). The length of the shaft averaged 22.55 cm, and it was characterized by upper, middle-, and lower-part circumferences, measuring 22.15 cm, 15.95 cm, and 16.9 cm, respectively (Table 2). This complex configuration is distinct from other species and could reflect adaptations related to locomotion and muscle attachment.

Musculospiral Groove and Medial Surface

The lateral surface of the humerus featured a shallow spiral groove, referred to as the musculospiral groove, designated for the attachment of the brachialis muscle (Figure 5). This groove encircled the cranial surface and connected proximally to the caudal surface. Comparatively, this groove exhibited variations among different species, with shallow features in cattle, a more spiral and deeper groove in horses [5], and minimal presence in giraffes [14, 15]. The medial surface of the humerus blended with the cranial and caudal surfaces, appearing rounded from side to side. The teres major tuberosity was positioned proximal to the midpoint, similar to observations in horses and cattle [5], while being absent in giraffes [14, 15]. This distinct arrangement likely contributes to the functional capabilities of the humerus within the common eland's limb structure. The crest of the humerus served as a demarcation between the cranial and lateral surfaces of the bone. The lateral surface, extending proximally, exhibited a smooth and wide configuration, which gradually transformed into a rough and narrow texture distally (Figure 5). In comparison to cattle [24], the deltoid tuberosity of the common eland's humerus, where the deltoideus

Table 2.
Morphometric data for various features of the humerus (N = 2)

Parameters		Measurements (cm)	
		Right	Left
Total length		35.8	36.0
Shaft:			
	Length	22.5	22.6
	Circumference of the upper part	22.3	22.0
	Circumference of the middle part	16.1	15.8
	Circumference of the lower part	17.0	16.8
Circumference of head		26.6	27.0
Proximal extremity:			
	Circumference	39.5	41.0
	Width	12.2(cr-cau) 12.5(L-M)	12.3(cr-cau) 12.7(L-M)
Distal extremity			
	Circumference	29.0	28.5
	Width	9	8.9

muscle attached, was less prominent. This contrasts with horses, where the deltoid tuberosity was more pronounced [5]. Additionally, a small prominence known as the teres minor tuberosity was located proximal to the deltoid tuberosity, further enhancing the muscle attachment site (Figure 4, 5). Notably, these tuberosities were absent in giraffes [14, 15], differing from the observations in the present study. In sheep, the deltoid tuberosity was less prominent and closer to the proximal end [5], showcasing variations in muscle attachment across species. The lateral head of the triceps brachii muscle was attached to the humeral neck on the lateral surface by a curving line proximal to the deltoid tuberosity. The cranial surface of the shaft of the humerus ended at the radial fossa distally. The caudal surface is smooth, almost straight, rounded from side to side, and ends distally at the olecranon fossa (Figure 4). The nutrient foramen was located almost at the middle of the caudal surface of the humerus (Figure 4) like sheep [5], and Black Bengal goats [21]. According to [5], the nutrient foramen of cattle is placed in the distal third of the caudal surface of the humerus, but that of horses is positioned in the distal third of the medial surface of the humerus. In Asiatic cheetah, there were two main nutrient foramina on the shaft of the humerus: one was on the roof of the olecranon fossa, and the other was proximal to the supratrochlear foramen on the medial surface [26]. The head, neck, two tuberosities, and intertuberal groove made up the proximal extremity. The neck of the humerus was clearly defined caudally.

Figure 3.
Distal aspect of Scapula. 1= Glenoid cavity, 2= Supraglenoid tuberosity, 3= Coracoid process, 4= Glenoid notch.

Proximal Extremity and Tuberosities

The proximal extremity of the humerus encompassed several key features, including the head, neck, two tuberosities (greater and lesser), and the intertuberal groove (Figure 4). The head, with its rounded

Figure 4.
Humerus (caudal view); 1= Head of humerus, 2= Neck of humerus, 3= Major (lateral) tuberosity, 4= Body of humerus, 5= Deltoid tuberosity, 6= Olecranon fossa, 7= Medial epicondyle, 8= Lateral epicondyle, 9= Nutrient foramen.

Figure 5.
Humerus (cranial view); 1= Major (lateral) tuberosity, 2= Minor (medial) tuberosity, 3= Intertuberal groove, 4= Deltoid tuberosity, 5= Radial fossa, 6= Trochlea, 7= Capitulum, 8= Musculospiral groove for brachialis, 9= Teres major tubercle.

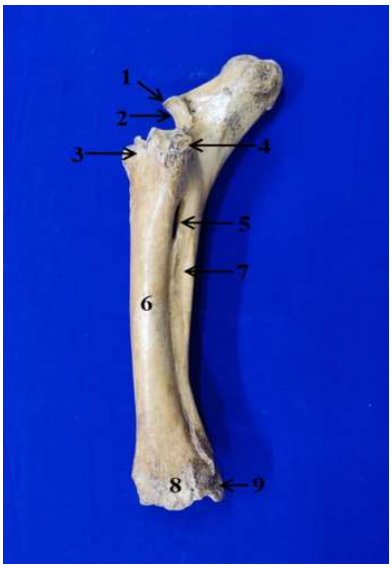

Figure 6.
Radius and ulna (lateral view); 1= Anconeal process, 2=Trochlear notch, 3= Radial tuberosity, 4= Tuberosity for attachment of the lateral collateral ligament of the elbow joint, and extensor digitorum communis and lateralis, 5= Proximal interosseous space, 6= Shaft of radius, 7=Shaft of ulna, 8= Tuberosity for attachment of lateral collateral ligament of carpal joint, 9= Styloid process of ulna.

shape and convex articular surface, facilitated articulation with the glenoid cavity of the scapula. The greater tubercle, positioned cranio-laterally, extended approximately 2-3 cm beyond the head level, forming the shoulder point. This tubercle was divided into cranial and caudal parts, which played a role in delineating the lateral limit of the intertuberal groove (Figure 4). In contrast, the lesser tubercle was less prominent, possessing both cranial and caudal portions. The intertuberal groove, positioned cranially and undivided, represented a notable feature of the humerus. This arrangement differed from species like horses, Asiatic cheetahs, and giraffes, which exhibited an intermediate tubercle that divided the intertuberal groove [5, 14-15, 26].

Distal Extremity and Articulation Surfaces

The distal extremity of the humerus featured the medial epicondyle and lateral epicondyle, alongside radial and olecranon fossae (Figure 4). The trochlea (medial condyle) and capitulum (lateral condyle) provided oblique articular surfaces for articulation with the radius and ulna (Figure 5). The trochlea, larger than the capitulum, displayed a sagittal groove that extended up to the olecranon fossa. This deep fossa accommodated the ulna's anconeal process, ensuring functional integration with the radius-ulna complex (Figure 5). The radial fossa, situated cranially proximal to the trochlea's sagittal groove, was consistent with patterns observed in other species. All these characteristics are like cattle, horses, sheep [5], Asiatic cheetahs and Asian elephants [26], and giraffes [14, 15]. Black Bengal goats had shallow radial and olecranon fossae [21], whereas, in dogs, these two fossae frequently communicate with one another through a wide supratrochlear foramen [9]. The craniocaudal diameter of the proximal and distal extremities was 12.25 cm and 8.95 cm (average), respectively (Table 2).

Radius and Ulna

The forearm of the common eland is composed of two bones: the radius and ulna. The weights of the right and left radius-ulna were measured at 934 gm and 986 gm, respectively (Table 3). While the shafts of both the radius and ulna were largely fused, except for the proximal and distal interosseous spaces, there were notable differences in their relative lengths. Specifically, the ulnar bone was longer than the radius in the common eland, a characteristic shared with cattle [5], Black Bengal goats [21], and giraffes [14, 15]. This contrasts with horses, where the radius tends to be larger than the ulna. The radius was positioned craniolaterally to the ulna, articulating with the humerus at its proximal end and craniomedially at the carpal joint (Figure 6). Both bones exhibited convex cranial surfaces and concave caudal surfaces, with the ulna being thinner and fused cranially to the caudal surface of the radius, apart from the interosseous spaces. This anatomical arrangement shared similarities with cattle [5], Black Bengal goats [21], and giraffes [14, 15].

Radius Characteristics

The radius, shorter and broader than the ulna, displayed an oblique orientation, being flattened craniocaudally. It consisted of a shaft and two extremities: proximal and distal (Figure 6). The shaft's average length was 33.9 cm, with a middle part circumference of 14.15 cm (Table 3). Longitudinally curved, the radius featured a smooth, convex cranial surface and a concave caudal surface along its length. Notably, two distinct borders, lateral and medial, were observed. This contrasts with the giraffe's elongated linear-shaped radius [14, 15] and is consistent with the morphology of domestic ruminants like cows [24] and the common eland. A shallow, smooth groove was observed along the caudal border, forming the proximal interosseous space in coordination with the ulna. The distal interosseous space was notably narrow and elongated in the distal part of the radius shaft, differing from horses where this space is absent [5]. Furthermore, the distal interosseous space was small and indistinct in Black Bengal goats [21], whereas it was entirely absent in Asian elephants [25].

Table 3.
Morphometric data for various features of radius (N = 2)

Parameters		Measurements (cm)	
		Right	Left
Total length		34.0	33.8
Proximal extremity:			
	Circumference	25.9	25.8
	Width	10.0(L-M) 5.3(Cr-Cau)	9.8(L-M) 5.4(Cr-Cau)
Distal extremity:			
	Circumference	23.1	23.5
	Width	7.8(L-M) 6.2(Cr-Cau)	7.8(L-M) 6.1(Cr-Cau)
Circumference of shaft:			
	Upper	16.5	16.2
	Middle	14.1	14.2
	Lower	15.4	15.0

The proximal extremity, or head, of the radius, was transversely widened and flattened craniocaudally. It featured two proximal attachments: one with the humerus and another with the medial ulnar surface. A concave articular surface, merging with a notch for articulation with the humeral condyle, was evident on the proximal extremity. Below the edge of this articular surface, the medial and lateral tuberosities were located. A raised, rough area on the cranial surface of the proximal extremity, termed the radial tuberosity, was also observed (Figure 6). The distal extremity was similarly flattened craniocaudally, forming the trochlea. It presented three articular surfaces that articulated with the proximal row of radial, intermediate, and ulnar carpal bones. This configuration corresponded with observations in domestic ruminants [5] and Black Bengal goats [21].

Ulna Characteristics

The ulna, constituting the longest bone with an average length of 41.6 cm in the forearm of the common eland (Table 4), exhibited a relatively underdeveloped structure that was fused with the radius along its caudolateral side. The ulnar shaft, triangular, featured three surfaces and tapered distally (Figure 7). The proximal end of the ulna was notably prominent, possessing a larger circumference (average of 15.3 cm) compared to the distal end (average of 7.2 cm) (Table 4). At the proximal end of the ulna, the olecranon projected beyond the radius, and its free end expanded to form the olecranon tuber (Figure 7). The olecranon process was composed of two surfaces and two borders. The medial surface was concave, while the lateral surface was convex. The anconeal process, characterized by a sharp border, protruded cranially proximal to the articular surface. The trochlear notch, wider at its cranial part, was positioned proximally. On either side of the articular surface, the lateral and medial coronoid processes extended distally, creating a concave surface for articulation, and continued the trochlear notch proximally. In the distal extremity of the ulna, articulation with the accessory carpal bone and the ulnar carpal bone was observed in the common eland. The ulnar carpal bone's lateral facet was formed by extending the distal end downward to create the lateral styloid process, a feature consistent with findings in cattle, sheep [5], Black Bengal goats [21], West African giraffes [14, 15], Asiatic cheetahs, and Asian elephants [26]. However, this characteristic was absent in horses [5]. Contrary to horses [5], in the common eland of this study, the ulna extended to the distal extremity of the radius. This alignment corresponded with observations in cattle [24], sheep [5], Black Bengal goats [21], West African giraffes [14, 15], Asiatic cheetahs, and Asian elephants [26]. In

Table 4.
Morphometric data for various features of the ulna (N = 2)

Parameters		Measurements (cm)	
		Right	Left
length		41.7	41.5
Proximal extremity:			
	Circumference	15.4	15.2
	Width	6.2	6.5
Distal extremity:			
	Circumference	7.2	7.1
Shaft:			
	Upper	6.2	6.1
	Middle	7.2	7.1
	Lower	4.8	4.8

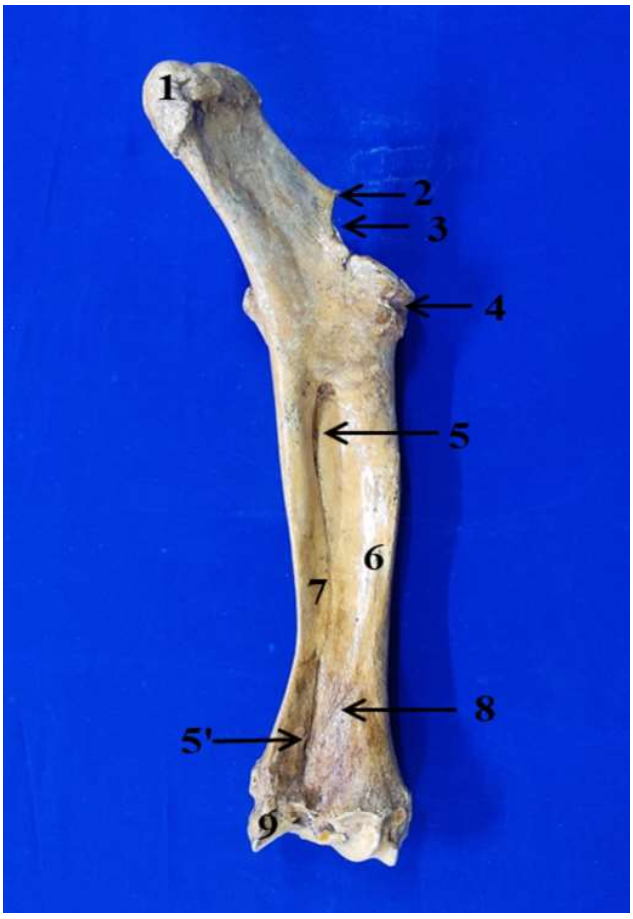

Figure 7.
Radius and ulna (caudomedial view); 1= Olecranon, 2= Anconeal process, 3=Trochlear notch, 4=Proximal extremity of radius, 5, 5'= Proximal and distal interosseous spaces, 6= Shaft of radius, 7=Shaft of ulna, 8= Vascular groove, 9= Styloid process of the ulna.

contrast, the ulna was fused with the proximal third of the radius in horses [5].

Materials and Methods

Specimen Collection

This common eland, aged 16 years and weighing 475 kg, died due to age-related complications at the Bangladesh National Zoo, Dhaka, in November 2020. The bones were collected from the deep burial with aseptic measure from National Zoo, Dhaka, Bangladesh. The bones were transported under aseptic conditions to the laboratory of the Department of Anatomy and Histology, Faculty of Veterinary Science, Bangladesh Agricultural University. The objective was to prepare bones for museum specimens and educational purposes, with official authorization from the director of Bangladesh National Zoo (Order on.01/2021/217).

Preparation of Bones

The bones were collected in a sequential manner, processed by removing the mud with a brush, and washed under running tap water, followed by dipping in detergent water for 2 hours. Bones were then subjected to boiling in 5% soda water for 1 hour, followed by a 7-day immersion in the same solution. This process aimed to facilitate the complete digestion of extra muscles, tendons, ligaments, and associated structures, drawing from the methodology outlined by [18]. The container housing the bones was hermetically sealed, placed in a shaded area at room temperature, and left undisturbed throughout this period. Post 7 days, bones of the forelimbs (scapula, humerus, radius, and ulna) were retrieved, with excess tissues meticulously removed using a blade. The collected bones underwent a thorough cleaning process using detergent and were subsequently rinsed extensively under running water. Cleaned bones were immersed in a container filled with a 5% hydrogen peroxide solution. Repeated rinsing under running water ensued until the bones reached the desired level of whiteness. This step aimed to enhance bone aesthetics. A 10% bleaching water solution was used to submerge the bones for two hours, thereby forestalling potential microbial degradation. Sun exposure facilitated the complete drying of the bones.

Biometric Study

The morphological parameters of each forelimb bone were meticulously examined from various angles. A digital balance was employed to measure the bone weight in grams (gm). Additionally, metallic calibrated scales facilitated precise measurements of bone length, width, height, and circumference in (cm). To document the findings, individual photographs of the prepared bones were captured using a digital camera.

Conclusion

In conclusion, this comprehensive study focused on the gross anatomy of the forelimbs of the common eland has elucidated distinct characteristics, as well as differences and similarities with other mammals. Through meticulous examination of morphometrical and morphological features, valuable insights were gained into the structural attributes of the bones. This knowledge bears significance for diverse applications, including bone identification, radiographic interpretation, and forensic investigations involving the common eland. Furthermore, these findings pave the way for a deeper comprehension of the precise anatomical parameters, ultimately contributing to the enhancement of veterinary care practices.

Authors' Contributions

M.R.I., I.H. conceived and planned the experiments. M.R.I., I.H. carried out the experiments. M.R.I., I.H., contributed to sample preparation. L.A., contributed to the interpretation of the results. M.R.I., L.A., A.S. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare they have no conflict of interest associated with this work.

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RESEARCH ARTICLE

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Histopathological assessment of wound healing after using adipose-tissue derived mesenchymal stem cells with Tragacanth gum hydrogel and human amniotic membrane as dressing

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ABSTRACT

Wound healing and finding a solution for fast healing are among of the major issues of today's world. This study aimed to assess the effect of Tragacanth gum hydrogel as a three-dimensional scaffold of MSCs along with a wound dressing of human amniotic membrane in the healing of full-thickness skin wounds in rat. In this study, 54 Albino female rats (150 g) were divided into control, hydrogel, and hydrogel+stem cell groups. Under general anesthesia, two bilateral full-thickness wounds were created on the dorsal area by a 9.8-millimeter biopsy punch. Rats were euthanized on days 3, 10, and 21 for histopathology and cell tracking by PCR evaluation of tissue samples. The histopathological results showed that no significant difference was seen on days 3 and 21, and there were significant differences only on day 10. In terms of epithelialization, the treatment groups were significantly different from the control group hydrogel+MSCs had a statistically significant difference with the control group in terms of granulation tissue formation. Cell tracking results with PCR on days 3, 10, and 21 in the hydrogel+MSCs group showed that MSCs were found only on day 3. The results of the present study showed that the use of stem cells together with the Tragacanth gum hydrogel as a scaffold and the use of human amniotic membrane as a dressing can cause fast healing of full-thickness wounds.

Keywords

Amniotic membrane, Mesenchymal stem cells, Rat, Scaffold, Wound healing

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Abbreviations

MSC: mesenchymal stem cell

PCR: Polymerase chain reaction

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Introduction

The skin acts as the first defense barrier of the body against external injuries, pathogens, and water loss, and it is also a thermoregulator [1,2]. In the management of wounds, the best method should be selected according to the size of the wound, the amount of tissue damage, the presence of infection, and the length of time since the wound was caused [3]. Naturally, the body's speed in responding to injury and wound healing should be a way that prevents the infection and severe inflammation or organ failure [4]. In addition, open wounds can be treated by two methods: primary (suturing) and secondary healing. Sometimes, due to the large size of the wound, the amount of tissue damage, infection, and economic conditions, the wound is treated with secondary healing methods (contraction and reepithelization), which would be a more appropriate and practical method [5]. Although a variety of local treatments with different substances are used for secondary wound healing, today, cell therapy is used as a new treatment with minimal side effects in the treatment of various diseases, including wound healing. Among the cells with different origins, MSCs have the potential to be used for wound cell therapy. MSCs are found in most tissues with different sources and have been used to manage the wound healing and regeneration process [6]. Studies showed that adipose-derived MSCs (AD-MSC) have effective properties such as easier separation, affordability, and lack of ethical issues, and they have high differentiation ability into different types of cells, including skin cells and myocytes [7].

The second solution that can be used in wound healing is the use of biological scaffolds as a carrier for the transfer of cells, drugs, medications and various compounds. One of these biological scaffolds is hydrogels, which can be made from various natural polysaccharides. Natural polysaccharides, as abundant polymers, are hydrophilic and environmentally friendly. They are more similar to the extracellular matrix and rarely cause inflammatory responses and cytotoxicity due to their structure and high hydrophilicity [8,9]. Tragacanth gum, as an anionic polysaccharide, is a natural polymer extracted from renewable sources of agrochemicals. Its advantages include wound healing and drug delivery to control and improve the microstructure, texture, stability, and viscosity of cosmetics, biological products, food, and pharmaceutical formulations. In addition, they are used as emulsifiers and thickeners [10–12]. Tragacanth gum hydrogels are hydrophilic polymers with three-dimensional structures that have a high capacity to absorb water and are used as a controlled drug release and wound dressing [13–15]. Moreover, they have biocompatibility features in the presence of different fluids and tissues of the body. They are similar to the native ECM in terms of the lack of toxicity, subtlety, non-allergenic power, and rubbery nature [16,17].

One of the protective dressings, that is widely used in wound healing, and its effectiveness has been confirmed in various studies, is HAM. The HAM triggers the migration and proliferation of the epithelial cells of the epidermis [18]. It has anti-inflammatory, antibacterial, and non-immunological properties and causes the wound to contract faster. It is impregnated with several essential growth factors [19].

Considering that all these valuable substances alone are effective in wound healing, in this study, the simultaneous effect of MSCs and Tragacanth gum hydrogel on wound healing and the use of HAM as dressing were investigated.

Abbreviations Cont'd

ECM: Extracellular Matrix
HAM: Human Amniotic Membrane
PBS: Phosphate-buffered saline
DMEM: Dulbecco's modified Eagle's medium

Results

MSCs Characterization and Differentiation

In passage 0, the cell colonies of MSCs were heterogeneously visible. As the passage numbers increased, the cells became more homogeneous and morphologically elongated (Figure 1A, B).

In this study, the cells were placed in the third passage in the differentiation media, inducing fat and bone cells. After 21 days of culture for differentiation into fat and 14 - 17 days for differentiation into bone, their successful differentiation towards fat and bone cells was proved by Oile Red O (bright red fat vacuoles) and Alizarin Red (Calcium ions deposited in the external matrix of cells) staining methods, respectively. Moreover, examining the activity of alkaline phosphatase was indicative of the differentiation of these cells to bone cells (Figure 1C-H).

DAPI staining of HAM

The results of this staining showed that cell removal was complete. The membrane was entirely uniformly black under the fluorescent microscope (Figure 2A-C).

Histological wound healing assessment

Epidermal cells regeneration

The process of wound healing 3 days after surgery in the rats of the control and hydrogel groups was observed only as the thickening of the epidermis tissue

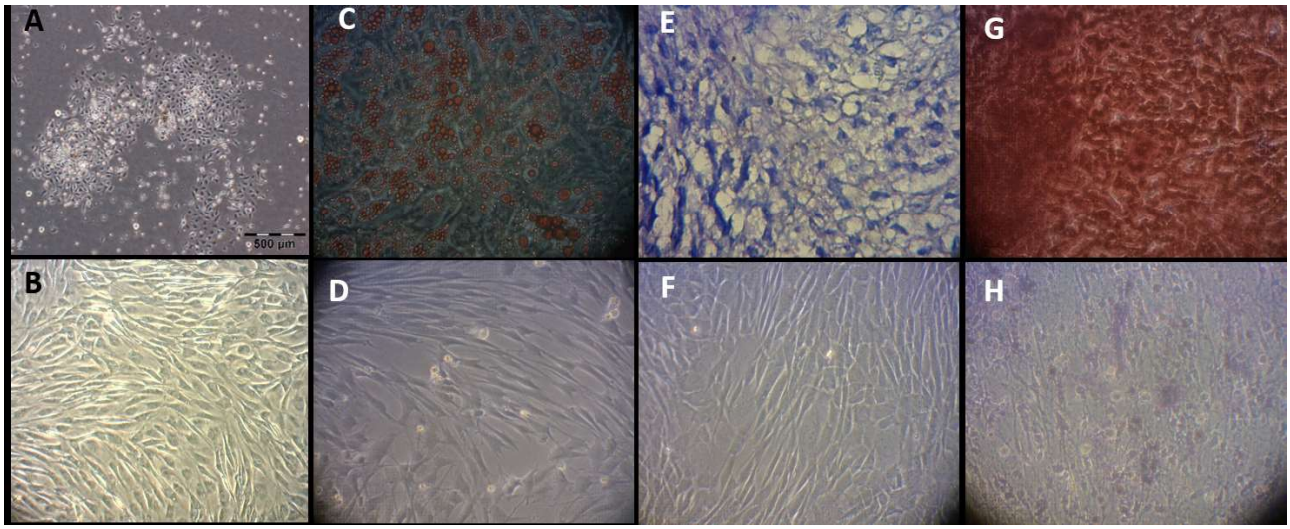

Figure 1.
Characteristics of rat adipose tissue-derived mesenchymal stem cells (adMSCs). Phase contrast photomicrographs show the morphology of colonies of rat adMSCs at passage 0 (A) and expanded rat adMSCs at passage 1 (B). The cells represent spindle-like morphology. Rat adMSCs at passages 1-3 were characterized by adipogenic differentiation via Oil Red O staining that represents the lipid vesicles within differentiated cells (C) versus the control cell without adipogenic induction media (D). The osteogenic differentiation potential of rat adMSCs was also examined by Alkaline phosphatase activity assay (E) and Alizarin red staining (G) against their respective controls (F and H). All the images except A were taken by ×100 magnification.

