University Of Mosul
College Of Veterinary Medicine

Comparative Histomorphometrical and Immunohistochemical study of Cecum in adult Rabbit (*Oryctolagus cuniculus*) and Hamster (*Mesocricetus auratus*)

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MSc /Thesis
Veterinary Medicine / Veterinary Anatomy

Supervised by:
Assistant professor
Dr. Ammar Ghanim AlHaaik

1446 A.H **2024 A.D**

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A Thesis Submitted

By

Doaa Saad Ameen Al nuaimi

To

The Council of the College of Veterinary Medicine

University of Mosul

In

Partial Fulfillment of Requirements

For the Degree of Master of science

In

Veterinary Medicine / Veterinary Anatomy

Supervised by

Assistant Professor

Dr. Ammar Ghanim AlHaaik

1446 A.H **2024 A.D**

قل لَّوْ كَانَ الْبَحْرُ مِدَادًا لِّكَلِمَاتِ رَبِّي لَنَفِدَ الْبَحْرُ قَبْلَ أَن تَنفَدَ كَلِمَاتُ رَبِّي وَلَوْ جِئْنَا بِمِثْلِهِ مَدَدًا ۞

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I certify that this thesis entitled " **Comparative Histomorphometrical and Immunohistochemical study of Cecum in adult Rabbit (*Oryctolagus cuniculus*) and Hamster (*Mesocricetus auratus*) "** was prepared under my supervision at the College of Veterinary Medicine / University of Mosul, as a partial fulfillment of the requirements for the degree of Master in Veterinary Medicine/Veterinary Anatomy.

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Based on the supervisor and linguistics recommendations, I forward this thesis for the defense.

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Date: / /2024

Postgraduate Committee Director Certification

Based on the supervisor, linguistics and the Head of the Department of Anatomy recommendations, I forward this thesis for the defense.

Signature:
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Date: / /2024

Examination Committee Decision

We, the members of the Evaluation and Discussion Committee, have reviewed this thesis and discussed the student **Doaa Saad Ameen** in its contents on / /2024, and certify that the deserves the degree of Master in Veterinary Medicine/ Veterinary Anatomy.

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The College of Veterinary Medicine Council was met, the meeting , on / /2024, and decided to award him a degree of Master in Veterinary anatomy.

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Dr. Dhafer Mohammad Aziz
Dean

Thanks and appreciation

First of all, I would like to acknowledge and thank my supervisor Asst. Prof. Dr. Ammar Alhaaik who made this work possible, his guidance and advice carried me through all the stages of writing my thesis.

To the dearest and closest people to my heart ,my parents, for their love, support, and preparing me for my future.

Words cannot express my gratitude to my husband and my children for their love, understanding and continuing support.

I am also grateful to my husband´ family, especially my father in law for their support and care.

Finally, I would also like to give special thanks to the Department of Anatomy, University of Mosul as a whole for their support.

Thank you all for your assistance and support

Doaa

Summary

The present study aimed to explore the morphological, histochemical and immunohistochemical study of cecum structure in the local breed of rabbits and Syrian hamster. To obtain this aim, specimens from cecum with its three parts including base, body and apex were collected from 15 adult rabbits and the same number was also collected from hamsters. Routine histology stain (H&E) as well as Massons trichrome stain besides PAS-AB $_{pH2,5}$ technique, additionally, immunohistochemical markers (GP2 and Vimentin) used for cecal M-cell (microfolded cell) expression were used to attain our objectives. The Microscopic examinations of cecal wall revealed that the wall of cecum in the three portions (base, body and apex) in both animals have identical known four intestinal layers but the folds of appendix of rabbit had well developed wide apices and narrow bases which appeared as (leaf-like). These folds lined with one layer of columnar epithelium and abundant goblet cells among them and the latter was characterized by its cylindrical shape with wide apical border and basally situated nuclei, which after staining with H&E appeared with clear cytoplasm. Also, the appendix of rabbit possess a well-developed lymphoid tissue in the shape of lymphoid nodules, every one of them comprehend four dissimilar locations which are: the dome region, middle germinal center, a region of the peripheral, and a widespread inter follicular area between the lymphoid follicles. The dome was also delimited by a specific FAE. This type of epithelium consists of simple columnar epithelium with numerous modified cells called M-cell. This epithelium was ordinarily lacking of goblet cells, yet there were isolated goblet cells in some spots.