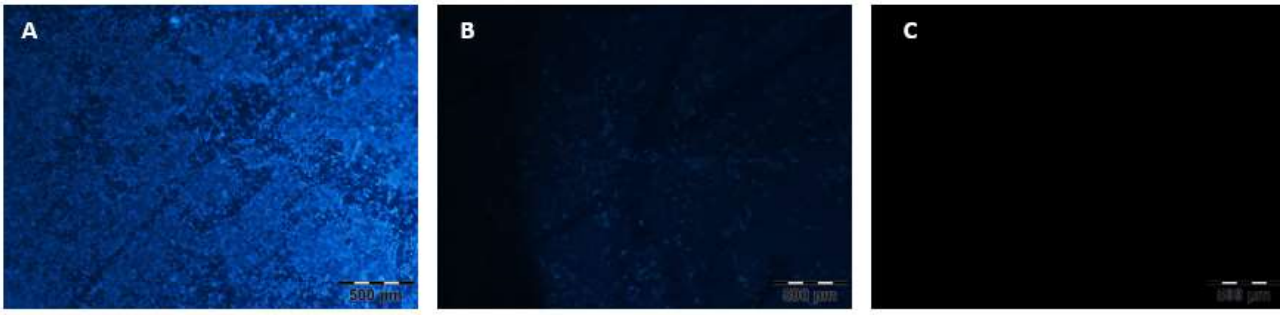

Figure 2.
Confirmation amniotic membrane decellularization by DAPI fluorescence dye. A: Amniotic membrane before decellularization, B: amniotic membrane during decellularization, C: amniotic membrane after decellularization. In this staining, the blue dots represent the nucleus of the amniotic membrane cells, and the black parts indicate the decellularized amniotic membrane.

at the cut edges. In some rats of the hydrogel+MSCs group, the epidermis was completely regenerated, and the entire wound was covered with keratinocyte cells. In addition, the amniotic membrane was observed as a serous layer covered with squamous cells on the wound scab in this group. In general, between the treatment groups and the control group were significant ($p < 0.05$) on the 10th day, and at this time, complete regeneration of the epidermis was observed only in the hydrogel+MSCs group (Figure 3A, 4A-D).

Inflammation response

According to our results, granulomatous inflammation was severe in all groups on days 3 and 10. On the 10th, in the hydrogel and control groups, the presence of more granulomatous inflammation and giant cells was confirmed. On the 21st day, the hydrogel+MSCs group had the lowest grade of granulomatous inflammation and inflammatory cells, but these differences were not significant ($p > 0.05$) (Figure 3B, 4E-G).

Granulation tissue formation

According to the total scores of granulation tissue formation on days 3 and 10, the samples of the hydrogel+MSCs group showed the highest rate of granulation tissue formation compared to the other groups, but the difference with the control group was significant only on day 10 ($p < 0.05$). On the 21st day, this criteria was higher in the hydrogel and control groups than hydrogel+MSCs but the difference was not significant (Figure 3C, 4H-J).

PCR analysis of male DNA in female tissue

Sex mismatch between donor and recipient an-

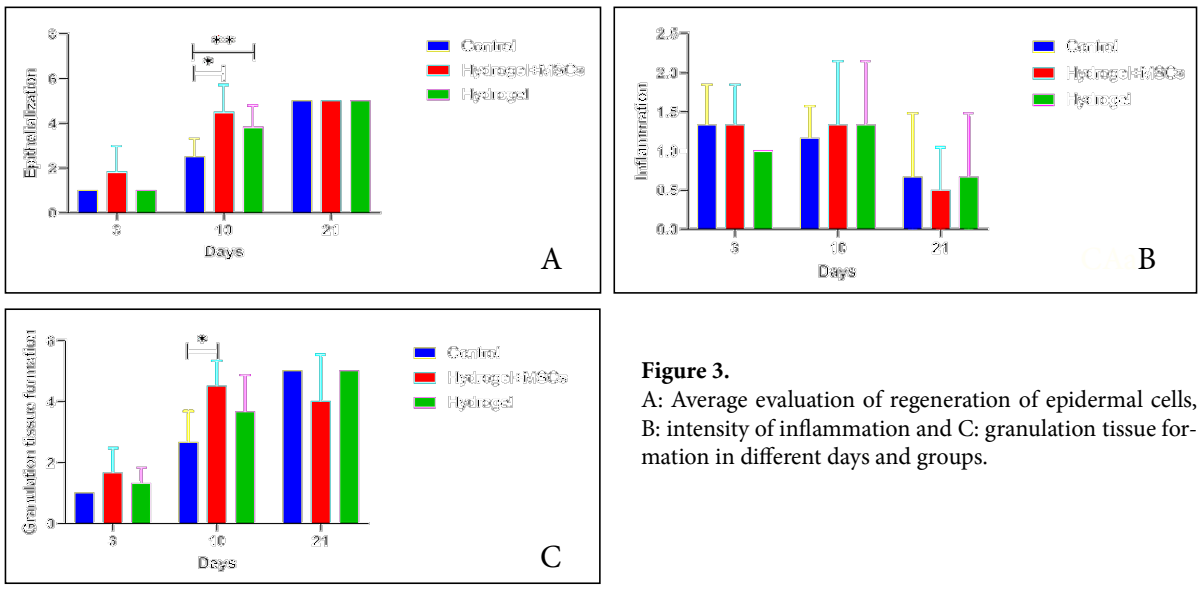

Figure 3.
A: Average evaluation of regeneration of epidermal cells, B: intensity of inflammation and C: granulation tissue formation in different days and groups.

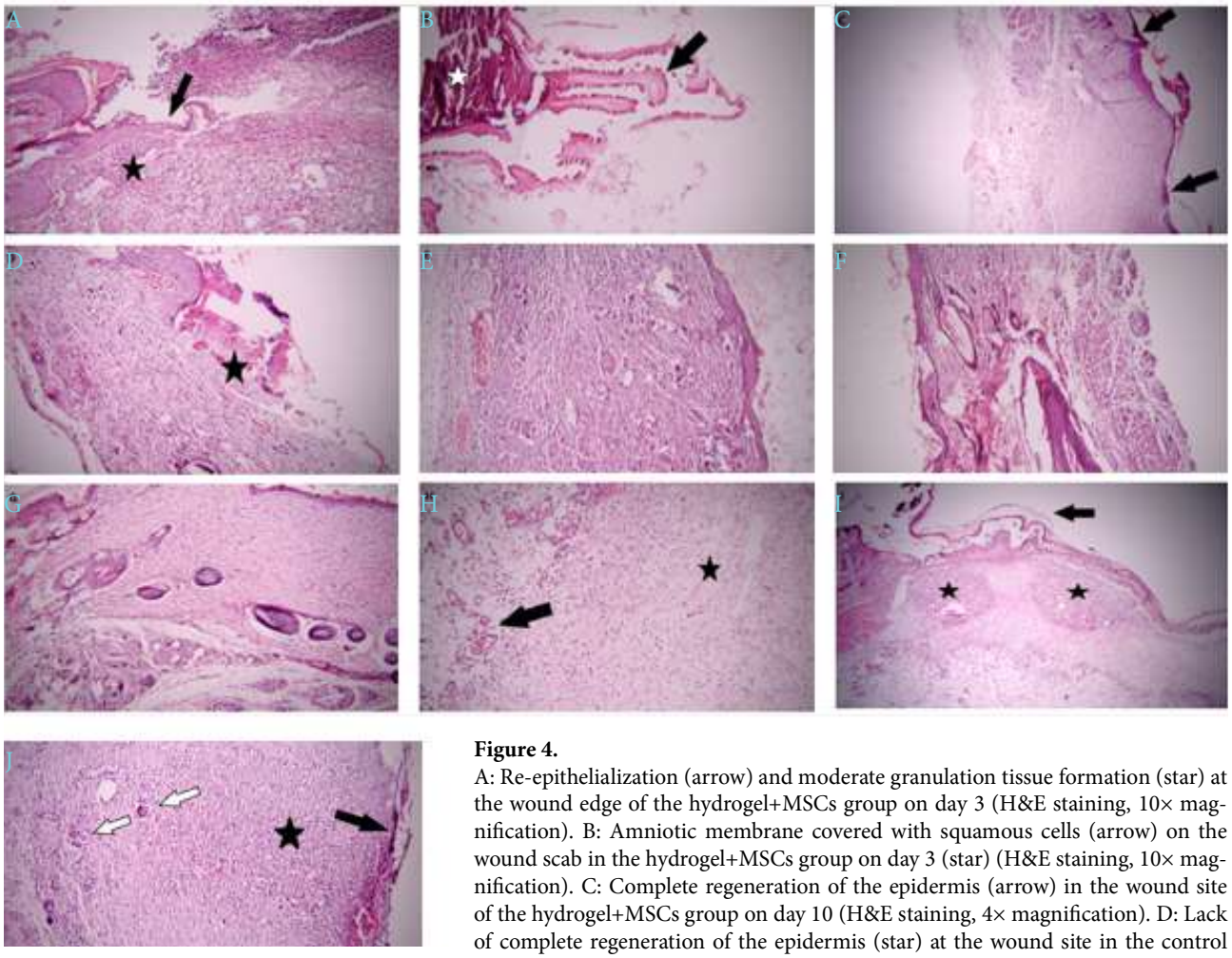

Figure 4.
A: Re-epithelialization (arrow) and moderate granulation tissue formation (star) at the wound edge of the hydrogel+MSCs group on day 3 (H&E staining, 10× magnification). B: Amniotic membrane covered with squamous cells (arrow) on the wound scab in the hydrogel+MSCs group on day 3 (star) (H&E staining, 10× magnification). C: Complete regeneration of the epidermis (arrow) in the wound site of the hydrogel+MSCs group on day 10 (H&E staining, 4× magnification). D: Lack of complete regeneration of the epidermis (star) at the wound site in the control group on day 10 (H&E staining, 10× magnification). E: Diffuse granulomatous inflammation in the dermis area at the wound repair site in the hydrogel+MSCs on day 10 (H&E staining, 10× magnification). F: Diffuse granulomatous reaction at the base of the wound in the hydrogel group on day 21. (H&E staining, 10× magnification). G: Granulomatous reaction around the unrecognizable structure in the control group on day 21. (H&E staining, 10× magnification). H: Hyperemia and the beginning of the angiogenesis process at the base of the wound (arrow) and the absence of granulation tissue formation (star) in the control group on day 3 (H&E staining, 10× magnification). I: Two foci of granulation tissue formation in the center of the wound (star) and the amnion membrane on the surface of the wound in the hydrogel+MSCs group on day 3 (arrow) (H&E staining, 4× magnification). J: Lack of complete regeneration of the epidermis and the presence of a scab on the surface of the wound (black arrow), the formation of abundant granulation tissue and angiogenesis (star), and the presence of granulomatous reaction in the base of the wound (white arrow) (H&E staining, magnification ×10).

imals was designed to determine the persistence of MSCs in the wound tissue. Female rats were injected with a hydrogel containing AD-MSCs of male rats at the wound site. The results obtained from PCR, revealed that MSCs were present until the 3rd day only in the wound of the group receiving hydrogel+MSCs. However, no bands were observed on the 10th and 21st days and in the two other groups (Figure 6).

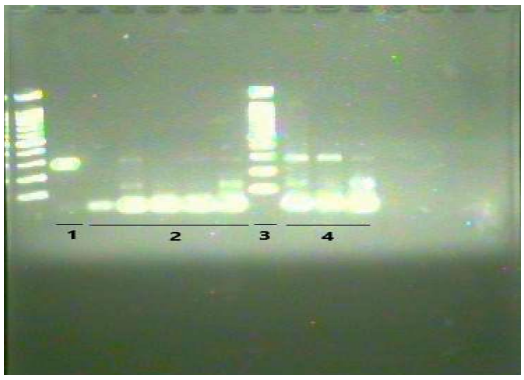

Figure 5.
Representation of the gel electrophoresis to trace the SRY gene in order to check the presence of MSCs in the wound healing site on day 3. From left to right: 1) MSCs isolated from the fat of male rats. 2) Samples were isolated from the hydrogel+MSCs group on day 3. 3) ladder. 4) Replication of three samples from the same group with higher DNA concentration.

Discussion

MSCs can differentiate into other cells and secrete or suppress the growth hormones or essential cytokines in the wound environment. AD-MSCs in large quantities are easily isolated and cultured and have great potential in therapeutic applications [20]. In the present study, the regeneration of the epithelium was completed on day 3 in the hydrogel+MSCs group, and in the other groups, it was completed on day 10. In different studies, the start of epithelialization was different, and most of them had significant differences with the control group. It has been shown in a research that AD-MSCs increase blood supply and the rate of granulation tissue formation in wounds, survive in the wound for up to 14 days, and have lasting effects on the wound [21].

Contrary to the above studies, one investigation showed that between the control and treatment groups, there was no significant difference in terms of the amount of collagen, epithelialization, angiogenesis, and number of fibroblasts and macrophages. They showed that AD-MSCs had a significant effect in reducing the size of the wound, but their effect on the severity of skin lesions and pathological factors was not confirmed. Compared to BMSCs (Bone MSCs), they have a lower ability to differentiate into endothelial cells [22]. Furthermore, Karimi et al.(2014) reported that AD-MSCs had no significant improvement in acute burn wound healing [23].

The present study showed that the hydrogel+MSCs group had the highest amount of granulation tissue and angiogenesis on days 3 and 10, which decreased on day 21. Lotfi et al. (2019) stated that the granulation tissue thickness in the keratinocyte/MSCs/scaffold group rose in the first week, and declined significantly in the second week compared to other groups [24]. In the present study and the research by Lotfi et al., polymers made from natural materials such as hydrogels provided a suitable environment and direct cell contact. In the current investigation, the hydrogel group had the highest amount of granulation tissue on day 21 compared to the hydrogel+MSCs group, which could indicate the critical role of stem cells that have paracrine signaling properties, which reduce inflammation, and promote angiogenesis and cell proliferation [24].

In this study, we used allogenic AD-MSCs. According to the literature, autologous MSCs have more accelerated cicatrization than allogeneic MSCs. However, in burn injuries, allogeneic MSCs can be the only available option [25]. Research showed that the intradermal injection of allogenic AD-MSCs in burn wounds caused a significant difference on the 14th days with the control group [16].

In the detection of the SYR gene by PCR, the band of this gene was observed only on day 3 in the hydrogel+MSCs group. Hanson et al. (2016) injected allogenic male AD-MSCs intradermally in the partial-thickness of female minipigs. In female tissues, male DNA content was evaluated by the PCR amplification of a 377 bp segment from chromosome Y. They observed Y chromosome bands with a decreasing trend on days 0, 7, and 10 [26]. Based on this study, it would have been better to include the cell tracking investigations of day 7 in the present study to better understand this decreasing process . The difference between the present study and the above study may be attributed to the type of animal modeling, method, and amount of stem cell injection. The reason for the decrease in the presence of MSCs in the wound site is unknown. However, it can be because of MSCs migration from the wound site, MSCs phagocytosis by macrophages, or mechanisms involved in cell processing and tissue regeneration [26].

Tragacanth gum hydrogel can be a suitable scaffold for AD-MSCs. It accelerates the proliferation and differentiation of cells and provides a suitable space for the support and adhesion of cells. It is also capable of expressing genes for up to 21 days and maintains the original morphology of cells. In the present study,

the hydrogel and control groups had significantly different epithelialization on day 10. Although no significant difference was observed in inflammation and granulation tissue formation between the hydrogel and other groups, on the 10th and 21st days, the rate of granulation tissue formation in the hydrogel group was higher than in other groups. One of the reasons is the high concentration of hydrogel, and the reduction of inflammation in the stem cell group is may result from the presence of MSCs. A study similar to the current research showed that wound closure occurs faster in the PCL-GT-stem cells group than in the PCL-GT scaffolds group. Granulation tissue, collagen synthesis, and angiogenesis were improved in the PCL-GT-stem cells group. They stated that GT accelerates the transition from the inflammatory and germinal phases as well as the maturation of scar tissue [27–29]. Researchers demonstrated that creams made from Tragacanth gum at a concentration of 6% had the highest effect on rabbit wound healing compared to the control group [27]. In another study, with the daily application of Tragacanth gel, a significant difference was observed in terms of epithelialization, inflammation, and granulation tissue on the 10th day compared to the control group. Similar to the present study, they showed that Tragacanth gum caused most of the wounds to close on the 10th day by accelerating wound contraction [28].

A proper dressing should enhance epithelial regeneration, control the amount of exudate, prevent material leakage, reduce inflammation and infection, and be comfortable for the patient. In this study, amniotic membranes, as an economically reasonable alternative biomaterial were used to benefit from the above characteristics and also prevent hydrogel leakage. In some studies, the use of amniotic membranes alone in wound healing was ineffective [30], but in others, it had no significant difference with the control group or other treatment groups [31]. Studies have shown that using the amniotic membrane alone is effective for shallow wounds while a more effective solution is required in wide and deep wounds, such as full-thickness wounds and third-degree burns [32].

Studies showed that the application of MSCs with cellular/acellular amniotic membrane multiplies the rate of wound healing compared to utilizing amniotic membrane alone [33]. In this study, on the 3rd days in the stem cell group, acellular human amniotic membrane was observed as a serous layer covered with squamous cells on the wound scab, which is a sign of the effective role of MSCs.

In summary, in this study, the synergism effect of MSCs, Tragacanth gum hydrogel, and human amniotic membrane as a dressing was investigated. Histopathology results showed that the combination of SCs and Tragacanth gum hydrogel was influential in the immediate wound closure, and the human amniotic membrane played a supporting role.

Materials and Methods

Ethical statement

All procedures and experiments were performed on animals according to the guidelines of the Animal Care Committee and were approved by the Research Ethics Committees of Ferdowsi University of Mashhad (IR.UM.REC.1400.333).

Isolation and Cell Expansion of ADSCs

Adipose tissue source was prepared from a male Wistar rat (8 weeks old). After general anesthesia with ketamine 10% (50 mg kg-1, Bremer Pharma GmbH, Warburg, Germany) and xylazine 2% (5 mg/kg, Alfasan, Woerden, Netherlands), laparotomy was performed under aseptic conditions. A volume of. 2-3 ml of accumulated fat from the retroperitoneal, perirenal, and inguinal regions was dissected bilaterally and collected with at least manipulation. These samples were transferred to the laboratory in sterile conditions and in the shortest time for the subsequent steps. Adipose tissue was washed with PBS (Gibco, Eggenstein, Germany) containing 1% of the penicillin–streptomycin (Gibco, Eggenstein, Germany) to separate the vessels and connective tissues attached to the fat pieces as much as possible and the process was repeated if necessary. For enzymatic digestion, 1 mg/ml of collagenase type IA [Sigma-Aldrich (St. Louis, MO)] and 10 mg of bovine serum albumin (Invitrogen (Carlsbad, CA)) were dissolved in PBS. After adding 2 mM CaCl2 to this solution, it was sterilized using 0.2 micron filters (Jet Biofil/ Orange, Canada). The collagenase solution was added to 3 ml of adipose tissue fragments obtained in the previous step. The mixture of fat and collagenase was incubated at 37°C for 1 h in a shaking water bath (Memmert. Germany). After the enzyme digestion step, the adipose tissue was diluted at 1:3 with PBS and was centrifuged (800g, 10 min). After centrifuging, four phases were visible. Next, the suspended fats fragments, adipose tissue, and PBS in the three upper phases were removed from the test tube with a pipette without disturbing the cells. In the DMEM / low glucose medium (DENAzist Asia's DMEM-LG (Low Glucose)), the cell pellet was resuspended, and centrifugation (at 400 g for 6 min) of the solution was done. The Sediment (the pellet, which contained MSCs) was resuspended with DMEM/LG and cultured in DMEM-LG with 10% fetal bovine serum and 1% penicillin/ streptomycin. Then, it was transferred to a 75T flask and maintained at a temperature of 37˚C with 95% humidity and 5% CO_2. After 48 hrs, the culture medium was replaced and changed each day until the confluence of cells in the flask reached 80-90%. Then, the previous culture medium was removed, and cells were washed with PBS. Cells were then detached by the application of trypsin–EDTA for subculture [24,34–37]. Briefly, after adding trypsin to the flask, it was incubated for 5 minutes, a new culture medium containing 10% FBS was added to the flask to neutralize the effect of trypsin, and the contents of the flask centrifuged at 400 g for 6 minutes. The supernatant medium was removed, a new medium was added to it, and several times pipetting with a sampler was done until the cells were suspended. After cell counting using trypan blue, 75×10^4 cells were transferred into a new 75T flask, and after adding culture medium, the flask was transferred to the incubator.

Characterization of MSCs

The capability of the cells to differentiate into osteoblasts and

adipocytes was analyzed. Flow cytometry was performed in the previous study to assay the immunophenotype [24].

Adipogenic and Osteogenic Differentiation of MSCs

AD-MSCs 80-90% confluency, after 3 passages, were cultured in the adipogenic differentiation medium (DMEM supplemented with 10% FBS, 100 µmol/L indomethacin, 10 mM β-Glycerol phosphate, and 1 µM dexamethasone) (Sigma, Germany)) for 21 days. After changing this medium, at an interval of 2-3 days, the cells were stained with Oil red O at the end of this differentiation period. Also, for osteogenic differentiation, the cells were cultured in the osteogenic differentiation medium (DMEM supplemented with 10% FBS, 10 mM ascorbic acid, 10 mM β-Glycerol phosphate, and 0.1 µM dexamethasone) (Sigma, Germany)) for 14-17 days and then this induction medium was refreshed at an interval of every 2-3 days and cells were stained with Alizarin red solution. All these steps were performed on MSCs without induction by differentiation medium as a control. After staining, the cells were observed under the iX70 inverted microscope (Olympus, Japan)[37].

Alkaline phosphatase assay

The level of alkaline phosphatase activity increases with the differentiation of MSCs into osteoblast cells. Therefore, alkaline phosphatase activity can be used as an indicator to investigate the differentiation of these cells that can be easily detected by the substrate of this enzyme, i.e. BCIP/NBT. Briefly, after differentiation, the cells were washed with PBS buffer and fixed in 4% paraformaldehyde. The paraformaldehyde solution was removed and washed, then a BCIP/NBT tablet ((5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (Roche, USA) was dissolved in 10 ml of deionized water and 1 ml was poured on the differentiated cells and the control group and kept at room temperature for 5 to 10 minutes. For better penetration of the substrate into the cells, 0.05% Tween-20 was added to the PBS buffer. Then, they were washed with PBS buffer and examined with an inverted light microscope [37].

Preparation of Tragacanth gum hydrogel

To prepare Tragacanth gum hydrogel 6 % (6g/100 mL), after passing sterilization steps under the hood and UV lamp, 1/5 g of Tragacanth powder (Missouri, United States) is mixed with 25 ml of culture medium of DMEM-LG containing FBS and then it was shaken for at least 2hrs.

Preparation of Human Amniotic Membrane (HAM)

The Ethics Committee of the Ferdowsi University of Mashhad approved the use of HAM for this research. After obtaining written informed consent, placentas were obtained during elective cesarean section. Viral diseases were negative for all donors in the serological results. DMEM; Gibco, Scotland contained 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B solution (Biosera UK) was used to place the placental tissue in it. The placenta was washed several times in PBS with an antibiotic/antimycotic solution. Blunt dissection was performed to detach the HAM from the chorion. HAM was divided into approximately 2.5 × 2.5 cm pieces. To perform de-epithelialization, 0.5 M NaOH (Sigma-Aldrich) was used with a cotton-tipped applicator to rub the cells, followed by washing with sterile PBS in a shaker for 10–15 min. To confirm the cell removal of the amniotic membrane, DAPA staining (2µg/ml PBS) was done [38,39].

In vivo wound healing experiments in an animal model

In total, 54 female rats aged 1.5 months and weighting ~150 g were randomly divided into control, Tragacanth gum hydrogel (hydrogel) and Tragacanth gum hydrogel+MSCs (hydrogel+MSCs) groups. The rats were anesthetized by an intraperitoneal injection of 50 mg/kg ketamine and 5 mg/kg xylazine. To evaluate

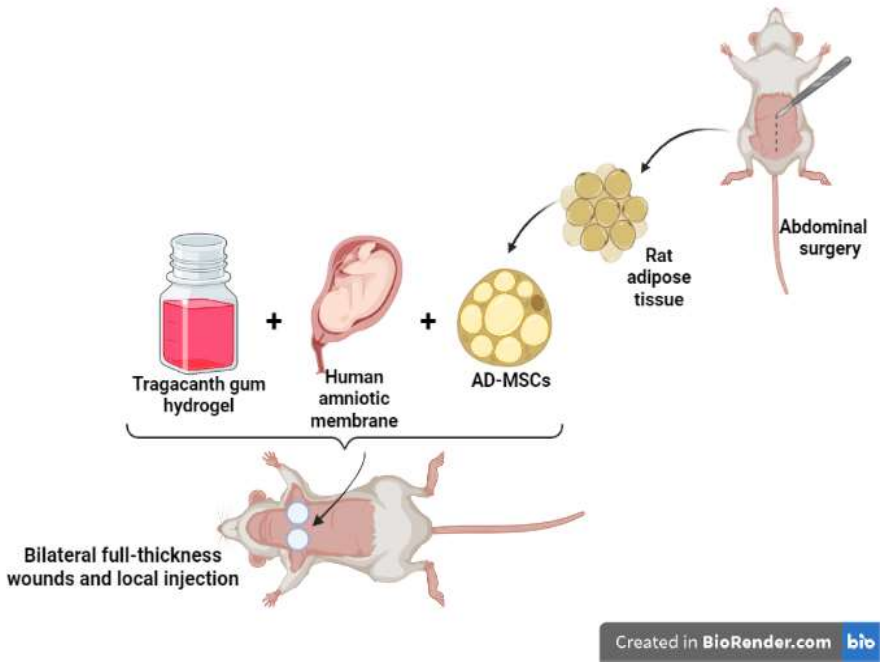

Figure 6.
Schematic representation of the study. Created with BioRender.com.

the effects of these compounds on wound healing, two full-thickness circular wounds of equal sizes (8.7 × 8.7 mm2) were aseptically generated using biopsy punches on either side of the dorsal area of each rat. The rats in the control group were treated with a hydrofilm dressing; in the hydrogel group, the amniotic membrane was sutured on the wound, 0.1 ml hydrogel was injected, and hydrofilm dressing was placed on them. In the hydrogel+MSCs group, following the suturing of the amniotic membrane, 0.1 ml gel-containing cells (3×10^5) were placed on the wound, and finally, the hydrofilm dressing was glued on the set (Figure 6).

During this research, the rats were kept in the animal house. They were placed individually in cages a temperature of 24˚C and a cycle of 12 hrs of light and 12 hrs of darkness with free access to water and food.

Histopathology

On days 3, 10, and 21 after surgery, six rats were selected, and after euthanasia with chloroform, sampling was conducted under sterile conditions. One of the two wounds was selected randomly, and the skin wound and surrounding skin were fixed in 10% formaldehyde, embedded in paraffin, and cut into 5 µm sections. Standard H&E staining was performed, and the samples were evaluated quantitatively and qualitatively in terms of epithelium of regeneration of the epithelium, the number of inflammatory cells in the area, and the amount of granulation tissue formation. The evaluated factors were scored as follows: regeneration of the epithelial tissue (beginning of the regeneration of the epithelial tissue or thickening of the cut edges = 1, coverage of less than half of the wound = 2, coverage of more than half of the wound = 3, coverage of the entire wound with low thickness = 4 , coverage of the entire wound with keratinization and normal thickness = 5); degree of inflammation (absence of inflammation = 0, mild inflammation = 1, moderate inflammation = 2, severe inflammation = 3); degree of formation and maturation of granulation tissue (absence of granulation tissue = 0, low granulation tissue formation = 1, moderate granulation tissue formation = 2, abundant granulation tissue formation = 3, abundant granulation tissue formation and with tissue maturity = 4)[40].

PCR

To evaluate the persistence of MSCs injected in the local tissue wound bed, tissue samples were taken in sterile conditions and were frozen (-80˚C). As a pre-extraction step, these sections were crushed in liquid nitrogen using sterile pestles and mortars, and the obtained powder was used for DNA extraction. Genomic DNA was isolated using the Animal DNA Isolation Kit (DENAzist Asia Co., Mashhad, Iran) according to the manufacturer's instructions. To trace the MSCs of male rat adipose used in the wound site, the Y chromosome was selected by the SYR gene [41]. Because living rats are female, the mentioned gene could be traced. Primers that were used to amplify the SRY gene (PCR product size: 273 bp; Gene ID: 103694554) were 5'- GTAGGTTGTTGTCCCATTGC-3' and 5'-GAGAGAGGCACAAGTTGGC-3'. The PCR protocol consisted of an initial denaturation step at 94°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 20 sec. A final extension of 5m at 72°C was applied at the end of the PCR reaction. The extracted DNA was quantified by a spectrophotometer at wavelengths of 260 nm and 280 nm. After DNA extraction, a 1.5% agarose gel was used, which consisted of 0.75 g of agarose powder, a solution containing 1 ml of TAE buffer, 49 ml of distilled water, and 1µL of ethidium bromide. For preparing of the Master mix solution, 20 ng per PCR sample was taken from the extracted DNA, and the final volume was adjusted to 10.5 µL with distilled water. The samples were then spun, and 14.5 µL of the Master mix and the primer solution were added to each sample. After another round of spinning, the samples were ready for use.

Statistical analysis

Statistical analyses were performed using SPSS (Ver. 26). Appropriate tests (Kruskal-Wallis Test and Mann–Whitney U-test) were used to analyze the data. $p < 0.05$ was considered significant.

Authors' Contributions

Conceptualization and Methodology: Hossein Kazemi Mehrjerdi, Hojjat Naderi-Meshkin, Hossein Nourani, Jeiran Rahvarian; Formal analysis and investigation: Hossein Kazemi Mehrjerdi, Shiva Amanollahi; Writing - original draft preparation: Shiva Amanollahi, Jeiran Rahvarian; Writing - review and editing: Shiva Amanollahi and Hossein Kazemi Mehrjerdi; Funding acquisition and Supervision: Hossein Kazemi Mehrjerdi, Hojjat Naderi-Meshkin.

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Competing Interests

The authors have no financial conflicts of interest.

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Online supplemental material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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RESEARCH ARTICLE

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Effect of Single-dose Pimobendan on Echocardiographic Parameters in Healthy New Zealand White Rabbits

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ABSTRACT

keeping rabbits as pets and their use in laboratory research increased the need for studying heart diseases and treatments in rabbits. Pimobendan is one of the most common medications used in cardiac diseases and is anecdotal in rabbits. The first step toward the approval of pimobendan in rabbits is assessing the potential for beneficial effects on cardiac function through echocardiographic functional parameters.This study aimed to determine the effects of pimobendan on echocardiographic parameters. Eleven rabbits were included in this study. Echocardiographic examinations were performed before and after pimobendan administration for each rabbit. The LV morphological and functional parameters were compared between study time points. Pimobendan resulted in changes in several echocardiographic variables in the rabbits, including FS and an increase in EF, SV, LVPWs, and LA end-systolic ($p = 0.0001$, $p = 0.0001$, $p = 0.0284$, $p = 0.0272$, and $p = 0.0007$, respectively). Moreover, LVIDs and end-systolic volume decreased ($p = 0.0343$ and $p = 0.038$). The changes in some parameters were not significant, such as LVIDd, LVPWd, end-diastolic volume, LA max, Mitral annulus diameter, and LA/Ao. FS, which indicates an increase in the power of heart contraction and consequently an improvement in heart function, increased in this study after pimobendan administration. Therefore, it can be concluded that pimobendan improves cardiac functions. Further studies are required to investigate whether pimobendan has similar effects in rabbits with cardiac diseases.

Keywords

Echocardiography, Pimobendan, Rabbits, Cardiac function

Number of Figures: 2
Number of Tables: 1
Number of References:: 26
Number of Pages: 6

Abbreviations

LVIDs: Left ventricular internal diameter end-systole

LVIDd: Left ventricular internal diameter end-di-

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Introduction

Heart disease in rabbits can result in CHF, characterized by an excess of fluid volume resembling the signs of left-sided failure, such as pulmonary edema and pleural effusion, as well as the signs of right-sided failure, including abdominal effusion, hepatomegaly, and splenomegaly. The initial symptoms of heart disease include reduced activity, weight loss, alterations in eating patterns, and breathing difficulties.

Pimobendan, a benzimidazole pyridazinone medication, has demonstrated significant advantages in CHF, preclinical DCM, and preclinical degenerative valve disease in dogs [1, 2]. Pimobendan is used to treat dogs with CHF secondary to DCM. It has positive inotropic and vasodilatory effects via phosphodiesterase 3-inhibition and calcium sensitization [1]. It has other effects, such as increasing coronary blood flow, positive effects on myocardial oxygen consumption, and satisfactory effects on myocardial compliance [2].

Rabbits have been widely used as animal models to study various cardiac diseases, namely endocarditis and cardiomyopathies [3, 4]. The most common types of cardiovascular disease in rabbits include myocarditis, arteriosclerosis, and cardiomyopathy. Electrocardiograms [5, 6], blood pressure measurements, and echocardiograms [7, 8] can be used for diagnostic purposes in rabbits. The extra-label use of pimobendan in rabbits with CHF has garnered a lot of attention in the past decade. The positive inotropic effect of the medicine has been reported in rabbits without any negative impacts on morbidity and mortality [9].

In 2020, Ozawa et al. evaluated the pharmacokinetics of oral pimobendan administrated to healthy New Zealand White rabbits. According to their findings [10], the half-life of pimobendan was 3.54 ± 1.32 h, the plasma concentrations were detected for up to 24 hours, and desmethyl pimobendan, which is the active metabolite of pimobendan, was detectable for 24-36 h [10].

The present study was designed to address the knowledge gap by investigating the echocardiographic effects of pimobendan administered orally to healthy New Zealand White rabbits.

Abbreviations Cont'd

astole
LVPWs: Left ventricular posterior wall end-systole
LVPWd: Left ventricular posterior wall end-diastole
IVSs: Interventricular septum end-diastole
IVSd: Interventricular septum end-systole
EDV: End-diastolic volume
ESV: End-systolic volume
EF: Ejection fraction
SV: Stroke volume
FS: Fractional shortening
LA max: Left atrium maximum dimension
LA min: Left atrium minimum dimension
MVA max: Mitral valve maximum area
MVA min: Mitral valve minimum area
LA/Ao: Left atrium to Aorta ratio
CHF: Cardiac heart failure
DCM: Dilated cardiomyopathy
LV: Left ventricle
LA: Left atrium

Results

Pimobendan caused a significant increase in SV, EF, FS, LVPWs, and the minimum size of LA compared to the pre-drug group ($p < 0.05$), whereas LVIDs and ESV decreased significantly ($p = 0.0343$ and $p = 0.038$, respectively). No significant change was observed in some parameters, including LVIDd, LVPW thickness in diastole (LVPWd), EDV, LA max, Mitral annulus diameter, and LA/Ao (Figure 1,2).

Table 1 summarizes the changes from the base time (before administration) up to 3 h after the oral administration of pimobendan.

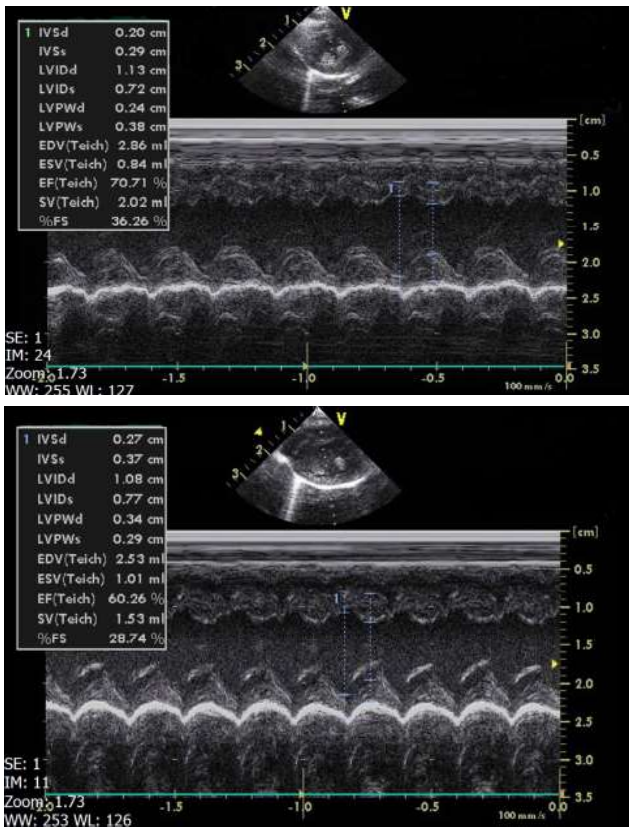

Figure 1.
Echocardiographic parameters in a rabbit before (lower image) and 3 hours after (upper image) the oral administration of pimobendan at 0.3 mg/kg

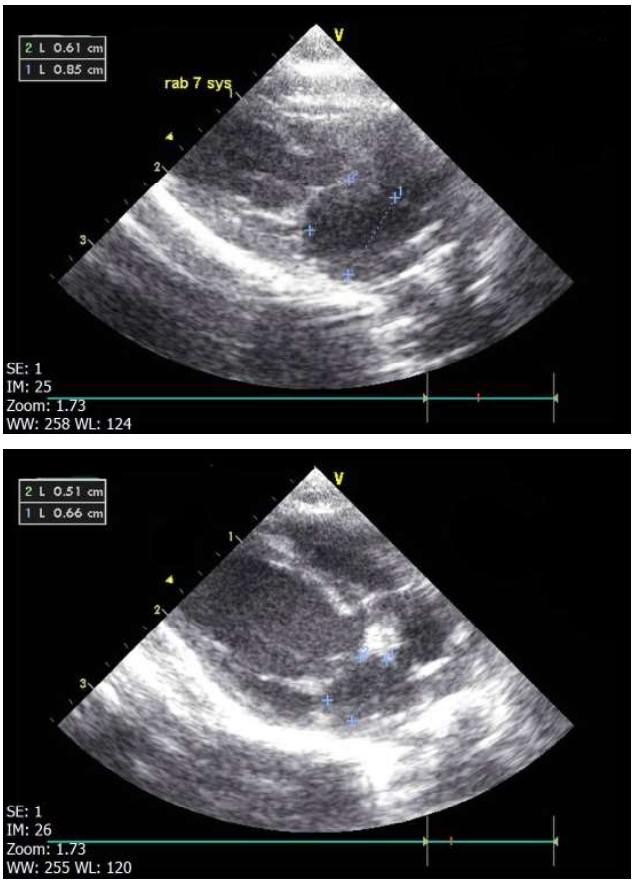

Figure 2.
Left atrium size and mitral annulus diameter during systole (upper image) and diastole (lower image)

Table 1.
Comparison of echocardiographic parameters before and after pimobendan administration

Variables	Pre-drug	Post-drug	*p*-value
IVSd	0.23 ± 0.02	0.22 ± 0.01	0.2158
IVSs	0.31 ± 0.01	0.32 ± 0.01	0.1388
LVIDd	1.05 ±0.01	1.09 ± 0.02	0.2537
LVIDs	0.74 ±0.02	0.73 ± 0.02	0.0343
LVPWd	0.29 ± 0.01	0.29 ± 0.01	0.4537
LVPWs	0.28 ± 0.01	0.30 ± 0.01	0.0007
EDV	2.36 ± 0.58	2.61 ± 0.13	0.2632
ESV	0.92 ± 0.16	0.89 ± 0.05	0.038
FS%	29.4 ± 0.64	32.95 ± 0.76	0.0001
EF%	60.99 ± 0.47	65.94 ± 0.99	0.0001
SV	1.4 ± 0.26	1.71 ± 0.09	0.0284
LA max	0.76 ± 0.02	0.78 ± 0.01	0.2736
LA min	0.57 ± 0.01	0.60 ± 0.01	0.0272
MVA max	0.52 ± 0.01	0.53 ± 0.01	0.7082
MVA min	0.46 ± 0.02	0.47 ± 0.01	0.3111
LA/Ao	1.12 ± 0.02	1.12 ± 0.02	0.7919

Discussion

This study found results about the effects of pimobendan on the LV function of healthy adult rabbits. The LV function increase by pimobendan was confirmed by some echocardiographic LV variables.

There was no significant change in LVID during diastole, while LVID during systole decreased after 3 h. Pimobendan had the same impact in a study on dogs with asymptomatic mitral valve disease and another research on beagle dogs [11, 12]. Moreover, in studies conducted by Boswood et al. and Haggstrom et al. on dogs with myxomatous Mitral valve disease both LVIDd and LVIDs decreased. In another investigation by Kinel et al. (2021) on dogs with Mitral valve disease, pimobendan only reduced the size of LV during diastole [13–15].

The LV posterior wall was slightly increased during systole. This finding was found in two studies by Yata et al. on healthy dogs and healthy cats [1, 2]. The main cavity of the heart is LV and the blood in LV is pumped in the aorta to deliver oxygenated blood to all body tissues. Therefore, a rise in LVPW can interfere with the ability of the heart to pump blood in the aorta. However, the increase in LVPWs in this study was very mild and remained in the normal range of LV posterior wall thickness in rabbits. As a result, it did not have destructive effects on the LV function.

The FS reflects the LV systolic function. Our study showed increased left ventricular FS after pimobendan administration, which means a rise in LV function. Left ventricular FS has been used in veterinary medicine [16] and humans [17] to assess LV systolic function. Prior studies have shown increased left ventricular FS in other animals. For example, a study evaluated cardiovascular effects after a single dose of pimobendan in healthy cats. The same result was found by Ro et al. after the oral administration of a pimobendan-pentoxifylline mixture in dogs [1, 18]. Changes in some echocardiographic parameters that indicate systolic function, such as the increase in FS and decrease in LVIDs, indicate the positive inotropic effect of pimobendan in rabbits.

In the current study, we observed a reduction in ESV after 3 h, but there was no significant change in the blood volume at the end of diastole. This finding is similar to that of Sengklab et al. (2022) [12]. Many studies of M-mode in veterinary medicine, showed a strong relationship between cardiac volume measurements (ESV and EDV) and cardiac output (19). In the present study, SV rose after 30 min of pimobendan administration. Despite the lack of change in cardiac output, it can be concluded that pimobendan can augment cardiac output, thereby improving cardiac function in diseases, such as heart failure in which cardiac output decreases. In similar studies on

dogs, different results had been found; SV increased after pimobendan administration in dogs with DCM, while in another study there was no change in SV in dogs with Mitral regurgitation [20, 21]. While SV rose after pimobendan administration in some studies on dogs, there was no change in SV in other research on dogs with Mitral valve regurgitation [20, 21].

The EF, as the gold standard index in evaluating LV, significantly increased after taking pimobendan. It depends on preload, afterload, and heart contractility. In patients with CHF, it can be reduced, which means the heart pumps less than before. With a significant increase in EF in our study, it can be concluded that pimobendan can be used in rabbits with CHF or any other diseases that can decrease EF. Similar results were found in an investigation on 24 dogs with Mitral valve disease [11] and another study on dogs with DCM treated with pimobendan [20].

There are a few studies about LA size after pimobendan administration. We observed that left atrial size at the end of the diastole increased slightly after 30 min and did not change since then. However, a study on dogs with cardiomyopathy showed that pimobendan did not cause a change in the size of the LA [22]. In addition, in a study on the left ventricular function of myxomatous Mitral valve disease in dogs treated with pimobendan, a similar result was obtained, and no change in the size of the LA was reported [23]. Pimobendan had similar effects in a study on the effects of pimobendan on left atrial transport function in cats [24].

Considering the discrepancy in the findings concerning the effects of pimobendan on LA in rabbits, further research is required. The reason for increasing the size of the LA in our study was not found.

Conclusion

Echocardiographic results after pimobendan administration indicated that pimobendan has useful effects on LV function in healthy rabbits. Further evaluations are needed to find out whether pimobendan is effective in rabbits with cardiac disease.

Materials and Methods

Ethical statement

Islamic Azad University, Karaj Branch approved all the procedures used in the current study for the care and treatment of animals (IR.IAU.K.REC.1401.018).

Animals

Eleven White New Zealand rabbits (24-30 weeks old and weighing 2.1 ± 0.3 kg) were purchased from the Pasteur Institute of Iran (Tehran, Iran) for this study. All the rabbits were adopted under supervision after the experiments were finished.

Medication

Pimobendan (0.3 mg/kg, Vetmedin 5 mg chewable tablet, Boehringer Ingelhei, Germany) was dissolved in distilled water and administered orally [10]. The medication dosage was calculated according to the weight of each rabbit, and an appropriate amount of powdered pimobendan, depending on the weight of each animal, was separately dissolved in 1 cc of water and gently administered from the corner of their mouth.

Echocardiography protocol

Echocardiography was carried out under spontaneous respiration, and without any anesthesia. It was performed by a single board-certified radiologist using a GE Vivid 7 ultrasound machine equipped with a phased-array (S10) probe (5-10 MHz). To obtain the right parasternal window, rabbits were positioned in the right lateral recumbency, and an ultrasound probe was placed on the cranial aspect of the thoracic wall, which had been shaved before, through a gap in the echocardiography table.

For a short axis view from the right parasternal, M-mode imaging was made at the level of the papillary muscles. Measurements of IVSs, IVSd, LVIDs, LVIDd, left ventricular free wall in systole (LVFWs), and in diastole (LVFWd) were performed. The right parasternal short-axis view with M-mode was used for measuring the aortic and left atrial diameters at the level of the aortic valve. M-mode parameters were measured by the leading-edge method of the American Society of Echocardiography. Left ventricular EF and FS were calculated by the following formulas:

$FS = [(LVIDd - LVIDS) / LVIDd] \times 100$

$EF = (SV / EDV) \times 100$

$SV = EDV - ESV$

Study design

Rabbits were healthy based on physical, radiologic, echocardiographic, and hematologic examinations.

Chest radiography for possible respiratory disorders was obtained for all subjects. The animals were fed with water and pellet. They were housed in steel cages at temperatures of 21°C-24°C, with a 12:12 h light-dark cycle.

Each rabbit went through five echocardiographic examinations. On day 0 the first examination was performed, and 1 hour apart possible valvular blood regurgitation and thickening were checked by color Doppler and M-mode imaging. The third examination, the baseline, was done on day 1; the fourth and fifth evaluations were completed 30 min and 3 h after a single dose of pimobendan was administered, respectively [26]. The planning of the echocardiographic assessment time depended on a pharmacokinetics investigation of pimobendan in rabbits [10].

Statistical analysis

To compare the echocardiographic data in pre-drug and post-drug conditions, the average records were collected during three quantitative assessments of pre-drug. The records were investigated between time points using SAS-9.2 software. In this research, the primary descriptive statistics of the data and the primary statistical distributions of the data were obtained and investigated. To examine the normal distribution of data and variance independence, the Kolmogorov-Smirnov test was performed at a statistical level of 5%. Moreover, an analysis of variance was performed.

The mean values and standard deviations for each experimental group, including the pre-drug, 30 min post-drug, and 3 hours post-drug, for each parameter extracted, and comparisons between the means were performed by Duncan's multiple range test. The coefficient of variation, mean value, and total standard deviation with the level of significance were calculated. Differenc-

es at $p < 0.05$ were considered significant.

Authors' Contributions

Ariana Askari Ghalehi, Ali Moradganjeh, Vria Tohidi, and Ali Roustaei conceived and planned the experiments. Ariana Askari Ghalehi, and Ali Roustaei carried out the experiments. Ariana Askari Ghalehi planned and carried out the simulations. Ariana Askari Ghalehi and Ali Roustaei contributed to sample preparation. Ariana Askari Ghalehi contributed to the interpretation of the results. Ali Moradganjeh took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Antidiabetic effects of the heat-killed *Actinomycetales* species in the liver and kidney of diabetic rats

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ABSTRACT

Type 1 diabetes mellitus (T1DM) occurs due to the decrease in insulin secretion following the destruction of pancreatic beta cells. This disease is increasing worldwide, especially among children under the age of 5 years, which is usually associated with irreversible complications such as hepatopathy and nephropathy. The present study aimed to investigate the antidiabetic effect of the heat-killed Actinomycetales species, including Gordonia bronchialis (Gb), and Tsukamurella inchonensis (Ti) in streptozotocin-diabetic rats by oral administration. This experiment was performed in six groups, including healthy control, diabetic control, low-dose Gb (G1), high-dose Gb (G2), low-dose-Ti (T1), and high-dose Ti (T2). Subsequently; the levels of ALT, AST, total protein, albumin, BUN, creatinine, CRP, IL-1β, and IL-2 were measured in the serum samples in the 14th and 21st days. Besides, histopathological lesions were studied in the liver and kidney. Our findings showed that Gb and Ti could alter the examined serum parameters, particularly in the T2 groups. Also, histological examination revealed a remarkable attenuation in the pathological lesions such as focal necrosis, vascular congestion, and hemorrhage in the liver and kidney of the treated rats by Gb and Ti. Here, it is concluded that oral administration of the heat-killed Actinomycetales species, particularly with a high dose of Ti, could beneficially improve the progression of T1DM and its various complications, which can be used to treat T1DM in the future.

Keywords

Type 1 diabetes mellitus, Gordonia bronchialis, Tsukamurella inchonensis, hepatopathy, nephropathy

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Abbreviations

DM: Diabetes Mellitus
T1DM: Type 1 DM
T2DM: Type 2 DM
Gb: Gordonia bronchialis
Ti: Tsukamurella inchonensis
CRP: C- Reactive protein

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Introduction

Diabetes Mellitus (DM) is not a single disease but a general term that describes a collection of metabolic conditions, that result in high blood glucose levels due to defects in insulin function or secretion or both [1, 2]. Increasing evidence reported that it has affected approximately 285 million individuals globally, and this number is anticipated to increase to 439 million in 2030 [3], which is associated with severe and irreversible complications, such as nephropathy and hepatopathy [4]. Type 1 DM (T1DM) and Type 2 DM (T2DM) are the two primary forms of diabetes [5]. T1DM, formerly known as insulin-dependent diabetes mellitus (IDDM) [2]. The annual incidence of T1DM varies widely in different countries (from less than one person in 100,000 in Asia to more than 41 cases in 100,000 people in Europe). Children are newly diagnosed with this disease [5]. This disease is increasing worldwide, especially among children under the age of 5 years [1, 5-7]. Chemokines play a crucial role in both the immune system and inflammatory processes, which have been suggested as inducers of β-cell damage in human insulin-dependent diabetes mellitus [1].

Actinomycetales species can switch off pre-existing Th2 preponderance and stimulate Th1-mediated mechanisms. Recently, some aerobic Actinomycetales species, like Gordonia bronchialis and Tsukamurella inchonensis are capable of exerting subtly different adjuvant or immunomodulatory activities [7, 8]. In this regard, it has been revealed that subcutaneous injection of these killed bacteria improves T2DM and obesity in mice animal models [8]. Also, our previous reports presented the improvement impacts of the heat-killed Actinomycetales species in the pancreas [9], testes [10], and intestine [11] of diabetic rats. Thus, in the present study, the beneficial effects of the heat-killed Actinomycetales species, including Gordonia bronchialis (Gb) and Tsukamurella inchonensis (Ti), were investigated in streptozotocin-diabetic rats by oral administration. For this purpose; the liver and kidney biochemical indicators such as ALT, AST, total protein, albumin, blood urea, and creatinine were evaluated in the serum samples. Besides; the C-reactive protein (CRP), IL-1β, and IL-2 levels were measured and associated with histopathological evaluation of the liver and kidney.

Abbreviations Cont'd

STZ: Streptozotocin
AST: aspartate aminotransferase
ALT: alanine aminotransferase

Results

Biochemical findings

Lower levels of serum insulin along with elevated glucose values were detected in control diabetic rats compared to the other treated groups (supplementary file). Interestingly, there were no significant differences ($p > 0.05$) in glucose values between the diabetic rats and the treated groups in a dose-dependent manner. Moreover, lower insulin levels were observed in the diabetic rats, which improved significantly in the treated groups by using the bacteria, especially in Ti-recipient groups (Figure supplementary 1).