The M-cell differ from enterocyte by its large size, pale

basolateral located nuclei and exhibit pocket that contained several lymphocytes which explain the participation of these cells in the improvement of immunity reply .

The appendix appeared as a very developed lymphoid structure in the rabbit. M cells were identified after applying GP2 antibody marker in brown color in its cytoplasm as well as in the apical and basolateral cell membrane especially in the FAE of the dome shaped structure.

The cecum of hamsters and rabbits presented evidence of two different forms of autonomic nerve plexuses, the first, Meissner nerve plexuses which was small and located in the submucosal tunica next to circular inner muscle layer of muscular coat and it was more noticed in hamster cecum than rabbit. Between the two sheets of tunica muscularis, the second plexus (Auerbach's) situated as well as developing structures comprising glial and neurons cells. All parts of the cecal wall have Auerbach's plexuses, with the hamster having a very enormous and plentiful number of them.

Pale (clear) large goblet cells appeared with H&E whereas they appeared in magenta color when stained by PAS and blue color by Alcian blue. The largest number of goblet cells in both animals with its three parts was recorded in rabbits appendix whereas the least number was recorded in rabbit body. Carbohydrate profile of cecum revealed dominance of acidic mucin in the crypts whereas the predominant type of mucin in the surface epithelium and folds was neutral type.

List of abbreviations

M cell	Microfolded cell
FAE	Follicle associated epithelium
GALT	Gut associated lymphoid tissue
MALT	Mucosa associated lymphoid tissue
IHC	Immunohistochemistry
PAS	Periodic acid- Schiff
AB	Alcian blue
CHO	Carbohydrate
IEL	Intraepithelial lymphocytes
IEC	Intestinal epithelial cells
DCs	Dendritic cells
LP	Lamina propria
GP2	Glycoprotein 2

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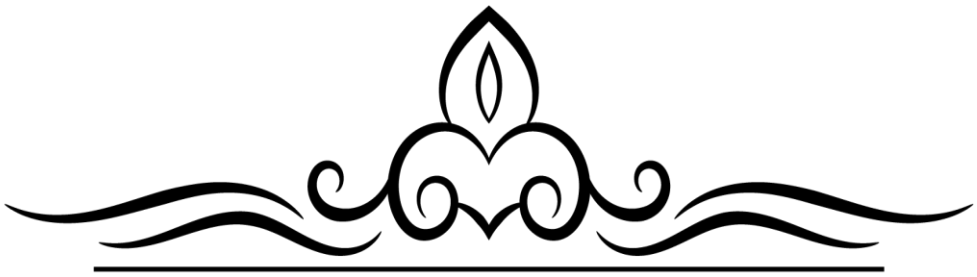
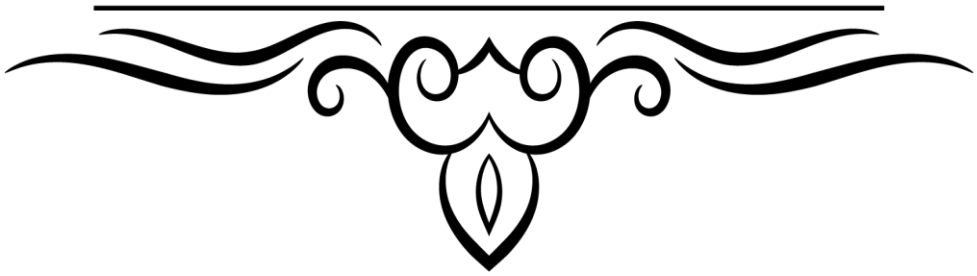


Chapter One

Introduction



Introduction

The cecum is generally labeled as first segment of large intestine as a closed end tubular structure among the ascendant colon and ileum (Barone, 1997). It is an extremely developed organ possess sac-like constrictions and several leaf-like spiral folding intended for fermentation (Ranjan and Das, 2021). The cecum collects the ileal contents then mixes them with the contents of the cecum for microbial fermentation (Björnhag and Snipes, 1999).

The caecum composes about 35 percent of entire size of the gastrointestinal tract in rabbit. It act as chief location of process of fermentation, but the proximal part of colon has a role in creation of Short-Chain Fatty Acids (SCFAs) too (Gidene and Perez, 2000).