The marked decreased values of serum albumin and total protein (figure 1A, B) were assessed in the diabetic animals when compared with other groups, which improved beneficially in all diabetic-treated groups. In albumin measurement, there were notable differences in healthy rats with other experimental groups, and also a marked difference ($p < 0.05$) was noted among the low-dose and high-dose Gb recipient groups on the 14th and 21st sampling days. In total protein data, both low and high-dose Gb and Ti recipient groups showed significant differences ($p < 0.05$) with the healthy and diabetic animals. Notably, the highest levels of both albumin and total protein were observed in the T1, G2, and G2 groups, on the 7th, 14th, and 21st sampling days.

Significantly decreased levels of blood urea and creatinine (figure 1C, D) ($p < 0.05$) were observed in all diabetic-treated groups as compared with the diabetic group. The significant difference in low-dose and high-dose groups was only in urea values on the 14-sampling day.

The activities of AST and ALT diminished in the diabetic rats (figure 1E, F) when compared with the healthy and diabetic-treated rats, particularly in Gb-recipient groups in a dose-dependent manner ($p < 0.05$).

CRP, IL-1β, and IL-2 serum levels

Here, remarkably higher levels of IL-1β and CRP inflammatory cytokines were found within the diabetic rats as compared to healthy animals, which improved in a dose-dependent manner in all diabetic-treated groups (figure 1G, H). On the other hand, considerably lower levels of IL-2 were observed in the diabetic animals when compared with the healthy rats. The serum levels of IL-2 significantly increased and improved in all diabetic-treated groups without a dose-dependent manner between Gb-recipient and Ti-recipient groups.

Histopathological findings

In the liver, healthy control rats presented a nor-

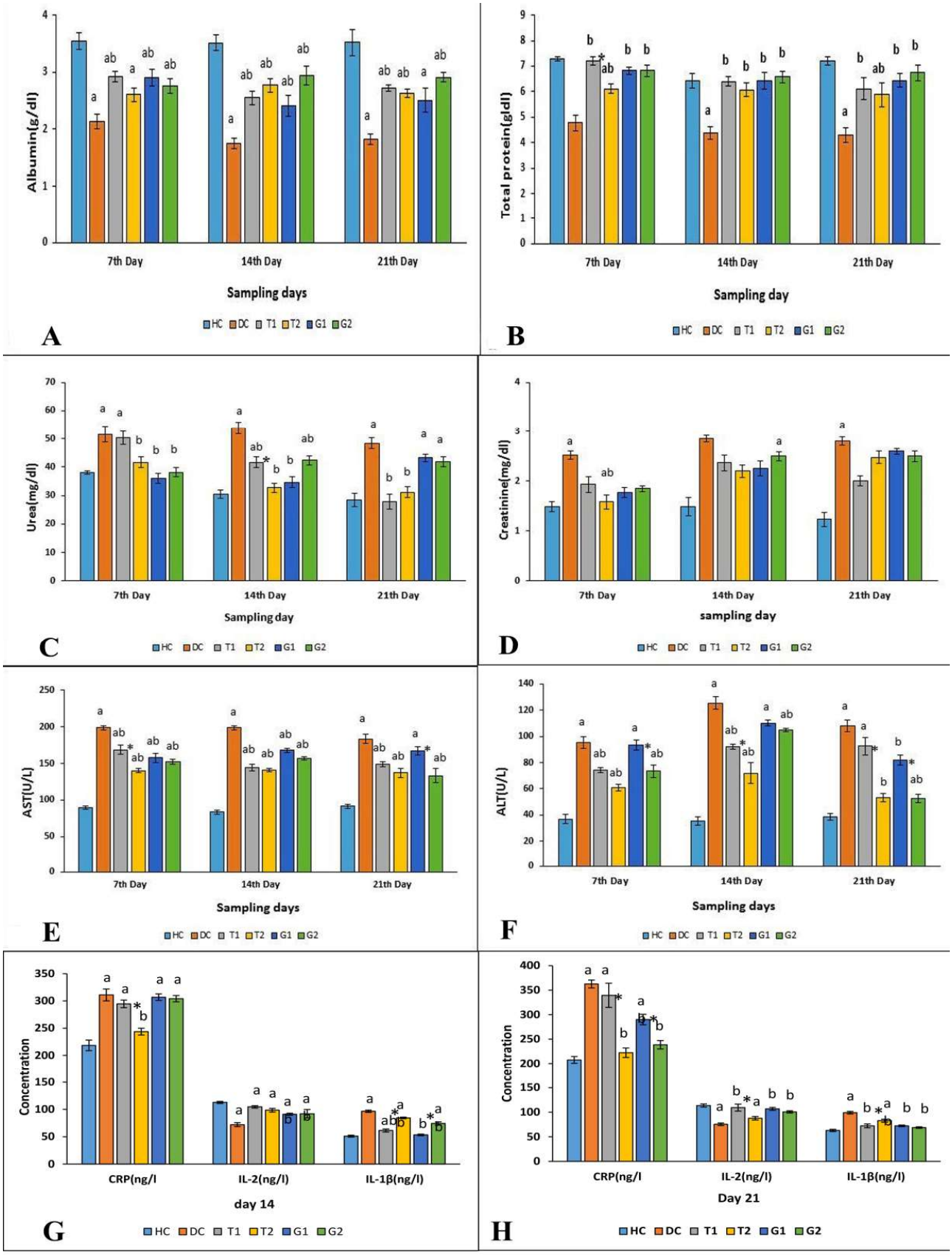

Figure 1.
The effects of oral administration of Actinomycetales species on the serum levels of albumin (A), total protein (B), urea (C), creatinine (D), AST (E), ALT (F), CRP/Il-2/IL-1β/day 14 (G), and CRP/Il-2/IL-1β/day 21 on STZ-induced diabetes. Data are presented as the mean ± SD. Differences were considered significant with $p < 0.05$. a: a significant difference with healthy control (HC); b: a significant difference with diabetic control (DC); *: a significant difference between low-dose and high-dose treated groups.

mal tissue structure consisting of evenly arranged polyhedral hepatocytes radiating outward from the central vein to the periphery. By contrast, there were severe to moderate pathological changes in the control diabetic group, including cell swelling and vacuolar degeneration of hepatocytes, particularly around the central veins, dilatation, and congestion of sinusoids, congestion in the central veins, focal single-cell necrosis, and mild hepatitis. Surprisingly, the livers of the animals in T1, T2, G1, and G2 groups exhibited marked improvements in all of the histopathological features (figure 2), particularly in the Ti high dose recipient group in the 21st after treatment with mild hepatocyte degeneration and vascular congestion.

In the kidney, a normal renal parenchymal structure (figure 3), together with well-defined glomeruli and tubules, was observed in the healthy control rats. In contrast, the diabetic animals with no treatment presented severe to moderate pathological changes comprising tubular epithelium degeneration, vacuolization and single-cell necrosis, vascular congestion, focal hemorrhage, focal interstitial nephritis, and atrophy with the congestion of glomeruli. Interestingly, all treated groups showed significant improvements in the renal lesions, mainly at each of both doses of Gb, which presented only mild vascular congestion and tubular hyaline casts.

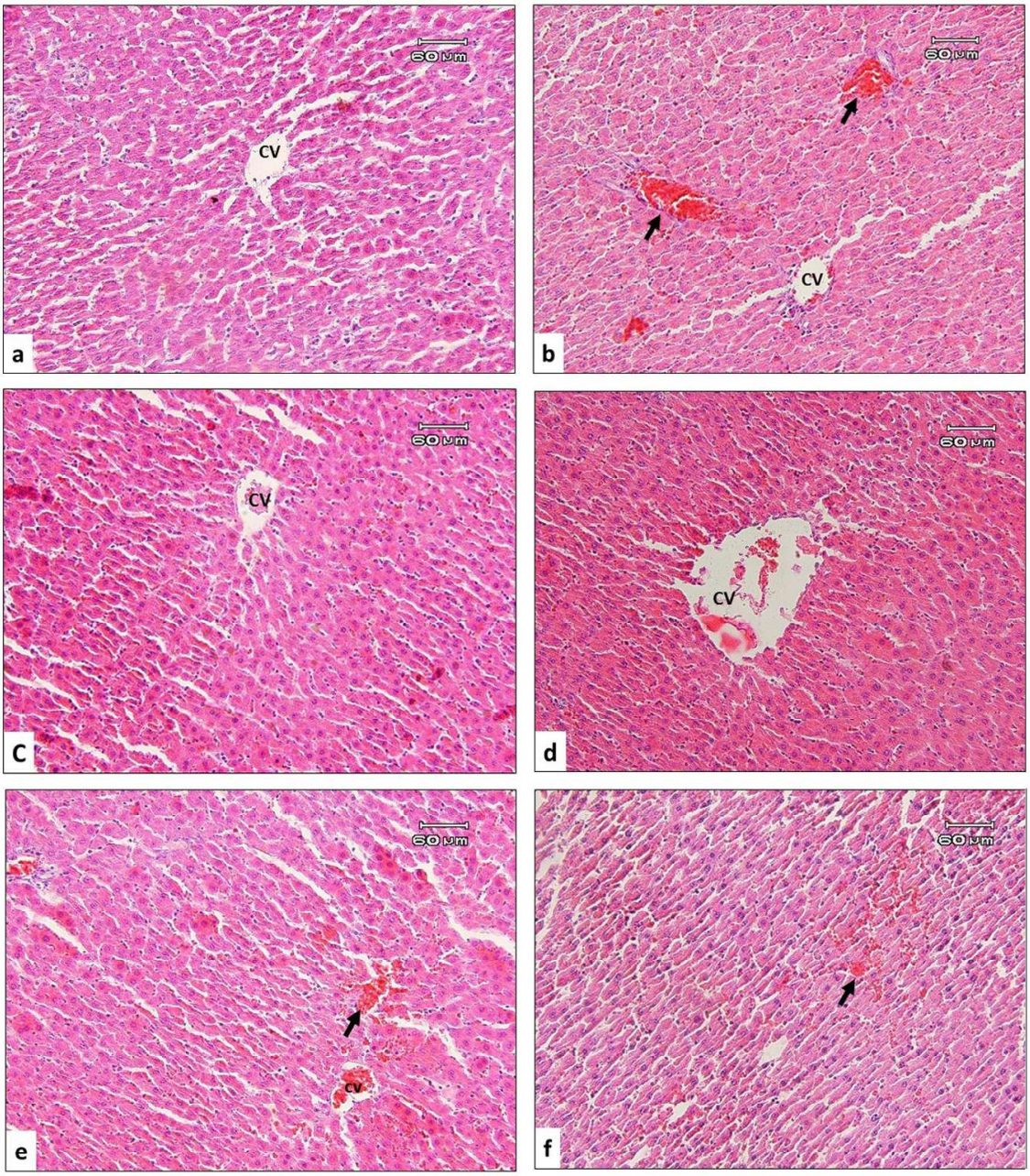

Figure 2.
Liver, rat, STZ-induced diabetes. a: healthy control with a normal liver structure; b: diabetic control with severe cell swelling and hemorrhage (arrows); c: high dose Ti-recipient group (T2) with mild cell swelling; d: low dose Ti- recipient group (T1) with mild to moderate cell swelling; e: low dose Gb- recipient group (G1) with mild to moderate cell swelling and hemorrhage (arrows); f: high dose Gb-recipient group (G2) with mild cell swelling and focal hemorrhage (arrow). H&E.

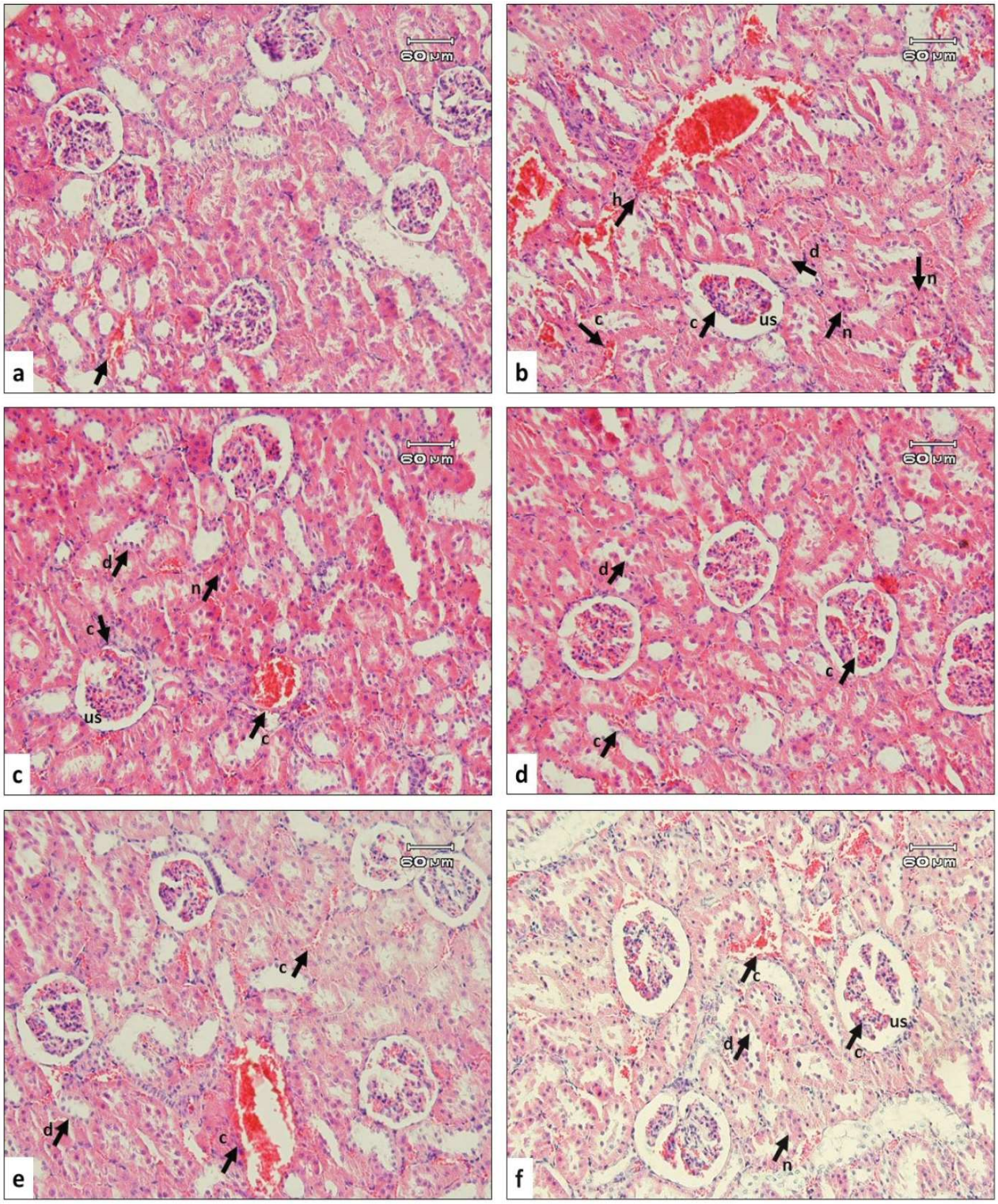

Figure 3.
Kidney, rat, STZ-induced diabetes. a: healthy control with a normal renal parenchymal structure; b: diabetic control showed severe to moderate tubular epithelium degeneration (d), vacuolization and cell necrosis (n), congestion (c) and hemorrhage (h) associated with enhancement of urinary space (us); c: low dose Gb-recipient group (G1) with mild congestion; d: high dose Gb- recipient group (G2) with mild congestion; e: low dose Ti- recipient group (T1) with mild to moderate congestion and cellular degeneration (arrows); f: high-dose Ti-recipient group (T2) with mild to moderate congestion and cellular degeneration (arrows). H&E.

Discussion

MSCs can differentiate into other cells and secrete or suppress the growth hormones or essential cytokines in the wound environment. AD-MSCs in large quantities are easily isolated and cultured and have great potential in therapeutic applications [20]. In the present study, the regeneration of the epithelium was completed on day 3 in the hydrogel+MSCs group, and in the other groups, it was completed on day 10. In different studies, the start of epithelialization was differ-

ent, and most of them had significant differences with the control group. It has been shown in a research that AD-MSCs increase blood supply and the rate of granulation tissue formation in wounds, survive in the wound for up to 14 days, and have lasting effects on the wound [21].

Contrary to the above studies, one investigation showed that between the control and treatment groups, there was no significant difference in terms of the amount of collagen, epithelialization, angiogenesis, and number of fibroblasts and macrophages. They showed that AD-MSCs had a significant effect in reducing the size of the wound, but their effect on the severity of skin lesions and pathological factors was not confirmed. Compared to BMSCs (Bone MSCs), they have a lower ability to differentiate into endothelial cells [22]. Furthermore, Karimi et al.(2014) reported that AD-MSCs had no significant improvement in acute burn wound healing [23].

The present study showed that the hydrogel+MSCs group had the highest amount of granulation tissue and angiogenesis on days 3 and 10, which decreased on day 21. Lotfi et al. (2019) stated that the granulation tissue thickness in the keratinocyte/MSCs/scaffold group rose in the first week, and declined significantly in the second week compared to other groups [24]. In the present study and the research by Lotfi et al., polymers made from natural materials such as hydrogels provided a suitable environment and direct cell contact. In the current investigation, the hydrogel group had the highest amount of granulation tissue on day 21 compared to the hydrogel+MSCs group, which could indicate the critical role of stem cells that have paracrine signaling properties, which reduce inflammation, and promote angiogenesis and cell proliferation [24].

In this study, we used allogenic AD-MSCs. According to the literature, autologous MSCs have more accelerated cicatrization than allogeneic MSCs. However, in burn injuries, allogeneic MSCs can be the only available option [25]. Research showed that the intradermal injection of allogenic AD-MSCs in burn wounds caused a significant difference on the 14th days with the control group [16].

In the detection of the SYR gene by PCR, the band of this gene was observed only on day 3 in the hydrogel+MSCs group. Hanson et al. (2016) injected allogenic male AD-MSCs intradermally in the partial-thickness of female minipigs. In female tissues, male DNA content was evaluated by the PCR amplification of a 377 bp segment from chromosome Y. They observed Y chromosome bands with a decreasing trend on days 0, 7, and 10 [26]. Based on this study, it would have been better to include the cell tracking investigations of day 7 in the present study to better understand this decreasing process . The difference between the present study and the above study may be attributed to the type of animal modeling, method, and amount of stem cell injection. The reason for the decrease in the presence of MSCs in the wound site is unknown. However, it can be because of MSCs migration from the wound site, MSCs phagocytosis by macrophages, or mechanisms involved in cell processing and tissue regeneration [26].

Tragacanth gum hydrogel can be a suitable scaffold for AD-MSCs. It accelerates the proliferation and differentiation of cells and provides a suitable space for the support and adhesion of cells. It is also capable of expressing genes for up to 21 days and maintains the original morphology of cells. In the present study, the hydrogel and control groups had significantly different epithelialization on day 10. Although no significant difference was observed in inflammation and granulation tissue formation between the hydrogel and other groups, on the 10th and 21st days, the rate of granulation tissue formation in the hydrogel group was higher than in other groups. One of the reasons is the high concentration of hydrogel, and the reduction of inflammation in the stem cell group is may result from the presence of MSCs. A study similar to the current research showed that wound closure occurs faster in the PCL-GT-stem cells group than in the PCL-GT scaffolds group. Granulation tissue, collagen synthesis, and angiogenesis were improved in the PCL-GT-stem cells group. They stated that GT accelerates the transition from the inflammatory and germinal phases as well as the maturation of scar tissue [27–29]. Researchers demonstrated that creams made from Tragacanth gum at a concentration of 6% had the highest effect on rabbit wound healing compared to the control group [27]. In another study, with the daily application of Tragacanth gel, a significant difference was observed in terms of epithelialization, inflammation, and granulation tissue on the 10th day compared to the control group. Similar to the present study, they showed that Tragacanth gum caused most of the wounds to close on the 10th day by accelerating wound contraction [28].

A proper dressing should enhance epithelial regeneration, control the amount of exudate, prevent material leakage, reduce inflammation and infection, and be comfortable for the patient. In this study, amniotic membranes, as an economically reasonable alternative biomaterial were used to benefit from the above characteristics and also prevent hydrogel leakage. In some studies, the use of amniotic membranes alone in wound healing was ineffective [30], but in others, it had no significant difference with the control group or other treatment groups [31]. Studies

have shown that using the amniotic membrane alone is effective for shallow wounds while a more effective solution is required in wide and deep wounds, such as full-thickness wounds and third-degree burns [32].

Studies showed that the application of MSCs with cellular/acellular amniotic membrane multiplies the rate of wound healing compared to utilizing amniotic membrane alone [33]. In this study, on the 3rd days in the stem cell group, acellular human amniotic membrane was observed as a serous layer covered with squamous cells on the wound scab, which is a sign of the effective role of MSCs.

In summary, in this study, the synergism effect of MSCs, Tragacanth gum hydrogel, and human amniotic membrane as a dressing was investigated. Histopathology results showed that the combination of SCs and Tragacanth gum hydrogel was influential in the immediate wound closure, and the human amniotic membrane played a supporting role.

Materials and Methods

Ethical approval

The experiment was authorized by the Research Ethics Committee, Tabriz University of Medical Sciences, Iran (ethical approval code: 5-4-1171).

Experimental design

Sixty healthy adult male Wistar rats weighing approximately 245–365g, were obtained and divided equally into six groups (Table 1). In five groups, T1DM was induced by an intraperitoneal (i.p) injection of Streptozotocin (STZ) (Sigma Aldrich Co.-USA) with a dosage of 55 mg/kg. Blood glucose levels were assessed three days later, the time point when treatments were initiated [9-11]. The treatments were managed according to Table 1 by two different doses (low dose and high dose) of two of the heat-killed Actinomycetales species, including G. bronchialis (Gb) and T. inchonensis (Ti), and also normal saline (for the diabetic and healthy control groups) [8-11], which was administered orally applying intragastric gavage technique for 14 consecutive days. The animals were monitored daily for 21 days. Blood specimens were collected after anesthesia (by i.p administration of 50 and 8 mg/kg BW of ketamine and xylazine, respectively) on the 7th, 14th, and 21st days. Sera were discreet at 750 × g for 15 min for upcoming biochemical and immunological assessments. Besides, five rats in each group were euthanized, and tissue specimens from the liver and kidney were collected for histopathological examination, which was fixed in 10% buffered formalin.

Biochemical assays

Serum biochemical indicators assessment

All of the examined biochemical indicators, such as blood glucose levels and serum insulin values, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), blood urea, and creatinine, albumin, and total protein were evaluated on the 7th, 14th and 21st sampling days of sampling by commercially available kits following the manufacture's instructions (Pars Azmoon, Tehran, Iran) and using a spectrophotometer (Photometer 5010, Berlin, Germany). The activities of AST and ALT were evaluated by a modified method of Reitman-Frankel at 340 nm [24]. The measurement of blood urea and creatinine was performed based on the methods of diacetyl monoxime (546 nm) and Jaffe (500 nm), respectively [24]. Besides, the evaluation of serum albumin and total protein was performed according to the methods of bromocresyl green (546 nm) and biuret (580 nm), respectively [24].

IL-1β, IL-2 and CRP evaluation

The levels of IL-1β, IL-2, and CRP were assessed in the preserved serum samples on the 14th and 21st sampling days using Rat ELISA commercial kits (Koma Biotech, Korea) following the manufacturer's instructions [25].

Histopathological examination

The formalin-fixed tissue samples underwent standard processing, sectioned, and stained with common hematoxylin and eosin (H&E), and then studied microscopically under a light microscope (CH-3, Olympus, Japan). The tissue sections were examined for pathological changes such as atrophy, necrosis, vascular congestion, and hemorrhage [26].

Statistical analysis

The provided data were analyzed using SPSS software (SPSS, version 16 for Windows, USA). More specifically, the ANOVA and non-parametric tests were employed to statistically analyze the serum parameters and pathological lesions across the different groups, respectively, and a $p < 0.05$ was deemed significant.

Table 1.
Different treatments were conducted in six groups of 10 rats each in the present study.

groups	Treatment for 14-continuous days
Low dose Gb	Diabetes treated with 105 CFU/rat* *G. bronchialis*
High dose Gb	Diabetes treated with 107 CFU/rat *G. bronchialis*
Low dose Ti	Diabetes treated with 105 CFU/rat *T. inchonensis*
High dose Ti	Diabetes treated with 107 CFU/rat *T. inchonensis*
Diabetic control	Diabetes treated with normal saline
Healthy control	No diabetes treated with normal saline

*CFU/rat: Colony Forming Unit).

Authors' Contributions

M. Kh. and M.M.A.: Conceptualization, Methodology, Writing - Review & Editing. K.N., and G.M.I.: Conceptualization and Methodology. S. Gh. And F.J.A.: Investigation, Writing - Original Draft. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors have no financial conflicts of interest.

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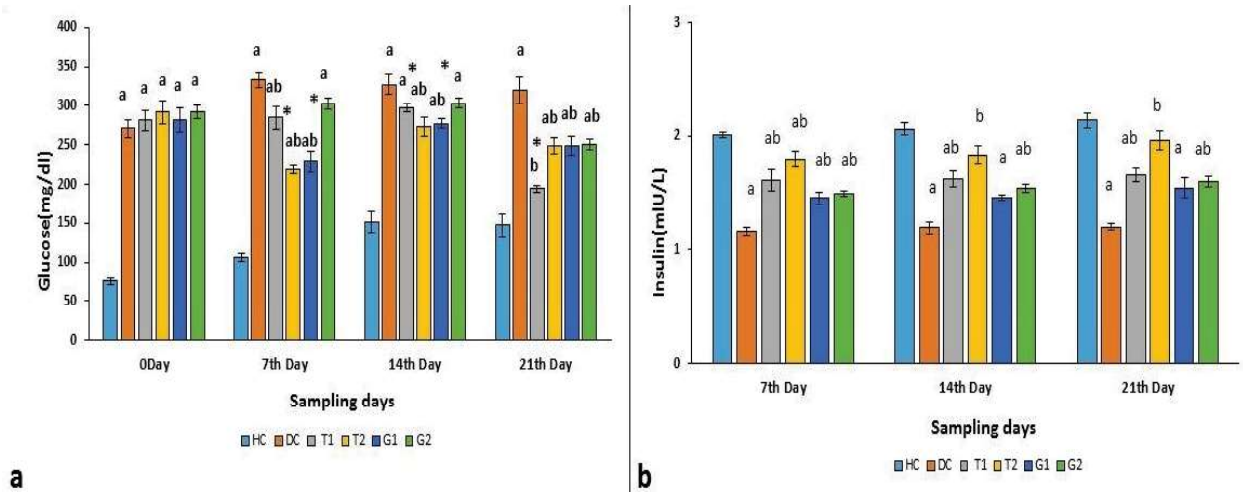

Figure S1.
The effects of oral administration of Actinomycetales species on the serum levels of glucose (A) and insulin (B) on STZ-induced diabetes. Data are presented as the mean ± SD. Differences were considered significant with $p < 0.05$. a: a significant difference with healthy control (HC); b: a significant difference with diabetic control (DC); *: a significant difference between low-dose and high-dose treated groups.

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RESEARCH ARTICLE

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Protective effects of pomegranate peel extract on the gill, liver, and kidney in experimental cadmium poisoning in common carp (*Cyprinus carpio*)

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ABSTRACT

This study aimed to investigate the protective effects of pomegranate peel (PoP) extract on the gill, liver, and kidney tissues of common carp exposed to cadmium (Cd). For this purpose, 150 common carp weighing 65± 0.85 g were randomly divided into five groups with three triplicates for each group (30 fish per group). The control group received a standard diet without cadmium chloride ($CdCl_2$), the Cd group was exposed to 0.5 mg/L $CdCl_2$, and the extract groups received PoP extract with concentrations of 1%, 2%, and 4% (percentage of food weight) along with 0.5 mg/L $CdCl_2$ in the water. After four weeks, tissue samples were collected from the gill, liver, and kidney and stained with hematoxylin and eosin for histopathological examination. In the gills of the Cd group, lesions included congestion, hemorrhage, clubbing or fusion of the secondary lamellae, and telangiectasia of the lamellae. The liver tissue of the Cd group exhibited severe degeneration and necrosis of hepatocytes, while hemorrhage, congestion, cellular degeneration or necrosis, and hyaline cast were visible in the kidney tissue of this group. The severity of the mentioned lesions was significantly reduced in the PoP extract groups, particularly at concentrations of 2% and 4% ($p < 0.05$). Based on the results, it can be concluded that PoP extract has significant protective effects on the gill, liver, and kidney tissues of common carp exposed to $CdCl_2$.

Keywords

Cadmium; Pomegranate peel extract; Liver; Kidney; Gill; Histopathology

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Number of Pages: 9

Abbreviations

Cd: Cadmium
$CdCl_2$: Cadmium chloride
PoP: Pomegranate peel
ROS: Reactive oxygen species
AgNPs: Silver nanoparticles
NO: Nitric oxide.

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Introduction

Heavy metals are significant pollutants in the aquaculture industry worldwide due to domestic and industrial activities [1,2]. Cadmium (Cd), along with other heavy metals like lead and mercury, poses a public health hazard. The United States Agency for Toxic Substances and Disease Registry ranks Cd as the seventh most dangerous agent [3]. Additionally, the International Organization for Cancer Research classified Cd as a human carcinogen in 1993 [4]. Human exposure to Cd occurs through food and inhalation, with cigarette smoke being a predominant source, containing approximately 1.5 to 2 µg of Cd per cigarette [5]. It has been shown that Cd can adversely affect various systems, including the respiratory, reproductive, nervous, immune, endocrine, cardiovascular systems, and the liver, and it is a potent carcinogenic agent [5-7].

In cases of fish poisoning with heavy metals, such as Cd, some organs like the gill, liver, and kidneys are known to be the main organs exposed to $CdCl_2$ in water through respiration and ingestion [2]. These organs are the primary. Numerous studies have demonstrated that Cd can accumulate in various tissues of fish, including muscles, which can have negative implications for human health [7-11]. Cd has been shown to disrupt iron metabolism, leading to anemia and alteration of blood parameters [13,13]. In addition, it suppresses antioxidant mechanisms, leading to lipid peroxidation and oxidative stress [14,15]. There is, therefore, a critical need to minimize or prevent the deleterious effects of Cd exposure.

The use of herbal medicines as supplements or alternatives is growing worldwide. Pomegranate (*Punica granatum L.*), widely cultivated in the Middle East, particularly in Iran, has a long history of use in Iranian herbal medicine [16]. During agricultural production and processing, wastes from pomegranates are generated. These by-products, such as pomegranate peel, offer economic potential as they are a rich source of bioactive substances, including phenolic acids and tannins [17]. One study has shown that the peels of fruits like pomegranates, oranges, apples, and peaches contain higher phenolic content compared to their edible fleshy parts [18]. Pomegranate peel (PoP) is particularly rich in flavonoids and phenolic compounds like tannins or tannic acids, making it a valuable source of bioactive substances [18-20]. Furthermore, it has been shown that PoP extracts have potent wound healing, antioxidant, anti-inflammatory, antibacterial, antiviral, antifungal, and anticancer properties, and they can decrease blood lipid levels [19,21-23].

Therefore, this research aimed to investigate the potential benefits of PoP extract as a natural remedy for alleviating Cd-induced toxicity in the gill, liver, and kidney tissues of common carp (*Cyprinus carpio*) through histopathological examination.

Results

Histopathological findings

Gill

Histopathological examination revealed various lesions in the Cd group, including congestion, telangiectasia of lamellae, hemorrhage, disruption of the gill structure, and hypertrophy and hyperplasia of the lamellar epithelium, leading to distal clubbing or fusion of the secondary lamellae (Figure 1). Treatment with different concentrations of PoP extract ameliorated the lesions caused by Cd administration (Figure 1).

Statistical analysis showed that the Cd group exhibited severe lesions, while all concentrations of the PoP extract reduced the severity of the lesions. This improvement was significant for all the mentioned lesions in the PoP extract groups ($P < 0.05$) (Figure 2). However, no significant difference was observed among the three PoP extract groups ($P > 0.05$). Although the PoP extract significantly improved the Cd-induced lesions, the normal and healthy gills in

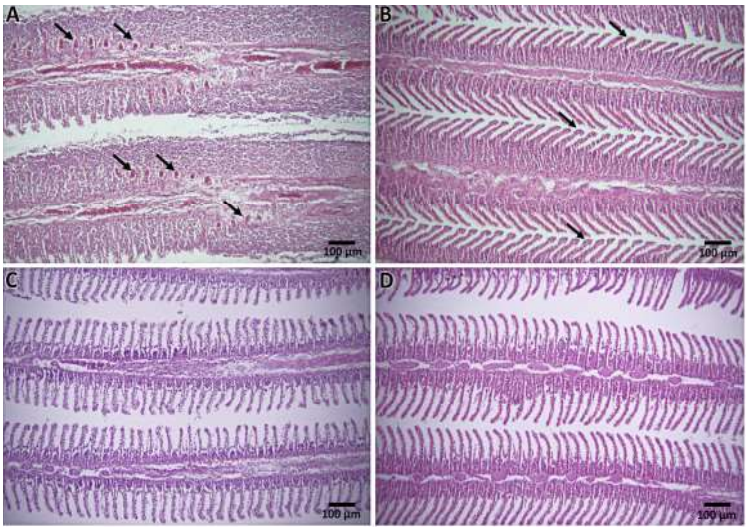

Figure 1.
Micrographs of the gills in common carp that were exposed to CdCl2 and subsequently treated with pomegranate peel extract (n = 9 for each group). In the Cd group (A), congestion, the severe fusion of secondary lamellae, and lamellar telangiectasia or aneurysm can be (arrows). There is club formation of secondary lamellae in the 1% PoP extract group (B). The 2% (C) and 4% (D) PoP extract groups exhibited slight distal clubbing of the lamellae. Hematoxylin and eosin (H & E) staining, scale bars = 100 µm for all.

the control group had lower scores compared to the PoP extract groups ($p < 0.05$).

Liver

In the tissue sections from the liver of the Cd group, severe degeneration and necrosis of hepatocytes were observed. The cytoplasm of the hepatocytes appeared hypertrophied, almost transparent, and clear, with only the cell membrane visible, along with a vesicular nucleus typically located centrally within the cell (Figure 3). Cell nuclei were lost in some hepatocytes, and they showed necrotic changes. In some cases, which were limited to the Cd group, infiltrations of inflammatory cells, predominantly lymphocytes (lymphocytic hepatitis and pancreatitis), were found (Figure 3).

In the groups that received $CdCl_2$ along with different amounts of PoP extract, particularly 2% and 4%, significant improvement in the lesions was observed ($p < 0.05$). However, the liver tissues in

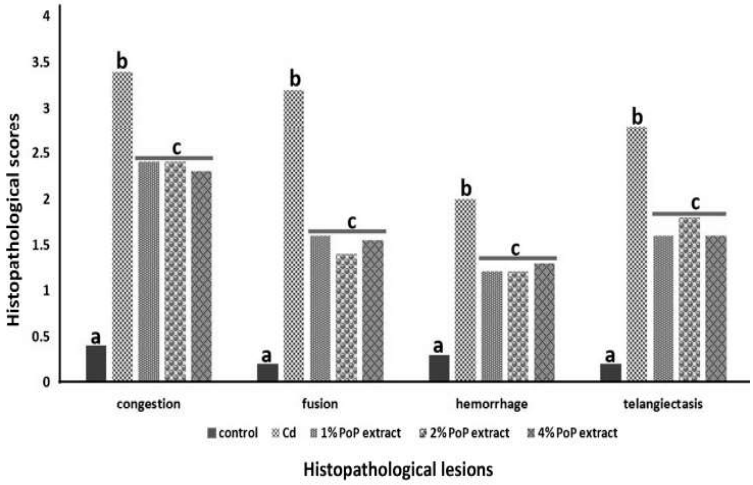

Figure 2.
Statistical analysis of the scores of various histopathological lesions, including congestion, fusion of the secondary lamellae, hemorrhage, and telangiectasis, in the gill tissue related to different groups after four weeks of exposure. The scores are reported as Mean ± SEM and analyzed using Kruskal-Wallis and Mann-Whitney tests. Different letters indicate a significant difference ($p < 0.05$)

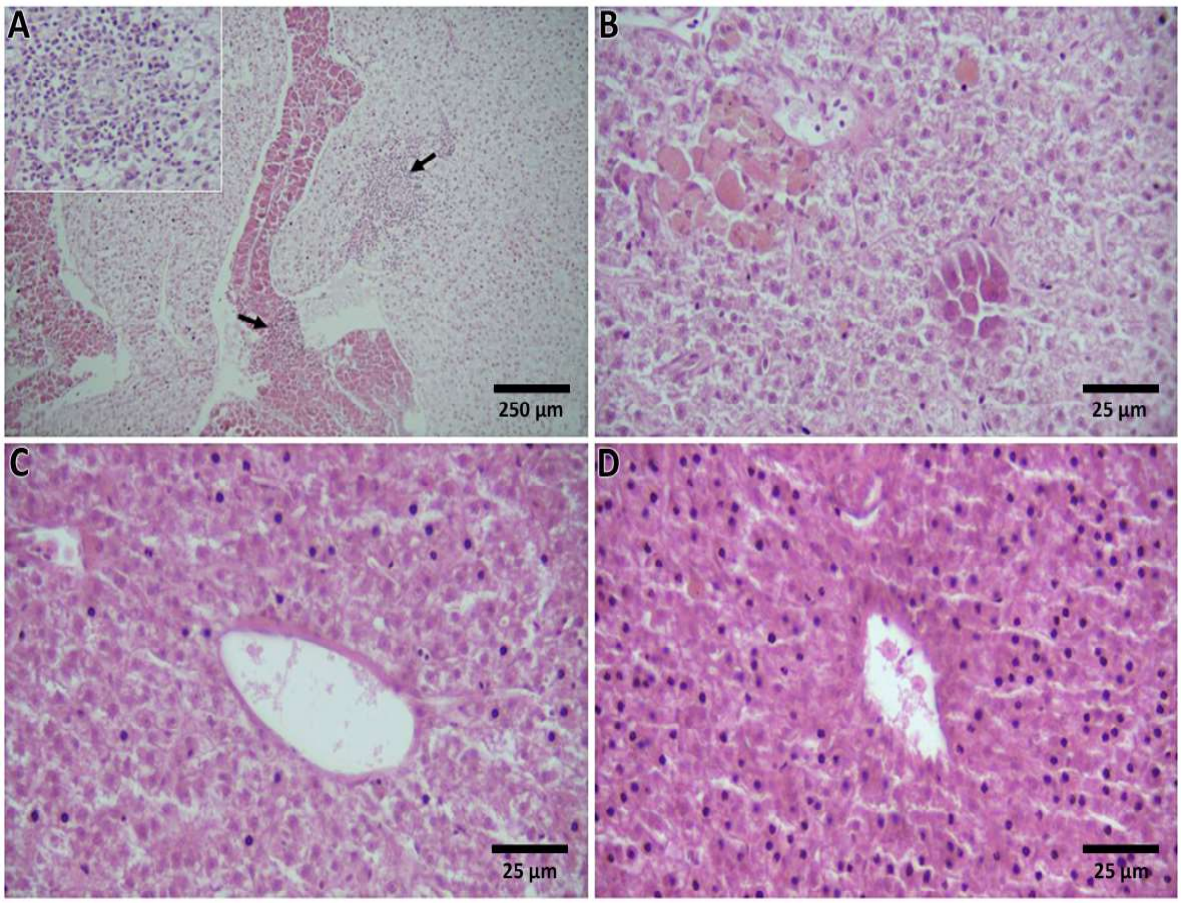

Figure 3.
Histopathological examination of the liver of common carp following CdCl2 poisoning and subsequent treatment with PoP extract (n = 9 for each group). In the Cd group (A), severe degeneration and necrosis of hepatocytes, as well as infiltration of mononuclear inflammatory cells with the predominance of lymphocytes can be observed (arrows) (H & E staining, scale bar = 250 µm). In the upper corner: infiltration of lymphocytes is shown at a higher magnification. In the 1% (B), 2% (C), and 4% (D) PoP extract groups, there is an improvement in cell degeneration and necrosis of hepatocytes. No evidence of inflammation is observed in the PoP extract groups. H & E staining, scale bars = 25 µm for B, C, and D.

these groups remained different from normal and healthy tissues in the control group. The 1% PoP extract group showed some recovery in the lesions, but it was not statistically significant (p = 0.095). Moreover, there was no significant difference between the treatment groups receiving PoP extract (Figure 4).

Kidney

The control group exhibited the normal structure of the kidney tissue, while the Cd group showed various lesions such as congestion, hemorrhage, degeneration of the tubular epithelium characterized by hydropic degeneration or cell swelling with narrowed tubular lumen, and cell necrosis (Figure 5). Eosinophilic hyaline casts were also observed in the tubular lumens in this group. These lesions were more severe in the Cd group, but PoP extracts reduced the CdCl2-induced lesions (Figure 5).

Compared to the Cd group, the PoP extract groups, especially 2% and 4%, showed significant improvements in the lesions, including hemorrhage, hyaline casts, and cell degeneration and necrosis (p < 0.05). However, there was no significant difference in the scores among the three PoP extract groups (p > 0.05) (Figure 6).

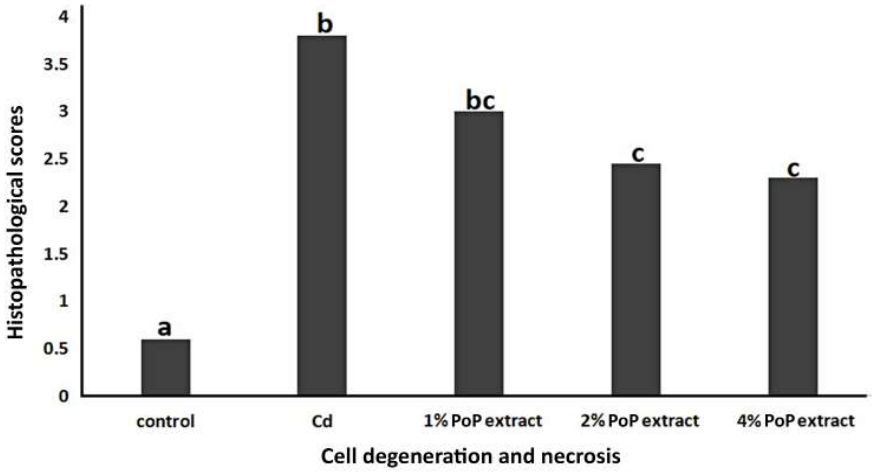

Figure 4.
Statistical analysis of the scores related to cell degeneration and necrosis in the liver tissue of the different groups after four weeks of exposure. The scores are reported as Mean ± SEM and analyzed using Kruskal-Wallis and Mann-Whitney tests. Different letters are significantly different (p < 0.05).

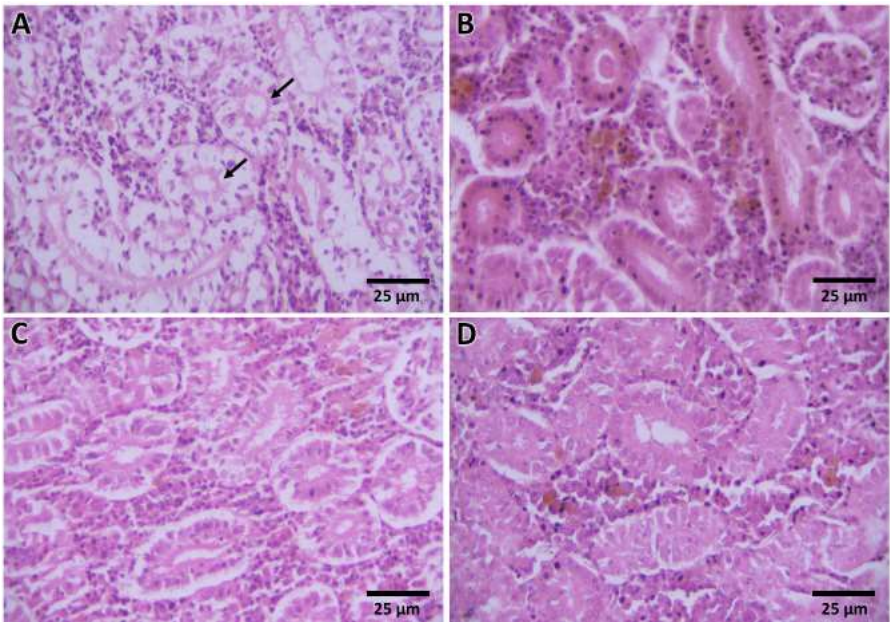

Figure 5.
Photomicrographs of the kidney tissues exposed to CdCl2 and treated with PoP extract (n = 9 for each group). In the Cd group (A), there are severe hydropic degenerations in the epithelial cells of the tubules, as well as narrowing of the lumen (arrows). In the 1% (B), 2% (C), and 4% (D) PoP extract groups, there is a recovery and reduction of hydropic degeneration in the tubular epithelial cells. H & E staining, scale bars = 25 µm for all.

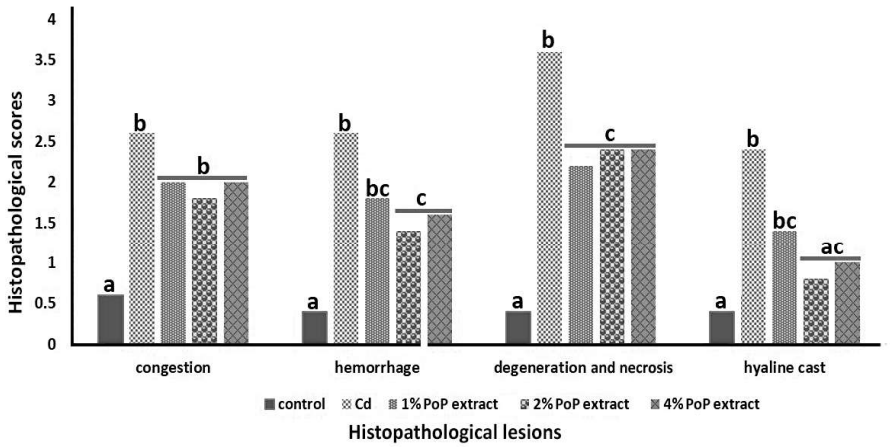

Figure 6.
Statistical analysis of the scores related to histopathological lesions, including congestion, hemorrhage, degeneration and necrosis, and hyaline cast, in the kidney tissue of different groups four weeks after the CdCl2 exposure. The scores are reported as Mean ± SEM and analyzed using Kruskal-Wallis and Mann-Whitney tests. Dissimilar letters are regarded as significantly different (p < 0.05).

Discussion

In the present study, CdCl2 caused various histopathological lesions in the gill, liver, and kidney tissues of common carp. Previous studies have also reported similar tissue damage induced by Cd exposure. For instance, Ahmed et al. [24] investigated the toxic effects of Cd in climbing perch (Anabas testudineus) and observed epithelial cell necrosis, separation of the epithelial layer, and fusion of secondary lamellae in the gill tissue. They also reported congestion, cell degeneration, and necrosis in the liver, as well as vacuolation in the kidney [25]. They showed that heavy metals like Cd can cause significant histopathological changes in various tissues of fish. Likewise, Peykanheraty et al. [24] found hyperplasia, clubbing, and fusion of the lamellae in the gill of Chondrostoma regium exposed to CdCl2, along with congestion and focal necrosis in the liver. They indicated that the gill and liver tissues can be regarded as the main organs exposed to the harmful effects of Cd.

Cd is known to cause cytotoxicity by binding to thiol groups in mitochondria, leading to mitochondrial dysfunction, cellular degeneration, and necrosis [5]. It also can increase lipid peroxidation, resulting in structural impairment and vacuolization of the liver and other tissues [2,5,24]. Cd may negatively affect the antioxidant system and generate free radicals, including reactive oxygen species (ROS) like superoxide (O2-), hydroxyl (OH-), hydrogen peroxide (H_2O_2), and nitric oxide (NO) in the body [5,7].

The findings achieved from the present study are fully aligned with previous studies, demonstrating that Cd exposure can cause structural disruptions in various tissues. These tissue injuries highlight the importance of implementing effective methods to prevent or mitigate the harmful effects of heavy metals like Cd.

Disposing the agricultural wastes, such as PoP, presents a significant challenge. However, there has been growing interest in extracting valuable nutrients, including phenols, from these agricultural waste materials as safe and affordable sources of natural antioxidants [26-28]. Several studies have reported that pomegranates possess significant antioxidant activity compared to other dietary plants or fruits [27,29,30]. PoP has received considerable attention due to its high content of bioactive substances and antioxidant capacity.

Numerous studies have investigated the effectiveness of PoP extracts in mitigating the cytotoxicity of heavy metals and toxic agents in various animal models [31-34]. For instance, Hamed and Abdel-Tawwab [30] demonstrated that PoP powder inclusion in the diet of Nile tilapia could alleviate the adverse effects induced by silver nanoparticles (AgNPs). In that study, PoP significantly increased antioxidant activity and reduced tissue damage in the liver and kidneys, which are caused due to AgNPs exposure [31]. Likewise, another study showed that PoP extract significantly reduced lipid peroxidation and improved tissue damage and apoptosis in the liver of the Wistar rats exposed to lead (Pb) [32].

Jafari et al. [34] found that PoP extract, particularly at concentrations of 1% and 2% of diet weight (compared to 4%), effectively reversed the decline in liver antioxidant enzyme activity and the increase in lipid peroxidation caused by the $CdCl_2$ exposure over a period of 140 days in fish. They showed that Cd could negatively affect fish activities and physiology, and the PoP extract improved tissue functions. Our study supports these findings, while greater improvements were observed with the 2% and 4% PoP extracts in our study. Jafari and coworkers claimed that the probable bitterness of water due to the higher concentration of 4% may reduce appetite, water consumption, and physiologic activities, leading to reduce the extract intake by the fish and its positive effects [33]. Moreover, the density of 20 fish per aquarium in that study was higher than our study (N=10), and the period of the study was longer (140 vs. 28 days), which could affect the results of the studies.

The antioxidant activity of PoP is mainly attributed to compounds such as vitamin C, flavonoids, quercetin, ellagic acid, gallic acid, tannins, ellagitannins, and gallotannins [18,19,26,27,30]. These compounds can increase antioxidant activities, reduce lipid peroxidation, chelate Cd, and inhibit Cd deposition [5,35,36].

It has been reported that flavonoids and ellagic acid in PoP can function as potent scavengers and chelating agents for O_{2^-} and OH free radicals produced through the metabolism of heavy metals [5,35]. The presence of hydrogen atoms in the structure of gallic acid can delocalize ROS [36,37]. In addition, gallic acid has been found to have anti-inflammatory potentials, and it can reduce the Cd-induced inflammatory markers, including myeloperoxidase, interleukin-6, and NO in the rat brain [36]. Winiarska-Mieczan et al. [38] demonstrated that tannic acid can reduce the Cd accumulation in the rat lung and heart. Moreover, PoP-activated carbon as an adsorbent has been successfully applied to remove $CdCl_2$, the most common form of Cd and highly soluble in water, from aqueous ecosystems [39].

Taken together, the positive effects of PoP extract on the $CdCl_2$-induced lesions in the gill, liver, and kidney tissues suggest its hepatoprotective and nephroprotective potentials and antioxidant properties.

In conclusion, the PoP extracts mitigated the histopathological lesions induced by CdCl2 in the gill,

liver, and kidney tissues of common carp. The extent of improvement was particularly significant at higher concentrations of the extract (2% and 4%), indicating a dose-dependent effect. Although the PoP extract significantly reduced the tissue damage caused by $CdCl_2$, the tissues did not fully recover their normal structure. The presence of active phytocompounds in PoP extract and its protective role against toxic substances like $CdCl_2$ suggest its potential in the field of aquatic toxicology and fish physiology.

Materials and Methods

Ethical statement

The present study was conducted according to the Animal Experimental Guidelines approved by the Institutional Animal Care and Use Committee at Ferdowsi University of Mashhad.

The ethical approval for this study was issued with the ethical code for grant number 3/58310 from the Committee on Research Ethics of IR.UM.REC.1401.133, based on the Ethical Guidelines of Research from Ferdowsi University of Mashhad. The study was performed in the Aquaculture Laboratory, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad.

Preparation of pomegranate peel extract

To prepare the PoP extract, the PoPs were washed with distilled water, dried in an oven at 40 °C for 10 days, and then ground. In the next step, the PoP powder (10 g) was extracted in a Soxhlet extraction apparatus using an equal mixture of four solvents: water, ethyl acetate, acetone, and ethanol. The extraction process was performed in three repetitions for 6 h each. The obtained extracts were then centrifuged at 4500 rpm for 3 min to remove fine particles and filtered using the Whatman paper (grade No. 41). Finally, the extract was concentrated in a vacuum oven at 40 °C, dried, and ground [40]. The PoP extract was ground, and the powder was stored at -18 °C until further use.

Study procedures

A total of 150 healthy common carp weighing approximately 65 ± 0.85 g with an average body length of 18 ± 1.5 cm and indeterminate sex were used in this study. The fish were randomly distributed among 15 glass aquaria, with a density of 10 fish per aquarium. After seven days of acclimatization period and feeding with a standard commercial fish diet, the fish were divided into five groups, with three replications for each group (30 fish in three aquaria per group).

The five groups (in three replicates) included as follows:

1- Control group: Fish received a standard diet without CdCl2, provided in four meals amounting to 2.5% of their body weight.

2- Cadmium (Cd) group: Fish were fed with the standard diet and exposed to a concentration of 0.5 mg/L of $CdCl_2$ (Merck, Germany) [34,41].

3- 1% PoP extract group: Fish were fed the same diet as the Cd group and received 0.35 g of the powdered PoP extract, which accounted for 1% of food weight [34].

4- 2% PoP extract group: In addition to the diet provided to the Cd group, fish received 0.7 g of PoP extract, representing 2% of the food weight.

5- 4% PoP extract group: Fish received 1.4 g of PoP extract (4% of food weight) along with the standard diet and 0.5 mg/L of $CdCl_2$.

After four weeks from the study, three fish from each aquarium (n = 9 for each group) were randomly selected and caught using an aquarium fish net. These fish (n = 45) were anesthetized with clove powder (0.5 g/L) [16, 34]. On necropsy of the fish, the gill, liver, and kidney tissues were removed, and the samples (with a size of 2 × 2 cm) from these tissues were taken for histopathological examinations.

It should be noted that the rest of the fish were kept for educational purposes in the Department of Aquaculture.

Histopathological examination

The tissue samples from the gill, liver, and kidneys were immediately placed and fixed in a 10% neutral buffered formalin solution. The formalin solution was changed after 24 h with a fresh formalin solution. The tissue samples were then dehydrated with varying degrees of ethanol, cleared with xylene, embedded in paraffin waxes, and cut into 5 µm-thickness sections in the laboratory of the Pathobiology Department. Finally, the sections were stained with hematoxylin and eosin dyes, and the prepared slides were examined under a light microscope equipped with a digital camera (Olympus, Japan) for any histological changes, including congestion, hemorrhage, cellular degeneration or necrosis, and other lesions. Ten fields of view at ×400 magnification (high-power fields) were examined for the histopathological lesions, and each lesion was scored for all groups based on Table 1 [42].

Statistical analysis

The Kruskal-Wallis and Mann-Whitney tests were used to analyze and compare the histopathological scores (Mean ± SEM) between the groups using the statistical package SPSS version 19.0 for Windows. P values lower than 0.05 ($p \leq 0.05$) were considered as significant.

Table 1.
The scoring system used for analysis of each histopathological lesion in the gill, liver and kidney of the fish.

Scoring of lesions	Description
0	Normal structure, with no lesion
1	Lesions in <25% the studied microscopic fields
2	Lesions in 25-50% the studied microscopic fields
3	Lesions in 50-75% the studied microscopic fields
4	Lesions in >75% the studied microscopic fields

Authors' Contributions

H.J. carried out the experiments and contributed to sample preparation. S.A and D.S conceived and planned the experiments, contributed to sample preparation, contributed to the interpretation of the results, and took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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The First Evaluation of Chlamydia abortus Infestation in the Iranian Dromedary Camel Population

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ABSTRACT

Chlamydiosis is an important disease in sheep, camel, goats, cats, birds, and cattle, which is caused by different species of the genus *Chlamydia*. Chlamydiosis of ruminants is a zoonosis and is especially worrying for pregnant women in contact with animal shelters. Chlamydiosis in camels can cause abortion, cervical adhesion, ovarian hydrobursitis, and reproductive failure in male camels. Chlamydia in camels can remain asymptomatic for a long time. Infected camels may play an important role in the transmission of *Chlamydia* to other animal species. Chlamydiaceae family members are currently placed in one genus and seven species. Among these seven species, *Chlamydia abortus* is of special importance in camels. It is possible to detect and distinguish chlamydial species by PCR and specific primers. The present study is the first study of *Chlamydia abortus* in the Iranian dromedary camel, which is very important. A total of 100 blood samples with anti-coagulant were taken from apparently healthy male and female camels in the south of Kerman province. Next, DNA was extracted from each blood sample using a blood DNA extraction kit according to the manufacturer's instructions. PCR was performed using rOMP90_3 specific primer to evaluate the presence of *Chlamydia abortus*. None of the samples were positive for *Chlamydia abortus*. According to the results, it can be said that *Chlamydia abortus* is probably not common in camels in the south of Kerman province of Iran.

Keywords

Chlamydia abortus, PCR, Camel, Iran

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Number of Pages: 6

Abbreviations

PCR: Polymerase chain reaction
ELISA: Enzyme-linked immunosorbent assay
EDTA: Ethylene diamine tetraacetic acid
rOMP: Recombinant outer membrane protein
DNA: Deoxyribonucleic acid

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Introduction

Chlamydiosis is an important disease with global distribution in animals, especially ruminants, which is caused by different species of *Chlamydia genus*. *Chlamydia* can be considered gram-negative bacteria without metabolic energy production mechanisms. Therefore, Chlamydia is an obligate intracellular parasite that must live inside the cell, where the host cell provides intermediate compounds rich in energy [1]. These bacteria are also called "energy parasites". One of the important consequences of chlamydiosis is abortion [2]. Chlamydia abortion occurs in the last 2-3 weeks of pregnancy. The fetus is born dead and the placenta is inflamed. Chlamydiosis of ruminants is a zoonosis, especially worrying for pregnant women in contact with animal shelters [3]. Studies in sheep and goats showed that infection is mainly transmitted through contact with abortion products, vaginal secretions, and aborted or dead fetuses. The same may be true for camels as well [4]. Chlamydiosis in camels can cause abortion, cervical adhesions, ovarian hydrobursitis, and reproductive failure in male camels [5–8]. Chlamydia in camels can remain asymptomatic for a long time [9], and infected camels may play an important role in the transmission of Chlamydia to other animal species [10]. The prevalence of camel chlamydiosis is higher in adult camels than in young camels and female camels than in male camels [5, 11–13]. Chlamydiaceae family members are currently placed in one genus and seven species. Among these seven species, Chlamydia abortus in camels is of special importance [14]. Using PCR, it is possible to detect and distinguish Chlamydial species by specific primers [15]. The present study is the first detection of Chlamydia abortus infection in the Iranian camel population by PCR, which is very important.

Results

In this study, based on the results of electrophoresis, all 100 blood samples tested were negative for Chlamydia abortus (Figure 1).

Discussion

Chlamydiosis is an important disease in a wide range of animals caused by different species of Chlamydia bacteria [2]. This disease in camels can cause various symptoms, including abortion, cervical adhesion, ovarian hydrobursitis, and reproductive failure [5–8]. Moreover, Chlamydia in camels can remain asymptomatic for a long time [9]. As a result, infected camels can transmit this bacterium to other animals [4]. The present study is the first evaluation of Chlamydia abortus in the dromedary camel population in Kerman, Iran, which is very important. None of the samples were positive for Chlamydia abortus. Therefore, it can be said that Chlamydia abortus is probably not common in camels in the south of Kerman province of Iran.

Studies similar to the current research have been conducted around the world. In a study conducted in Tunisia, blood and serum samples of 470 healthy dromedary camels from eight different provinces of Tunisia were collected to detect Chlamydia abortus. In the serological method (ELISA), 5.75% positive samples were detected, while no positive samples were observed in the molecular method (PCR) [16]. In another study, similar to the results of the present study, blood samples were collected from 82 camel herds (865 dromedary camels). Chlamydia abortus was detected in camel blood by ELISA at a very low percentage (2.5%) [17].

In a study, serum and milk samples were taken

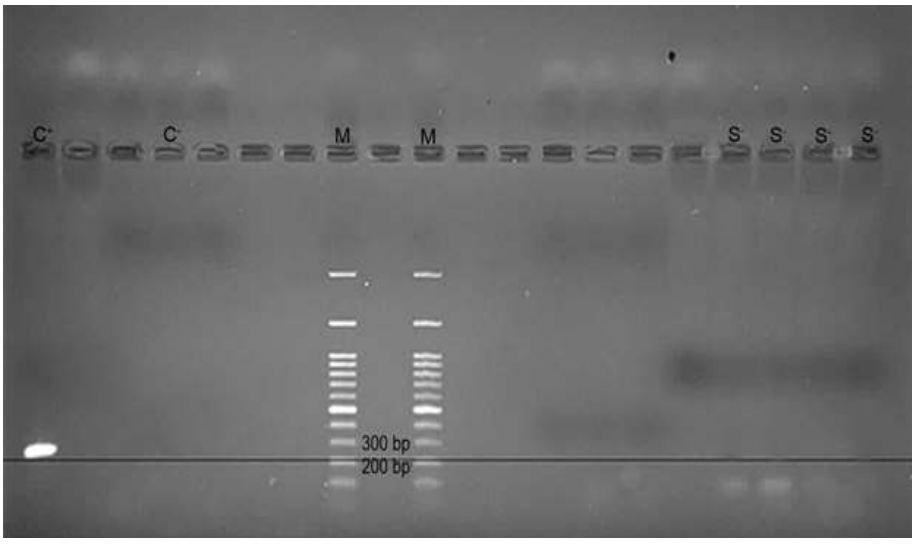

Figure 1.
Amplification of Chlamydia abortus gene using rOMP90_3 primers. Negative clinical samples (lanes 17-20), negative control (lane 4), positive control (lane 1), and molecular weight marker (lanes 8 and 10).

from 30 camels and 300 contact sheep belonging to six different farms in the western region of Saudi Arabia. In these farms, camels and sheep were kept together. Three of these farms had sheep abortions. Two methods, ELISA and PCR, were used in this study, showing that 18 camels and 142 sheep were positive with PCR, while 11 camels and 109 sheep were positive with ELISA. The findings showed that camels can be infected with Chlamydia, but most of the infected camels look healthy. Therefore, they can play an important role in the transmission of this infection to the animals in contact with them [18]. Among the reasons for the difference between the latter study and the current research, we can mention the type of sample, the way of keeping livestock, and the diagnosis method. In the mentioned article, the milk and serum samples were examined by molecular and serological methods, while in the present study, the study was performed exclusively on the blood samples of camels and only by molecular methods. In addition, the camels were next to the sheep in the field. It should also be noted that three of these sheep had abortions. In the present study, camels in the deserts were investigated. In another research, bursal tissue (n=5) and bursal fluid (n=6) samples were collected from 11 female camels with ovarian hydrobursitis. Real-time PCR was used for the initial detection of Chlamydia abortus in infected samples. The prepared samples were inoculated into embryonated chicken eggs. Subsequently, Giemsa staining and direct immunofluorescence were used to detect any chlamydial inclusions in infected yolk sacs. Next, the second real-time PCR was performed on infected yolk sacs. The Chlamydia abortus gene was found in 83.8% and 63.6% of infected bursa tissue and bursa fluid samples and infected yolk sacs, respectively. Moreover, all the yolk sac smears tested with direct immunofluorescence and Giemsa staining showed intracytoplasmic inclusion bodies [7]. The difference between the mentioned study and the present research is that in the above study, camels affected by ovarian hydrobursitis were evaluated, while in the present study, apparently healthy camels were assessed. Furthermore, in our study, the molecular technique was conventional PCR, while in this study,the molecular technique was real-time PCR. In another research, to evaluate the causes of abortion in Western camels, samples were taken from 34 camels older than 5 years and 19 camels younger than 5 years. Fifteen internal organs (liver, heart, lung, and spleen) from aborted camels and twenty vaginal swabs from aborted camels were collected for Chlamydia isolation through inoculation in embryonated chicken eggs. Chlamydia inclusion bodies were detected in 45% and 20% of vaginal swabs and internal organs, respectively [5]. The difference between the above study and the current research results from several reasons. They studied the internal organs and vaginal swabs of aborted camels, while we used the blood samples of healthy camels. Moreover, in the mentioned study, Chlamydia isolation through inoculation in embryonated chicken eggs was performed, while in the current investigation, the molecular test was performed. In another research, 1560 sheep and goat blood samples were collected from 130 flocks in five Kajiado counties. The samples were tested by PCR, and Chlamydia abortus DNA was detected in 20.3% and 28.1% of sheep and goats, respectively [19]. The difference between our study and this study may result from different species studied.

Research on Chlamydia abortus in the camels of different regions of the world has been conducted by ELISA. In a study, 245 blood samples were collected from Abu Dhabi female dromedary camels, aged 5-8 years, with a history of reproductive failure, including repeat breeder and abortion. The samples were tested by ELISA. The overall prevalence of chlamydiosis was 19.59%. The results showed that chlamydiosis was common among camels in Abu Dhabi [20]. Another study was conducted on 245 dromedary camels (205 females and 40 males) in different regions of western Libya. The animals varied in age from <1 to 20 years and were sampled randomly from both housed and nomadic herds. Blood serum samples of camels were tested by ELISA. The results showed that out of 245 camels tested, 30 camels were positive. The prevalence of chlamydiosis in females (14%) was twice males (5%) [11]. In a study, blood samples were taken from 60 dromedary camels (38 females and 22 males) aged 5-12 years in Iraq. These samples were tested by ELISA. All the male camel serum samples were negative for the presence of antibodies against Chlamydia abortus, while 18 of the 38 (47.36%) female camel samples were positive [21]. In another study, serum was collected from 378 female Mijaheem camels in different age groups from different parts of Saudi Arabia. The samples were tested by ELISA. They found the prevalence of chlamydiosis as 10.05% [13]. In a study, 141 infertile male dromedary camels (4-20 years) were used. Antibodies against Chlamydia abortus were detected by ELISA. The incidence rate of Chlamydia abortus was 13.48%. It could be concluded that Chlamydia abortus may play a role in causing reproductive failure in male camels [8].

Conclusions

According to the results of the present study, Chlamydia abortus is probably not common in camels in the south of Kerman province. However, further studies should be conducted to provide better conclusions.

Materials and Methods

Sample collection

One hundred blood samples with EDTA, as an anticoagulant, were taken from the jugular vein of apparently healthy mature (with an average age of four years and above) male and female camels with an average weight of 370 and 300 kg, respectively, in the south of Kerman province. It should be mentioned that camels in the deserts and far from other animals were used.

DNA extraction

DNA extraction was completed using the commercial blood DNA extraction kit according to the manufacturer's instructions (Parstous, Iran). The quality and quantity of extracted DNA were assessed using a Nanodrop spectrophotometer (Epoch, BioTek Instruments Inc., USA).

Conventional PCR

Chlamydia abortus was confirmed using PCR with a specific primer pair rOMP90_3 (Metabion, Germany) to identify the relevant gene with a weight of 220 bp [22] (Table 1). The PCR reaction mix was at a final volume of 20 µl, containing 2 µl template DNA, 0.5 µL of each of Chlamydia abortus specific F and R primers (0.25 µM), 7 µL of distilled water, and 10 µL of commercial master mix (Ampliqon, Denmark). Chlamydia abortus [23] and distilled water were also used as positive and negative controls, respectively. Subsequently, the samples were placed in a thermocycler (Biorad, USA) to amplify the target gene with the temperature program given in Table 2. PCR products were electrophoresed with a ladder (Ampliqon, Denmark) in agarose gel at a concentration of 1%. Following the electrophoresis, reading in the gel documentation system (Vilberlomart, France), photographing with the quantum capture software, and analyzing the results were performed.

Table 1.
The sequence of rOMP90_3 primers for the specific detection of Chlamydia abortus

Target	Primer name	Sequence(5-3)	Product size (bp)
rOMP90_3	rOMP90_3_F	5'-TTTTCAGGATCCTATTGTCCTCCAGGCA -3'	220
	rOMP90_3_R	5'-GTGAATTCAGCATAAATAGCCCCG-3'	

Table 2.
Temperature program of thermocycler device to amplify the target gene of Chlamydia abortus

Number of cycles	Temperature (centigrade)	Time	PCR step
1	95°C	3 min	Initial Denaturation
35	95°C	30 sec	Denaturation
	60°C	30 sec	Annealing
	72°C	60 sec	Elongation
1	72°C	10 min	Final elongation

Authors' Contributions

M.P. carried out the laboratory works, data analysis, and wrote the manuscript. E.M. designed the study, supervised the project, revised the data analysis, supervised the laboratory works, and revised all parts of the manuscript. M.G. supervised the project and revised the data analysis.

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Competing Interests

We pronounce that we have no irreconcilable situation.

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Helicobacter Detection in the Stomach of Apparently Normal Donkeys: Sampling, Diagnostic Methods, and Implications for Equine Glandular Gastric Disease and Serum Antioxidant Status

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ABSTRACT

The involvement of *Helicobacter*-like agents in EGGD is not clear. Much evidence supports the presence of *Helicobacter* in the horse's stomach, but it is not so clear about the donkey. The present study was conducted to evaluate the presence of *Helicobacter* in the stomach of donkeys and assess the best method for its identification and its possible participation in EGGD and serum antioxidant status. Gastric juice sampling and biopsy from NMP and PA were performed by gastroscopic method from 12 donkeys. Histopathology and RUT were used to identify *Helicobacter*. TAC, THIOL, NO, and DPPH were measured to assess the antioxidant status of serum. *Helicobacter* was detected only by the RUT method in one donkey (8.33%), but it is unlikely that this infection was effective in causing EGGD. The RUT results for the three samples of gastric juice, NMP, and PA were not different. Histopathology of NMP and PA did not show *Helicobacter* infection. *Helicobacter* is present in the stomach of a donkey but does not change the antioxidant status of serum. The RUT is more efficient than H&E histopathology for the assessment of *H. pylori* in the equine stomach, and the RUT of gastric juice is preferable to tissue samples because it is easy, fast, and non-invasive. In conclusion, further studies using more accurate methods are recommended to evaluate the effect of *Helicobacter* in the gastric diseases of donkeys and other equine.

Keywords

Equine Gastric Ulcer Syndrome, Donkey, Microbiome, Antioxidant

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Abbreviations

EGGD: Equine glandular gastric disease
NMP: Near the margo plicatus
PA: Pyloric antrum
RUT: Rapid urease test
H&E: Hematoxylin and eosin
TAC: Total antioxidant capacity

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Introduction

The EGGD is one of the most important gastrointestinal diseases in horses [1]. Gastric microbiota changes in EGGD, but the cause is not completely clear [2, 3]. However, there is no conclusive evidence that bacteria are always involved in the development of EGGD [4], and there is a possibility that microbiota changes are secondary and related to opportunistic bacteria. Opportunistic bacterial colonization has been observed in chronic EGGD [4, 5]. Bacteria, such as Escherichia fergusoni, Enterococcus faecium, Streptococcus bovis, and Sarcina can be associated with EGGD lesions [2, 6]. H. pylori is a suspected etiology in the pathogenesis of gastric ulcers in humans, dogs, and cats [7, 8]. However, the involvement of *Helicobacter* in EGGD is uncertain [9].

Helicobacter spp. belongs to the Campylobacter genus and is often believed not to be effective in the etiopathology of EGGD [4]. However, *Helicobacter* has been identified by different methods in the equine stomach [10–13] and feces [14]. Some studies did not find any evidence of the involvement of *Helicobacter* species in the pathogenesis of EGGD [2, 6, 15]. Although Helicobacter was found in the stomach of horses, it had no significant relationship with EGGD [3]. In some reports, the presence of *Helicobacter* has been associated with EGGD [16–18].

The RUT method has been used in some studies to identify Helicobacter in horses [10, 18]. Evaluation with RUT is based on the presence of bacterial urease enzyme and urea absorption from the culture medium. The sensitivity of RUT in horses was 40% compared to PCR [10] and 100% compared to histopathology [18]. Histopathological and immunohistochemical evaluation of the glandular and non-glandular gastric samples of horses showed 81% Helicobacter infection [12].

Hyperlipidemia, kidney disease, and grain overload increase the risk of EGUS in donkeys and can cause colic [19], but the importance of bacteria in the pathogenesis of EGUS in donkeys is unknown. However, some researchers consider Helicobacter to be a possible cause of EGUSs in donkeys [20, 21]. Moreover, people infected with *Helicobacter* have lower antioxidant levels than healthy people [22]. The present study gives a perspective on the presence of *Helicobacter* in the stomach of donkeys using RUT for the mucosal samples of gastric gland and gastric juice, as well as the HPE of biopsied tissue samples. It also explores the presence of EGGD and serum antioxidant levels in donkeys to evaluate the possibility of their relationship with the presence of *Helicobacter* in the stomachs of donkeys.

Abbreviations Cont'd

THIOL: Total thiol
NO: Nitric oxide
DPPH: Diphenyl-1-picrylhydrazyl
EGUS: Equine gastric ulcer disease
H. pylori: Helicobacter pylori
HPE: Histopathological evaluation

Results

Vital signs of animals, including body temperature, respiration rate, and heart rate were normal in clinical examination. The subjects had no clinical signs of diseases, such as alimentary disorders and colic. The results of the gastroscopy showed that the EGGD grade was zero in all donkeys and only one of the animals had a grade 1 EGGD.

Evaluation of RUT for gastric juice and glandular gastric tissue samples showed infection with urease = positive *Helicobacter*-like infections in only one of 12 animals (Figure 1). Both gastric juice RUT and glandular gastric (NMP and PA) mucosa RUT were positive in the same animal. In the gastroscopic evaluation of the animal with a positive RUT, there was no sign of even the mildest degree of EGGD.

Histopathological examination of NMP and PA gastric specimens did not confirm *Helicobacter*-like infection in any of the donkeys. No pathological changes were found in the histopathological samples of NMP and PA. There was no obvious difference between the antioxidant status of the animal that had positive RUT and the mean of other animals that had negative RUT (Table 1). The values obtained for the animal with a positive RUT were within the range of the changes of the values obtained for other animals with a negative RUT. Therefore, it may be concluded that the presence of bacteria and a positive test did not cause obvious differences between the two groups of animals (Table 1).

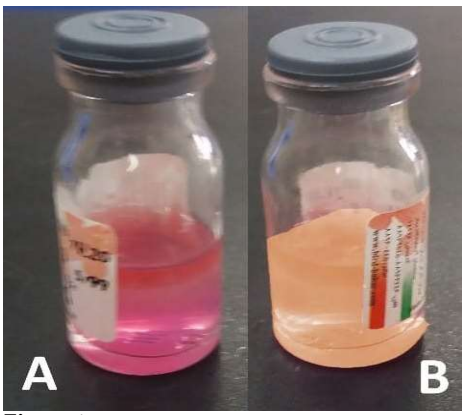

Figure 1.
Positive (A) and negative (B) RUT samples.

Table 1.
Comparison of serum antioxidant status in donkey with positive RUT and donkeys with negative RUT

Antioxidant test	RUT Negative (mean)	RUT Positive	CI
TAC (nmol/mg)	26.45	22.35	(1.21-6.98)
DPPH (nmol/mg)	46.89	48.67	(-6.56-3.01)
NO (nmol/mg)	28.80	26.37	(8.05-4.05)
THIOL (nmol/mg)	17.50	16.74	(-1.98-3.51)

total antioxidant capacity (TAC), total thiol (THIOL), nitric oxide (NO), diphenyl-1-picrylhydrazyl (DPPH)

Discussion

We evaluated the presence of *Helicobacter*-like bacteria in the donkey stomach by RUT and histopathology. The findings of the present study confirm the presence of Helicobacter in the donkey's stomach, but similar to the horse [4], the presence of *Helicobacter* is not related to the development of EGGD in the donkey.

Some studies emphasize the presence of *Helicobacter* in the equine stomach. In the genetic analysis conducted on the gastric mucosa of slaughtered Colombian horses, it was found that 23.3% of the samples were positive for *Helicobacter* species. A gene similar to H. heilmannii was identified. There was no significant relationship between the presence of Helicobacter and gastric ulcer [23]. Some species of *Helicobacter*, such as *H. equorum*, were able to multiply in the hindgut of horses in the experiments, but they did not cause any microscopic or clinical pathological complications [11]. The PCR evaluation of gastric mucosa biopsies of horses (93% with gastric lesions) showed that only 14% were positive for *H. pylori* and all the samples were negative for H. equorum. H. equorum was found in the fecal samples of only 8% of horses [24].

In some studies, no signs of *Helicobacter*-like agents have been found in the equine gastric. Fluorescence in situ hybridization and RUT were performed on healthy and unhealthy gastric mucosa of slaughtered Danish horses. There was no evidence of *Helicobacter* in the stomachs of healthy and unhealthy horses [6]. In a study on the cytology brush samples of horse glandular mucosa, the microbiota was analyzed by DNA sequencing method and no evidence of *Helicobacter* presence was found [2]. Moreover, Helicobacter was not reported in the microbiota of stable horses' feces [25]. Helicobacter was not found in the gastric mucosa of healthy American horses [26]. *Helicobacter* was not found in the gastric biopsies of Korean racing horses with gastric ulcer by PCR and culture, and only in two cases *H. pylori* and *H. ganmani* were isolated by next-generation sequencing techniques [15].

Studies have been conducted on the digestive microbiota of donkeys, but there were no clear signs of *Helicobacter* presence in the digestive system. Investigation of the digestive tract microbiota of slaughtered donkeys showed that diversity in the stomach pylorus is less than in the cecum and large intestines [27]. Growing evidence has shown that the diversity of the fecal microbiota of donkeys depends on age [28] and gender-dependent [29], and the microbial community composition in wild asses is more complex than in domestic donkeys [30].

In the present study, RUT of all the specimens of gastric juice and glandular gastric mucosa of NMP and PA revealed *Helicobacter* infection in the infected stomach. According to the results obtained in the present study, it is possible to use gastric mucosal RUT and gastric juice RUT to evaluate *Helicobacter*-like infections. However, the use of gastric juice is the preferred method because it is less invasive and can be completed with simpler equipment and less skill. As the results of the present study showed in equine, RUT is more sensitive than HPE for detecting *H. pylori* in humans [31]. A comparison of RUT and HPE in humans with gastritis showed that both tests have the same accuracy in detecting *H. pylori* infection. Since RUT is a cheap and fast technique, it can be a good alternative to HPE [32]. In humans, a new method for RUT has been used in which the mucosa is swept using a sweeping motion with an absorbent swab held with forceps. Compared to the conventional method of tissue sample collection, the sweeping RUT method had higher sensitivity and accuracy along with faster detection time for *H. pylori* diagnosis [33]. The findings of the current research showed no difference between the RUT of gastric juice, NMP, and PA gastric tissue samples.

In the previous study, the sensitivity of RUT in horses was 40% compared to detection by PCR as the gold standard [10]. Some *Helicobacter* isolates from horses were urease-negative [14] and had no pathological effect [11]. The use of RUT will not be suitable for detecting urease-negative *Helicobacter*. Therefore, RUT will not detect urease-negative H. pylori.

In the present study, H&E staining could not show *Helicobacter* infection in RUT-positive samples. Warthin-Starry special stain, Giemsa, and Blue Toluidine staining methods identify Helicobacter as well as RUT in horses [18]. Therefore, special staining techniques can be used to detect *Helicobacter* in equine. However, in one study, no *Helicobacter* con-

tamination was found in the histopathology of the postmortem horses' stomachs despite using special stains, including Gram, PAS, and Warthin Starry [34].

Histopathological and immunohistochemical evaluation of samples collected from the mucosa and submucosa of slaughter horses was performed in Araguari, Brazil. In this evaluation, 81% of the horses had Helicobacter species in both the glandular and non-glandular regions, and the highest contamination was observed in the margo plicatus region [12]. The results of the present study showed no difference between the histopathological results of NMP and PA.

The results of the present research showed that serum antioxidant levels in *Helicobacter*-positive donkeys do not differ from *Helicobacter*-negative donkeys. On the other hand, the serum antioxidant level in humans with *H. pylori* infection is different from healthy people and *H. pylori*-positive patients have lower total thiol, native thiol, and disulphide levels than negative cases [22].

Previously, it was believed that antibiotics should be used to treat EGUS because *Helicobacter* was considered to be involved in the development of this syndrome [35]. In some studies, the microbiota of horses with EGGD was not different from the microbiota of healthy horses. For example, Lactobacillus salivarius and Sarcina ventriculi have been found in the healthy and lesioned gastric mucosa of horses [6]. However, in one study, Sarcina was more in the mucosa involved with EGGD showing that it may play a role in its pathogenesis. Proteobacteria were more abundant in healthy mucosa than in EGGD [2].

The limitations of the present study were the small number of animals and the lack of more accurate Helicobacter diagnosis methods, such as PCR or specific staining for histopathology samples. In addition to the valuable findings of the present study on miniature donkeys, perhaps these results can be extended to horses as well. This study was not repeated in horses due to financial limitations for buying horses.

It was concluded that donkeys, similar to horses, may be positive for *Helicobacter*-like, but it is unlikely that this infection will be effective in the development of EGGD. Glandular gastric infection with *Helicobacter*-like did not change the antioxidant status of the serum. The results obtained from the RUT methods for all three gastric juice, NMP, and PA tissue samples were not different from each other. Histopathology with the H&E staining of glandular gastric tissue of NMP and PA could not show *Helicobacter*-like infection and the sampling sites did not make any difference to each other in this respect.

Materials and Methods

Animals

Miniature donkeys (n=12), including six females and six uncastrated males, were randomly obtained from West Azerbaijan province, Iran. The animals aged 3-7 years, weighed 150-200 kg, and had a body condition score of 3-4 out of 9 [36]. These animals received oral ivermectin (0.2 mg/kg, IVERGEN®, Laluk, Tehran, Iran) six weeks before the start of the study. The animals were housed in stables, were fed daily with alfalfa hay, and had constant access to water. Physical examination of the animals was performed and their clinical health was confirmed.

Experimental Design

In the present study, donkeys were subjected to gastroscopy and gastric juice samples were taken for RUT, and tissue samples of gastric glandular mucosa were taken for histological evaluations with H&E staining and RUT. Tissue samples were evaluated for the presence of *Helicobacter* spp. infection under a light microscope. The commercial RUT kit (Bahar Afshan, Tehran) was used to evaluate the presence of urease-positive bacteria.

Blood samples were collected from donkeys and a complete blood cell count was performed immediately after sampling. Serum was obtained after clotting and centrifugation at 5000 rpm for 10 min. The sera were kept at -20°C until measurements. To evaluate the oxidant-antioxidant status of serum, TAC, THIOL, NO, and DPPH were evaluated [37]. The researchers and technicians involved in the trial, including those who performed sampling and laboratory and histological analyses, were blinded to the previously obtained results.

Endoscopy

Food (12 hours) and water (4 hours) withholding were applied before gastroscopy [38]. Sedation was performed using intravenous acepromazine at a dose of 0.05 mg/kg and xylazine at a dose of 0.5 mg/kg (NEUROTRANQ®, Alfasan, Woerden, Holland) [39]. Before the gastroscopic examination, gastric fluid was sucked using an endoscope catheter tube (7 fr, 3.5 m, STORZ, Tuttlingen, Germany) for RUT (Figure 2). Gastroscopy (STORZ®, RP100, Tuttlingen, Germany) was performed by blowing air and washing food residues on the gastric mucosa. The entire stomach and the upper duodenum were examined, except for the part that was covered with a little water and food at the bottom of the stomach [40]. EGGD was evaluated with a grade of 0-4 by two expert investigators [41]. Glandular gastric mucosal biopsy of NMP and PA was performed using grasping forceps (3.5 m, STORZ, Tuttlingen, Germany). Prior to the biopsy, the sampling site was flushed with distilled water. Histopathological specimens were fixed in 10% formalin immediately after sampling.

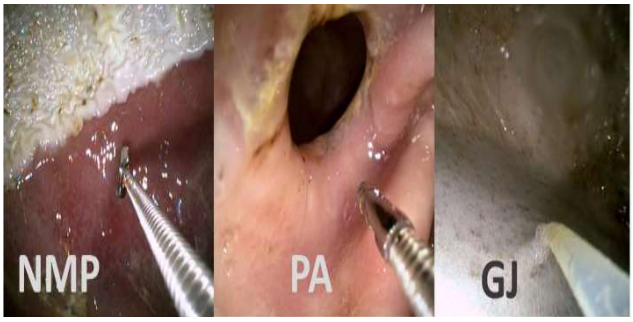

Figure 2.
Sampling of glandular gastric mucosa from near margo plicatus (NMP) and pyloric antrum (PA) for histopathology and RUT. Gastric juice (GJ) sampling for RUT.

Statistical analysis

Statistical analysis could not be performed due to the presence of only one animal with a positive rapid urease test, and only the descriptive statistics and range of parameters of other animals with a negative test were presented. The parameter values were calculated by the Excel software.

Authors' Contributions

MA contributed to concept design. MA and GJ collected all data. GJ performed the statistical analysis. MK performed the histopathological examinations. MA preparation of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version

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Competing Interests

The authors declare that there is no conflict of interest.

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Case Report

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Histopathologic aspects of pancreatic islet cell tumor in a dog

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ABSTRACT

A 12-year-old 4.3 kg intact female Terrier presented with a history of lethargy, anorexia, and melena for one month. The animal was dull and depressed. Dermatologic examination revealed some focal erythematous, crusty, and papulopustular lesions over the ventral abdomen. Ultrasonographic evaluation displayed an abnormal parenchymal pattern of the liver, which was noticed in the cranial part of the abdomen with diffuse hepatic involvement with irregular, hypoechoic, and heterogeneous ill-defined areas [a honeycomb-like echotexture]. Lateral thoracocervical radiograph showed numerous well-defined, small lytic lesions [polyostotic punched-out lesions] in the dorsal spinous process of axis bone in C2-C5 and pelvis that were likely metastatic lesions. Despite hospitalization and treatment, the dog died after 2 weeks and a necropsy was performed. At postmortem examination, a 5-cm mass was observed in the pancreas adjacent to duodenal loops which was finally diagnosed as a pancreatic tumor. Abnormal laboratory findings included elevated blood urea nitrogen, creatinine, cholesterol, total bilirubin, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyltranspeptidase, and calcium. Histopathological examination of the affected pancreas revealed neoplastic cells which were arranged mainly as solid nests or clusters and the amounts of the tumor stroma among the cellular clusters or individual neoplastic cells were scant. According to an immunohistochemical study which was positive for chromogranine A, the condition was diagnosed as a pancreatic islet cell tumor. Although rare, pancreatic islet tumors should be included in the differential diagnosis of abdominal discomforts, pancreatic inflammation, and hepatopathies. The final diagnosis of the tumor is achieved by combining imaging techniques and advanced histopathologic evaluations.

Keywords

Tumor, Pancreatic islet cell tumor, Histopathology, Immunohistochemistry, Dog

Abbreviations

C2-C5: Cervical vertebrae
GI: Gastrointestinal

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Number of Tables: 2
Number of References:: 12
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Introduction

Pancreatic endocrine tumors have been reported in various species including humans, dogs, cats, and ferrets [1]. In order to diagnose pancreatic endocrine tumors, the pathologist should confirm the neuroendocrine nature of the tumor cells. These tumors have various microscopic results, and immunohistochemical staining with different kinds of markers like chromogranin A, synaptophysin, and neuron-specific enolase, can usually confirm the neuroendocrine origin [2]. It can be difficult to accurately evaluate the degree of malignancy of pancreatic endocrine tumors but other features of the tumors, including local invasion and metastases to lymph nodes and distant organs, are helpful to explain their malignant nature [3]. There are different commonly recognized pancreatic endocrine tumors like gastrinomas, insulinomas, glucagonomas, and somatostatinomas. Although these different types of pancreatic endocrine tumors share some clinical features and histological aspects, they differ in their pathogenesis, hormonal syndromes produced, many aspects of biological behavior, and most importantly, in their response to chemotherapy and/or molecular targeted therapies. Here, we presented the histopathological appearance of a pancreatic islet cell tumor in an old Terrier dog. To the best of our knowledge, this is the first report of pancreatic tumors in Iran.

Case Presentation

A 12-year-old female Terrier dog weighing 4.3 Kg was presented to the Ferdowsi University of Mashhad Veterinary Teaching Hospital with a history of lethargy, anorexia, and melena for approximately one month. On physical examination, the animal was dull and depressed with a normal body condition score. Dermatologic examination revealed some focal erythematous, crusty, and papulopustular lesions over the ventral view of the abdomen. Differential diagnoses included endocrinopathies and malignancies. Complete blood count showed 1 % nucleated red blood cells and lymphopenia (520; Reference 1000-4800). Serum biochemistry revealed elevated blood urea nitrogen (88; Reference 10-28), creatinine (3.19; Reference 0.5-1.5), cholesterol (286; Reference 135-270), total bilirubin (0.43; Reference 0.1-0.4), aspartate aminotransferase (471; Reference 23-66), alkaline phosphatase (6470; Reference 20-156), gamma-glutamyltranspeptidase (45.5; Reference 1.2-6.4) and calcium (12.40; Reference 9-11.3). Other parameters, including C-reactive protein, were within normal ranges (Table 1, and 2).

Diagnostic imaging evaluations consisted of ultrasonography and radiology. Abdominal ultrasonography was performed with a 7.5 MHz linear transducer (Mindray, 6600 vets, China). The abnormal parenchymal pattern of the liver was noticed in the cranial part of the abdomen (Figure 1a). Diffuse hepatic involvement with irregular, hypoechoic, and heterogeneous ill-defined areas (a honeycomb-like echotexture) was observed that may be due to metastatic lesions or primary neoplasia. Lateral thoracocervical radiograph showed numerous well-defined, small lytic lesions (polyostotic punched-out lesions) in the dorsal spinous process of axis bone in C2-C5 and pelvis that are likely metastatic lesions (Figure 1b).

Characteristic ultrasound features of the evenly distributed hypoechoic nodular pattern, reflecting the neoplastic cells in the liver, which were consistent with the typical honeycomb pattern of superficial necrolytic dermatitis. Pancreatic mass was not detected by ultrasonography but later at necropsy, a mass was detected.

In spite of hospitalization and supportive treatment, the dog died after 2 weeks, and a necropsy was performed. At postmortem examination of the case, a 5 cm mass was observed in the pancreas adjacent to duodenal loops, which was finally diagnosed as a pancreatic tumor. The Liver was diffusely pale and had rounded margins and hepatomegaly was another prominent finding. Histopathological samples were taken from the liver and the suspected unknown mass

Table 1.
Haematological findings

Hematologic findings	patient	Reference valuesa
Hematocrit (%)	39	36-60
Hemoglobin (g/dl)	12.2	12.1-20.3
Red Blood Cell ($\times10^6$µl)	6.3	4.8-9.3
MCV (fl)	63	58-79
MCH (pg)	28	19-28
MCHC (g/dl)	37	30-38
Platelets($\times10^3$µl)	225	170-400
White blood cells ($\times10^3$µl)	11050	6.02-16.02
Mature neutrophils ($\times10^3$µl)	9750	2060-10600
Band neutrophils ($\times10^3$µl)	0	0-300
Lymphocytes($\times10^3$µl)	550	690-4500
Monocytes ($\times10^3$µl)	100	0-840
Eosinophils ($\times10^3$µl)	650	0-1200
Basophils ($\times10^3$µl)	0	0-150

James K. Klaassen, Reference Values in Veterinary Medicine LABORATORY MEDICINE VOLUME 30, NUMBER 3 MARCH 1999

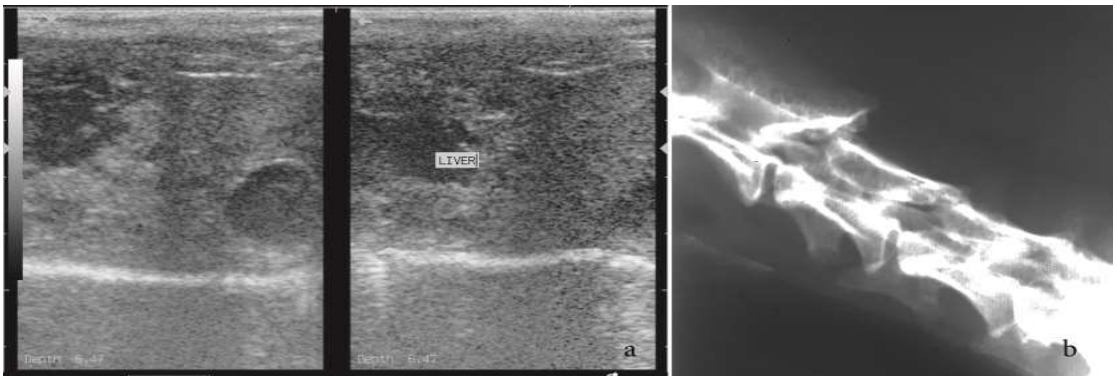

Fig 1.
(a) Sagittal images of liver ultrasonography showed diffuse hepatic involvement with irregular, hypoechoic and heterogeneous ill-defined areas. (b) Lateral cervical radiograph showed numerous well-defined, small lytic lesions [punched-out lesions] in the dorsal spinous process of C2.

in the pancreas and then, then fixed in 10% formalin before being embedded in paraffin. Some sections of the mass were used for immunohistochemical study for chromogranin A detection [4]. Histopathological examination of the liver revealed a severe and diffuse vacuolar change of hepatocytes. Most of the affected hepatocytes had clear and swollen cytoplasm (Figure 2a). In the affected pancreas, neoplastic cells were arranged mainly as solid nests or clusters, and the amounts of the tumor stroma among the cellular clusters or individual neoplastic cells were scant. Prominent and hyalinized collagenous connective tissue was observed between the neoplastic region and normal exocrine acini, and also in some parts of the tumor that formed a few separated microscopic areas within the tumor. An immunohistochemical study showed that the neoplastic cells were positive for chromogranine A (Figure 2b-2d). Based on postmortem, histopathological, and immunohistochemical findings, the condition was diagnosed as pancreatic islet cell tumor.

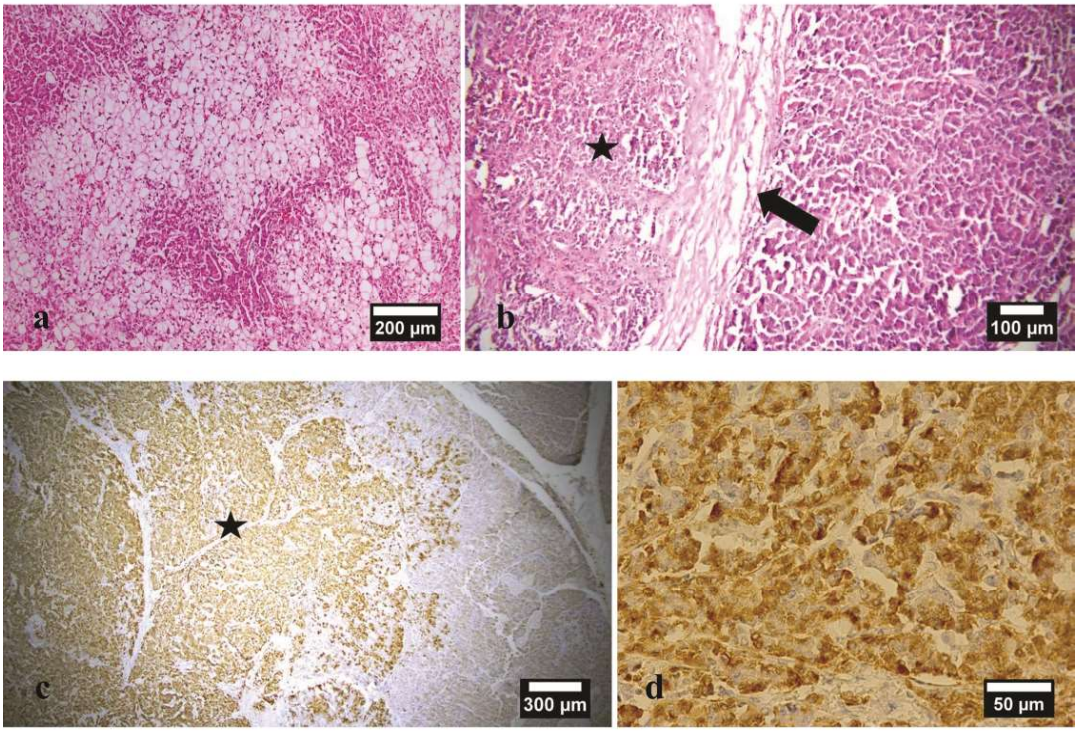

Fig 2.
(a) Severe vacuolar change of the hepatocytes in the affected case. (b) The neoplastic region [asterisk] is separated from the exocrine pancreatic tissue by a connective tissue capsule. (c) Immunohistochemical staining is positive for chromogranine A in the neoplastic area [asterisk]. (d) Higher magnification of the positive neoplastic cells for chromogranine A.

Table 2.
Serum biochemistry results

Biochemistry findings	patient	Reference valuesa
Total protein (g/dl)	7.5	5-7.4
Albumin (g/dl)	3.1	2.7-4.4
BUN (mg/dl)	88	4-27
Creatnine (mg/dl)	3.19	0.5-1.6
Glucose (mg/dl)	135	70-138
Cholestrol (mg/dl)	286	92-328
Bilirubin Total (mg/dl)	0.43	0.1-0.3
Alkaline phosphatase (IU/L)	6470	5-131
Alanine aminotransferase (IU/L)	156	12-118
Aspartate aminotransferase (IU/L)	471	15-66
Gamma-glutamyltranspeptidase	45.5	1.2-6.4
Calcium	12.4	9-11.3
Creatinine kinase (IU/L)	78	59-895

Result & Discussion

The islet cell tumors are immunohistochemically reactive to multiple hormones and can secrete different kinds of hormones including insulin, glucagon, somatostatin, pancreatic polypeptide, and gastrin, either singly or in combination [5].

Our case was a dog who presented with concurrent skin lesions, hepatic failure, and lytic bone involvement. The skin lesions are caused by degeneration of keratinocytes, resulting in epidermal edema and necrosis [6]. The precise mechanism underlying the development of skin disease in islet cell tumors remains unknown, but one of the proposed mechanisms is the occurrence of hypoaminoacidaemia, which may induce keratinocyte necrosis through epidermal protein deficiency [7]. Unfortunately, we were not able to take a skin biopsy sample during the examination and necropsy of delayed diagnosis of the tumor (missing data). Although not confirmed histopathologically, the presentation of the skin lesions in the present case resembles typical skin lesions of superficial necrolytic dermatitis [6, 8].

With pancreatic tumors, the signs can be vague or nonspecific (10); signs may include loss of appetite, vomiting, watery diarrhea, lethargy, pain in the abdomen, and weight loss. If the tumor has metastasized such as the bones, the clinician may notice lameness. Most of the affected dogs also have non-regenerative anemia, mild hyperglycemia, increased serum liver enzyme activities, and a honeycomb-appearing liver on abdominal ultrasonography. [6, 8]. The dog presented here had a history of lethargy, anorexia, and melena for approximately one month. These unremarkable signs might be due to concurrent pancreatitis, inflammation of the GI tract, and/or cholangiohepatitis.

Laboratory abnormalities observed in the present case indicate severe hepatocellular dysfunction. A wide variety of reasons, including, for instance, metastasis of pancreatic tumor, concurrent GI disease, and/or cholangiohepatis reported as reasons for these abnormal findings.

Bone lesions in the present case, might be due to metastatic invasion of the tumor, which was not confirmed histopathologically. Skeletal metastases including both osteolytic and osteoblastic lesions have been described. In people, a prevalence range of 5 to 20 percent of these lesions has been reported [9]. Most patients have widely metastatic disease at the time of diagnosis.

As emphasized in the present study, immunohistochemistry has become an essential ancillary examination for the identification and classification of these kinds of tumors. In the present case, the neoplastic cells were positive for chromogranin A, which is specific to endocrine cells. Chromogranin A, due to its primary expression throughout the neuroendocrine system, is a widely accepted biomarker for the assessment of neuroendocrine tumors [11].

Many authors believe that the prognosis of islet cell tumors is grave, although surgical removal of a pancreatic tumor may be curative in the unlikely scenario that metastasis has not occurred [11]. Clinicians should be aware of the uncommon early manifestations of islet cell tumors. Early diagnosis allows complete surgical removal of the neoplasm and provides the only chance of a cure. Additional case studies are needed to further characterize the cytomorphologic features and clinical presentation of pancreatic islet cell tumors in dogs.

Authors' Contributions

Javad Khoshnegah: Supervision, Conceptualization, Visualization, Resources, Writing- Reviewing and Editing.

Hossein Nourani: Methodology, Investigation, Writing- Reviewing and Editing.

Ali Mirshahi: Methodology, Investigation, Validation

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Competing Interests

The authors declare that there is no conflict of interest.

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Abstracts (in Persian)

ارزیابی آسیب‌شناسی التیام زخم پس از استفاده از سلول‌های بنیادی مزانشیمی گرفته شده از بافت چربی با هیدروژل صمغ کتیرا، و غشای آمنیوتیک انسانی به عنوان پانسمان

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چکیده

بهبود زخم و یافتن راه حلی برای بهبود سریع آن یکی از مسائل مهم دنیای امروز است. این مطالعه با هدف بررسی تأثیر استفاده از هیدروژل صمغ کتیرا به عنوان داربست سه بعدی سلول‌های بنیادی مزانشیمی همراه با پانسمان زخم غشای آمنیوتیک انسانی در ترمیم زخم‌های پوستی با ضخامت کامل در رت انجام شد. در این مطالعه ۵۴ رت ماده نژاد آلبینو (۱۵۰ گرم) به گروه‌های کنترل، هیدروژل و هیدروژل+سلول‌های بنیادی تقسیم شدند. تحت بیهوشی عمومی، دو زخم دو طرفه با ضخامت کامل در ناحیه پشتی توسط پانچ بیوپسی ۹/۸ میلی‌متری ایجاد شد. رت‌ها در روزهای ۳، ۱۰ و ۲۱ برای ارزیابی بافت‌شناسی و ردیابی سلول با ارزیابی PCR در نمونه‌های بافتی تحت آرام‌کشی قرار گرفتند. نتایج آسیب‌شناسی نشان داد که در روزهای ۳ و ۲۱ تفاوت معنی‌داری مشاهده نشد و تنها در روز ۱۰ تفاوت معنی‌داری وجود داشت. از نظر تشکیل بافت پوششی، بین گروه‌های درمان با گروه کنترل و از نظر تشکیل بافت جوانه‌ای، بین گروه هیدروژل+سلول‌های بنیادی مزانشیمی با گروه کنترل از نظر آماری معنی‌دار بود. نتایج ردیابی سلولی با PCR در روزهای ۳، ۱۰ و ۲۱ در گروه هیدروژل+سلول‌های بنیادی مزانشیمی نشان داد که تنها در روز ۳، سلول‌های بنیادی مزانشیمی یافت شدند. نتایج مطالعه حاضر نشان می‌دهد استفاده از سلول‌های بنیادی همراه با هیدروژل صمغ کتیرا به عنوان داربست، و استفاده از غشای آمنیوتیک انسانی به عنوان پانسمان، می‌تواند باعث ترمیم زودهنگام زخم‌های تمام ضخامت شود.

واژگان کلیدی

غشای آمنیوتیک، سلول‌های بنیادی مزانشیمی، رت‌ها، داربست، ترمیم زخم

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بررسی اثر تک دوز پیموبندان بر پارامتر های اکوکاردیوگرافی عملکرد قلب خرگوش های سالم نژاد سفید نیوزلندی

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چکیده

با توجه به روند رو به رشد نگهداری خرگوش بررسی بیماری های قلبی خرگوش و درمان آن ها الزامی می باشد. یکی از رایج ترین داروهای قلبی مورد استفاده در دامپزشکی پیموبندان است. پیموبندان در مدیریت درمان نارسایی قلبی پرکاربرد است. استفاده از این دارو در خرگوش صرفا بر اساس تجربه بالینی و تجربیات به دست آمده در سگ ها است و هنوز تایید نشده است. بنابراین اولین قدم تایید اثر این دارو بر عملکرد قلب است که یکی از راه های سنجیدن آن اکوکاردیوگرافی است.۱۱ خرگوش هرکدام ۵ بار برای اکوکاردیوگرافی فرستاده می شود. اولین و دومین بررسی اکوکاردیوگرافیک روز ۰ به فاصله یک ساعت از هم انجام می شود. در روز ۱، سومین بررسی اکوکاردیوگرافیک پیش از دادن پیموبندان و چهارمین و پنجمین بررسی، به ترتیب نیم ساعت و ۳ ساعت پس از دادن تک دوز پیموبندان (۰.۳ mg/kg) انجام می شود.تجویز خوراکی پیموبندان در خرگوش های سالم باعث ایجاد تغییراتی در برخی پارامتر های اکوکاردیوگرافی شد. از جمله، LVPWs، SV، EF، FS و اندازه دهلیز چپ در انتها دیاستول افزایش یافت (۰.۰۲۸۴ = p، ۰.۰۰۰۱ = p، ۰.۰۰۰۱ = p و ۰.۰۲۷۲ = p به ترتیب) در حالی که LVIDs و ESV کاهش یافت. (۰.۰۳۴۳ = p و ۰.۰۳۸ = p به ترتیب) این مطالعه نشان داد که پیموبندان اثرات مثبتی بر روی بطن چپ داشته و باعث بهبود عملکرد کلی قلب شد. مطالعات بیشتری لازم است تا مشخص شود که تاثیر پیموبندان در خرگوش ها با بیماری قلبی مشابه تاثیری است که در این مطالعه بر خرگوش های سالم گذاشته است یا خیر.

واژگان کلیدی

پیموبندان، اکوکاردیوگرافی، عملکرد قلبی، خرگوش، سالم

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اثرات ضد دیابتی گونه های اکتینومیستاله کشته شده با حرارت در کبد و کلیه موش های صحرایی دیابتی

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چکیده

بهدیابت نوع یک (T1DM) به دلیل کاهش ترشح انسولین به دنبال تخریب سلول های بتای پانکراس رخ می دهد. این بیماری در سراسر جهان به ویژه در کودکان زیر پنج سال رو به افزایش است که معمولاً با عوارض جبران ناپذیری مانند هپاتوپاتی و نفروپاتی همراه است. مطالعه حاضر با هدف بررسی اثر ضد دیابتی گونه های اکتینومیستاله کشته شده با حرارت، از جمله گوردونیا برونشیالیس (Gb) و تسوکومورلا اینکوننسیس (Ti) در موش های دیابتی شده با استرپتوزوتوسین، با تجویز خوراکی انجام شد. این آزمایش در شش گروه شامل کنترل سالم، کنترل دیابتی، Gb با دوز کم (G1)، Gb با دوز بالا (G2)، با دوز پایین (T1) و Ti با دوز بالا (T2) انجام شد. متعاقبا؛ سطوح AST، ALT، پروتئین تام، آلبومین، BUN، کراتینین، IL-1β، CRP و IL-2 در نمونه های سرم در روزهای ۱۴ و ۲۱ اندازه گیری شد. علاوه بر این، ضایعات هیستوپاتولوژیک در کبد و کلیه مورد مطالعه قرار گرفت. یافته-های حاضر نشان داد که Gb و Ti می توانند پارامترهای سرمی بررسی شده را به ویژه در گروه های T2 تغییر دهند. همچنین بررسی بافت‌شناسی کاهش قابل‌توجهی را در ضایعات پاتولوژیک مانند نکروز کانونی، پرخونی عروقی و خونریزی در کبد و کلیه موش‌های تحت درمان با Gb و Ti نشان داد. با توجه به نتایج پژوهش حاضر به نظر می رسد تجویز خوراکی گونه‌های اکتینومیستاله کشته شده با حرارت، به‌ویژه با دوز بالای Ti، می‌تواند به طور مفیدی پیشرفت T1DM و عوارض مختلف آن را بهبود بخشد، که با انجام تحقیقات تکمیلی می‌تواند برای درمان T1DM در آینده مورد استفاده قرار گیرد.

واژگان کلیدی

دیابت نوع یک، گوردونیا برونشیالیس، تسوکومورلا اینکوننسیس، هپاتوپاتی، نفروپاتی

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اثرات حفاظتی عصاره پوست انار بر بافت‌های آبشش، کبد، و کلیه در مسمومیت تجربی با کادمیوم در ماهی کپور (*Cyprinus carpio*)

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چکیده

این مطالعه با هدف بررسی اثرات محافظتی عصاره پوست انار (PoP) بر بافت های آبشش، کبد و کلیه در ماهی کپور معمولی مواجهه شده با کادمیوم (Cd) انجام شد. بدین منظور، ۱۵۰ ماهی کپور معمولی با وزن ۰.۸۵±۶۵ گرم به طور تصادفی به پنج گروه با سه تکرار برای هر گروه (۳۰ ماهی در هر گروه) تقسیم شدند. گروه کنترل جیره غذایی استاندارد بدون کادمیوم کلرید ($CdCl_2$) دریافت کرد، گروه Cd غلظت ۰/۵ میلی‌گرم در لیتر $CdCl_2$ دریافت کرد، و گروه های عصاره علاوه بر ۰/۵ میلی‌گرم در لیتر $CdCl_2$ ، عصاره PoP با غلظت های ۱٪، ۲٪ و ۴٪ (درصد وزن غذا) دریافت کردند. پس از چهار هفته، نمونه‌های بافتی از بافت‌های آبشش، کبد و کلیه گرفته شده و برای بررسی هیستوپاتولوژیک با هماتوکسیلین و ائوزین رنگ‌آمیزی شدند. در آبشش‌ها، ضایعات شامل احتقان، خونریزی، چماقی شدن یا همجوشی تیغه‌های ثانویه، و تلانژکتازی تیغه ها در گروه Cd بود. بافت کبد در گروه Cd دژنراسیون و نکروز شدید هپاتوسیت ها را نشان داد، در حالی که خونریزی، احتقان، دژنراسیون یا نکروز سلولی و قالب های هیالینی در بافت کلیه این گروه قابل مشاهده بودند. شدت ضایعات ذکر شده به طور معنی‌داری در گروه‌های عصاره، به‌ویژه ۲ و ۴ درصد، کاهش یافت ($p<0.05$). با توجه به نتایج، می توان نتیجه گرفت که عصاره PoP اثرات محافظتی قابل توجهی بر بافت های آبشش، کبد و کلیه در کپور معمولی مواجهه شده با $CdCl_2$ دارد.

واژگان کلیدی

کبد، کلیه، آبشش، هیستوپاتولوژی، کادمیوم، عصاره پوست انار

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اولین ارزیابی آلودگی کلامیدیا آبورتوس در جمعیت شتر ایران

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چکیده

کلامیدیوز یک بیماری مهم در گوسفند، شتر، بز، گربه، پرندگان و گاو است که توسط گونه های مختلف از جنس کلامیدیا ایجاد می شود. کلامیدیوز نشخوارکنندگان یک بیماری مشترک بین انسان و دام است و به ویژه برای زنان باردار در تماس با پناهگاه های حیوانات، نگران کننده است. کلامیدیوز در شتر می تواند باعث سقط جنین، چسبندگی گردن رحم، هیدروبورسیت تخمدان و اختلال در تولید مثل شتر نر شود. همچنین کلامیدیا در شتر می تواند برای مدت طولانی بدون علامت باقی بماند. شترهای آلوده ممکن است نقش مهمی در انتقال کلامیدیا به سایر گونه های جانوری داشته باشند. اعضای خانواده کلامیدیاسه در حال حاضر در یک جنس و هفت گونه قرار می گیرند. در بین این هفت گونه، کلامیدیا آبورتوس در شتر از اهمیت ویژه ای برخوردار است. با کمک تکنیک PCRمی توان گونه های کلامیدیا را با استفاده از پرایمرهای اختصاصی شناسایی و تفکیک کرد. مطالعه حاضر اولین مطالعه کلامیدیا آبورتوس در شترهای ایرانی است که بسیار حائز اهمیت است. بدین منظور از شترهای نر و ماده به ظاهر سالم در جنوب استان کرمان، تعداد صد نمونه خون با ماده ی ضد انعقاد گرفته شد. سپس از هر نمونه خون با استفاده از کیت استخراج DNA از خون، طبق دستورالعمل سازنده، DNA استخراج شد. در ادامه، روش PCR با استفاده از پرایمر اختصاصی rOMP90_3 برای بررسی حضور کلامیدیا آبورتوس انجام شد. نمونه ی مثبتی مشاهده نشد. بنابراین با توجه به این نتایج می توان گفت که کلامیدیا آبورتوس احتمالاً در شترهای جنوب استان کرمان در ایران، وجود ندارد.

واژگان کلیدی

شتر، ایران ، PCR ،کلامیدیا آبورتوس

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تشخیص هلیکوباکتر در معده الاغ‌های سالم انگاشته شده: نمونه‌برداری، روش‌ها و پیامدهای آن برای بیماری معده‌ی غده‌ای اسب‌سانان و وضعیت آنتی‌اکسیدانی سرم

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چکیده

دخالت هلیکوباکتر در بیماری معده غده اسب (EGGD) مشخص نیست. شواهد زیادی وجود هلیکوباکتر در معده اسب را تایید می کند، اما در مورد الاغ این موضوع چندان واضح نیست. مطالعه حاضر به منظور بررسی وجود هلیکوباکتر در معده الاغ و بررسی بهترین روش برای شناسایی آن و مشارکت احتمالی آن در وضعیت EGGD و آنتی اکسیدانی سرم انجام شد. نمونه برداری از شیره معده و بیوپسی از نزدیک مارگو پلیکاتوس (NMP) و آنتروم پیلور (PA) از ۱۲ الاغ به روش گاستروسکوپی انجام شد. هیستوپاتولوژی با رنگ آمیزی H&E و تست سریع اوره آز (RUT) برای شناسایی هلیکوباکتر استفاده شد. ظرفیت آنتی اکسیدانی تام (TAC)، تیول تام (THIOL)، نیتریک اکسید (NO) و دی فنیل۱--پیکریل هیدرازیل (DPPH) نیز برای ارزیابی وضعیت آنتی اکسیدانی سرم استفاده شد. هلیکوباکتر تنها با روش RUT در یک الاغ (۳۳/۸ درصد) شناسایی شد، اما بعید است که این عفونت در ایجاد EGGD موثر باشد. نتایج RUT برای هر سه نمونه شیره معده و NMP و PA تفاوتی با یکدیگر نداشتند. هیستوپاتولوژی NMP و PA با رنگ آمیزی H&E آلودگی به هلیکوباکتر را نشان ندادند. وجود هلیکوباکتر در معده الاغ وضعیت آنتی اکسیدانی سرم را به شکل واضحی تغییر نداد. RUT نسبت به هیستوپاتولوژی H&E برای ارزیابی هلیکوباکتر پیلوری در معده اسب کارآمدتر است و RUT شیره معده به دلیل آسان، سریع و غیر تهاجمی بودن به نمونه های بافتی ارجحیت دارد. در نهایت پیشنهاد می شود مطالعات بیشتری با روش های دقیق تر برای ارزیابی اثر هلیکوباکتر در بیماری های معده الاغ و سایر اسبسانان انجام شود.

واژگان کلیدی

سندرم زخم معده اسب، الاغ، میکروبیوم، آنتی اکسیدان

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شرح هیستوپاتولوژیک یک مورد تومور یاخته‌های جزایر درون ریز پانکراس در یک قلاده سگ

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چکیده

یک قلاده سگ تریر ۱۲ ساله ماده عقیم با وزن ۴.۳ کیلوگرم با شکایت تظاهرات جلدی روی شکم، بی حالی، بی اشتهایی، کاهش وزن و مِلنا در یک ماه گذشته، به بیمارستان آموزشی ما ارجاع داده شد. در معاینه فیزیکی، حیوان بی حال، افسرده و دارای نمره توده بدنی طبیعی بود. در ارزیابی درماتولوژیک جراحات جلدیِ اریتماتوز، دلمه ای و پاپولوپوستولار روی شکم بیمار دیده شدند. بررسی اولتراسونوگرافی نشان دهنده الگوی پارانشیمی غیرطبیعی کبد به صورت نواحی نامنظم هایپواکوژن و هتروژنِ پر از هوا بود. در رادیوگرافی جانبی، شمار زیادی ضایعات پلی اُستوتیک پانچی در زائده خاری مهره های گردنیِ شماره ۲ تا ۵ و در لگن مشاهده شد که احتمالاً ضایعات متاستاتیک بوده‌اند. به رغم درمان، بیمار زنده نماند و نکروپسی انجام شد. در کالبدگشایی، یک توده ۵ سانتی متری در پانکراس در کنار لوپ‌های دوازدهه مشاهده شد که نهایتاً تومور پانکراس تشخیص داده شد. یافته‌های آزمایشگاهی غیرطبیعی عبارت بودند از افزایش اوره، کراتینین، کلسترول، بیلی روبین تام، آنزیم‌های کبدی، گاما گلوتامیل تراتسفراز و کلسیم. اندازه کبد بزرگ شده و به طور وسیع رنگ پریده با لبه های گرد بود. بررسی هیستوپاتولوژیک کبد نشان دهنده تغییرات واکوئلار وسیع هپاتوسیت‌ها بود. در پانکراس درگیر، سلول های توموری بیشتر بصورت توده های سلولی توپور آرایش پیدا کرده بودند و مقدار استرومای تومور در بین توده های یاخته های توموری و یاخته های منفرد بسیار کم بود. بافت همبند کلاژنه هیالینه و مشخص بین ناحیه توموری و آسینی های طبیعی بخش برون ریز و همچنین در برخی بخش های تومور مشاهده شد که تعدادی کانون میکروسکوپی مجزا در داخل تومور تشکیل داده بود. ارزیابی ایمونوهیستوشیمی نشان داد که یاخته‌های سرطانی برای رنگ کروموگرانین A مثبت هستند. بر مبنای یافته‌های کالبدگشایی، هیستوپاتولوژی و ایمونوهیستوشیمی، عارضه بیمار، تومور یاخته های بخش درون ریز پانکراس تشخیص داده شد. گرچه این نوع تومور نادر است ولی لازم است در فهرست تشخیص‌های افتراقی مشکلات ناحیهٔ شکم، التهاب پانکراس و مشکلات کبدی جای گیرد. تشخیص نهایی نوع تومور با بهره گیری از روش های پیشرفته تصویر برداری و ارزیابی هیستوپاتولوژیک صورت می‌گیرد.

واژگان کلیدی

تومور، تومور پانکراس، هیستوپاتولوژی، ایمونوهیستوشیمی، سگ

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Protocol for DNA/RNA extraction, including quantification and determination of purity.

Reverse transcription (if used): amount of RNA, concentration of all reagents: primers concentration (either random primers or oligonucleotides), reverse transcriptase and master mix components.

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- Reviewers are expected to maintain the confidentiality of the manuscripts they review and not discuss any information from the manuscript with anyone other than the Editor-in-Chief, unless they have obtained explicit permission to do so. This also applies to invited reviewers who decline the review invitation.

- Reviewers are obligated to treat the manuscripts they receive for peer review as confidential and must not use any information obtained through this process for personal gain.

-Reviewers are expected to provide technical, professional, and objective comments on the manuscripts they are invited to review.

-Reviewers are expected to avoid personal biases in their comments and judgments, and express their views clearly with supporting arguments that assist the author in improving the manuscript.

-Reviewers are expected to identify any relevant published work that has not been cited by the authors. If a statement has been previously reported elsewhere, it should be accompanied by the appropriate citation.

-Reviewers should also bring to the attention of the Editor-in-Chief any significant similarity or overlap between the manuscript under consideration and any other publications of which they are personally aware.

The process has been explained in the section "Peer Review Process".

Ethical guidelines for Editor

-The editors should evaluate submitted manuscripts to determine if they fall within the scope of the journal. Additionally, the editors should recommend expert reviewers based on their integrated recognition of specialized reviewers.

-The Editor-in-Chief is responsible for deciding whether to accept or reject submitted manuscripts for the journal. This decision takes into consideration several factors, such as the judgment of the editorial board members, the validation of the work in question, its significance to researchers and readers, as well as any feedback from reviewers. Furthermore, the decision must also comply with legal requirements regarding libel, copyright infringement, and plagiarism, which are currently in force. The Editor-in-Chief works closely with other editors and reviewers to ensure that all submissions are fairly evaluated.

-The editors ought to uphold the anonymity of both reviewers and authors.

-The editors should disclose any potential conflicts of interest and make efforts to avoid them. If such circumstances arise, they are expected to delegate the handling of the manuscript to another member of the editorial board.

-The editors, particularly the Editor-in-Chief, should demonstrate a willingness to investigate cases of plagiarism and fraudulent data. When ethical concerns are raised about a submitted manuscript or published paper, the editors will take appropriate measures in response. Any reported incidents of unethical publishing behavior will be thoroughly examined, even if they come to light years after publication.

-When dealing with cases of suspected misconduct, the Editor-in-Chief follows the COPE Flowcharts. If an investigation supports the ethical concern, the journal will publish a correction, retraction, expression of concern, or any other relevant note.

-The editors must not share any information about submitted manuscripts with anyone until they are published, as appropriate.

-The Editor-in-Chief and members of the editorial board will not use unpublished materials disclosed in a submitted paper for their own research purposes without obtaining explicit writ-

ten consent from the author.

-Editors are expected to give fair consideration to all manuscripts submitted for publication, evaluating each on its own merits and without prejudice based on the author(s)' country, race, religion, nationality, sex, seniority or institutional affiliation. Decisions about editing and publishing are made solely based on the quality and relevance of the manuscript and are not influenced by external policies of governments or other agencies beyond the scope of this journal.

-The Editor-in-Chief has complete authority over the editorial content of the journal as well as the timing of its publication.

Ethical guidelines for Publisher

"Ferdowsi University of Mashhad press (FUM)" is promising to ensure that the decision on manuscript submissions is only made based on professional judgment and will not be affected by any commercial interests.

- FUM is committed to maintain the integrity of academic and research records.

- FUM is monitoring the ethics by Editor-in-Chief, Associate Editors, Editorial Board Members, Reviewers, Authors, and Readers.

- FUM, together with the Journal's editors, shall take reasonable steps to identify and prevent the publication of manuscripts where research misconduct has occurred, and under no circumstances encourage such misconduct or knowingly allow taking place.

- FUM is always checking the plagiarism and fraudulent data issues involving in the submitted manuscripts and willing to publish corrections, clarifications and retractions involving its publications as and when needed.

-FUM as the publisher supports the Journal for each published issue by paying a defined budget according to its published annual rank in the Portal of Scientific Journals of Iranian Ministry of Science, Research and Technology for costs including those pertaining to setup and maintenance of the publication infrastructure, routine operation of the Journal, processing of manuscripts through peer-reviews, editing, publishing, maintaining the scholarly record, and archiving.

Violation of Publication Ethics

The Editorial board of IJVST acknowledges that plagiarism is unacceptable in any of its forms:

Plagiarism:

Plagiarism is intentionally using someone else's ideas or other original material as if they are one's own. Copying even one sentence from someone else's manuscript, or even one of your own that has previously been published, without proper citation is considered by the JAM as plagiarism. All manuscripts under review or published with JAM are subject to screening using plagiarism prevention software (e.g. iThenticate). Thus, plagiarism is a serious violation of publication ethics.

Simultaneous Submission:

Care should be taken to ensure that the work has not been published elsewhere, in any language and is not simultaneously submitted to other journals.

Duplicate Publication:

Duplicate publication occurs when two or more articles, without full cross referencing, share essentially the same hypotheses, data, discussion points, and conclusions.

Redundant Publications:

Redundant publications involve the inappropriate division of study outcomes into several articles, most often consequent to the desire to plump academic vitae.

Data Fabrication:

Data fabrication means the researcher did not really carry out the study, but made up data or results and had recorded or reported the fabricated information. Data falsification means the researcher did the experiment, but manipulated, changed, or omitted data or results from the research findings.

Citation Manipulation:

Citation Manipulation implies excessive citations in the submitted manuscript that do not contribute to the scholarly content of the article and have been included solely for the purpose of increasing citations to a given author's work, or to articles published in a particular journal. This leads to misrepresenting the importance of the specific work and journal in which it appears and is thus a form of scientific misconduct.

Improper Author Contribution or Attribution:

All listed authors must have made a significant scientific contribution to the research in the manuscript and approved all its claims. Do not forget to list everyone who made a significant scientific contribution, including students and laboratory technicians.

Handling Misconduct Cases

The Editorial board of IJVST takes the necessary measures to examine the incoming papers on their originality, reliability of contained information and correct use of citations.

-If any of the unethical publishing behavior is detected by the Journal Editorial board or by one of the reviewers, the first action is to inform the Editor-in-chief by supplying copies of the relevant material and a draft letter to the corresponding author asking for an explanation in a nonjudgmental manner.

- If the infraction is less severe, the Editor, upon the advice of the Committee on Publication Ethics, sends the author a letter of reprimand and reminds the JAM publication policies; if the manuscript has been published, the Editor may request the author to publish an apology in the journal to correct the record.

- If the author's explanation is unacceptable and it seems that serious unethical conduct has taken place, the matter is referred to the Publication Committee via Editorial board. After deliberation, the Committee will decide whether the case is sufficiently serious to warrant a ban on future submissions.

Post-Publication Discussions and Corrections

This journal allows debate post publication on journal's site, through "Send comment about this article" section to the editor up to one month before final publication. Our mechanisms for correcting, revising or retracting articles after publication depends on the content of the received comment and if the sent comments are useful and applicable for readers/authors, they will be showed under reference section of the articles pages.

Publication Ethics

Complaint Policy

If the authors disagree with the editorial decision on their manuscripts, they have a right to appeal. Authors who wish to appeal an editorial decision should contact the Editor-in-Chief of the Iranian Journal of Veterinary Science and Technology. In such cases the Editor-in-Chief will review the manuscript, the editorial and peer reviewers' comments and gives his/her decision for accepting or rejecting a manuscript. Editor-in-Chief may, if so required, send the manuscript to a new handling editor for a fresh editorial review and to new reviewer for further peer reviewing. In such case, the final decision maker will be the Editorial board of the journal.

How to Make a Complaint

The procedure to make a complaint is quite simple. The complaint can be made by writing an e-mail to: ijvst@um.ac.ir. All complaints will be acknowledged within a week.

PEER REVIEW PROCESS

Iranian Journal of Veterinary Science and Technology peer reviews all submitted manuscripts with contents within the scope of the journal.

Initial assessment

The submitted manuscript will be subjected to a primary review by the editor or a member of the editorial board for suitability and relevance of the findings to the scope of the journal and quality of the science presented in the paper (sufficient originality, having a message that is important to the general field of Veterinary Medicine, quality of data, novelty, English language, and overall manuscript quality) within two weeks. If the paper is evaluated to be relevant to the scope of the journal and having enough scientific rigor and novelty, it will be sent for the next stage. Otherwise, those manuscripts which are evaluated as not-appropriate in the initial review will be rejected at this stage.

Initial screen

The initial screen will be performed by the editorial office for the structure and format of the manuscript.

Peer review (double-blind)

The manuscripts which are found to be appropriate after the initial screen will be sent for external review by experts in the related field. We have prepared a checklist for reviewers that summarizes their evaluation of the manuscript. The items in this checklist are:

1. TITLE is clear and adequate
2. ABSTRACT clearly presents objects, methods, and results.
3. INTRODUCTION well-structured and provides a rationale for the experiments described.
4. MATERIALS AND METHODS are sufficiently explained and is detailed enough to be reproduced.
5. RESULTS are clearly presented and supported by figures and tables.
6. DISCUSSION properly interprets the results and places the results into a larger research context, and contains all important references.
7. Conclusions are logically derived from the data presented.
8. English Language/style/grammar is clear, correct, and unambiguous.
9. Figures and tables are of good quality and well-designed and clearly illustrate the results of the study.
10. References are appropriate.
11. Regarding this article are you concerned about any issues relating to author misconduct such as plagiarism and unethical behavior.
12. Comments on the importance of the article.

Final Decision

Based on the reviewers' recommendations a final decision is made by the editor and if needed the help of a member of the editorial board (depending on the field of study). Decisions will include accept, minor revision, major revision with and without re-review, and reject. We aim to reach a final decision on each manuscript as soon as their review results are available.

Iranian Journal of Veterinary Science and Technology

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