Scientific Classification of European rabbit

- **Common Name**; Rabbits, old world rabbit, domesticated rabbit,
- **Kingdom**; Animalia
- **Phylum**; Chordata
- **Class**: Mammalia
- **Order**; Lagomorpha
- **Family**; Leporidae
- **Genus Species**; *Oryctolagus cuniculus*

1.1. The Rabbit (*Oryctolagus cuniculus*)

Rabbits are a member of the family Leporidae and they are herbivore animals which nourished mostly on the grass and presented excellently crafted GALT in contrast to other mammals (Herron, 2002; Cesta, 2006). Rabbits were used as a public model in research laboratory due to their comparatively big size and quiet nature, in addition to have a well-built gut associated lymphatic tissue (GALT) in contrast to different mammalian types (Sohn and Couto, 2012; Saleh, 2012).

Rabbits (*Oryctolagus cuniculus*) were categorized as hindgut fermenters that possess an extremely developed and fine distinguished large intestine (Amiry *et al.*, 2019).

As stated by pervious mentioned anatomical classical investigations on the rabbit's cecum, the rabbit's cecum is a model of a highly developed organ that is most likely allied to the previously mentioned process of the fermentation and coprophagy (Snipes, 1973).

The caecum in rabbits is divided anatomically to three portions by Snipes (1978): 1) bulbous ampulla coli, 2) corpus ceci, or body; and 3) caecum appendix vermiformis, or terminal section.

Generally, the microscopic structure of cecal wall of most mammals is made from four layers which are tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa (Mohamed *et al.*, 2018).

The appendix, which is a massive series of lymphatic tissues at the ileo-cecal valve, and sacculus rotundus, a large nodular Peyers' patch in the end of ileum, were unique to rabbits (Haley, 2003). Snipes (1978) submitted that sacculus rotundus and the appendix were specific lymphatic organs.

Furthermore, the microstructure of appendix vermiform had a noticeable major role as a primary organ for immune response prepared by this part of the gut-associated lymphatic tissue (GALT) of rabbit (Ranjan and Das, 2021).

1.2. The Hamster

The Syrian hamster (*Mesocricetus auratus*) was a broadly applied experimental animal model, It has unique anatomical and physiological specifications which make them favorite for research models (Valentine *et al*., 2012).

Scientific classification of hamster

- **Kingdom** ; Animalia
- **Phylum** ; Chordata
- **Subphylum** ; Vertebrata
- **Class** ; Mammalia
- **Order** ; Rodentia
- **Suborder** ; Myomorpha
- **Superfamily** ; Muroidea
- **Family** ; Cricetidae
- **Subfamily** ; Cricetinae
- **Genera** ; *Mesocricetus*
- **Species** ; *Mesocricetus auratus*

Hamsters were classified within Rodentia, the largest mammalian order. Historically, naturalists have hypothesized a close relationship between rodents and lagomorphs based on their shared characteristic of hypsodontic incisors adapted for gnawing (Meng and Wyss, 2005; Rose, 2006).

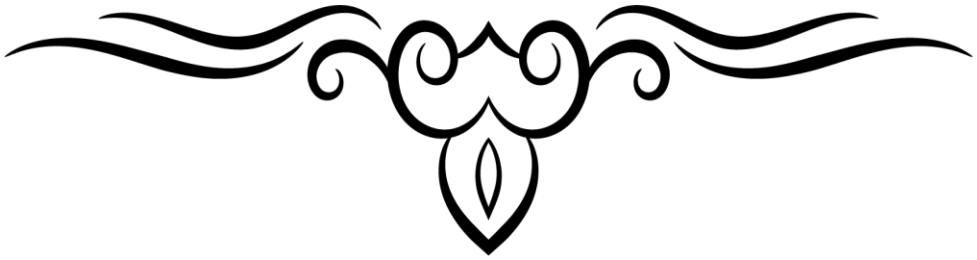
Aims of the study

This study is targeted to study the ceci differences between rabbits and hamsters through following parameters:

- Shape, location and dimensions of ceci.
- Histological architecture of the cecum wall.
- Micromorphometrical measurements of different structures in the cecal wall.
- Carbohydrate foundation of goblet cell in the ceci.
- IHC expression of M cell.

Chapter Two

Literature Review



Review of Literature

2.1. Cross- Review

2.1.1. Gross morphological approach

The most visible organ in the abdominal cavity is the cecum which is a tubular structure, typically contains 40% of the ingesta and about 49.75 cm in length, it doubles onto itself three times and has an internal surface made up of a long spiral fold that continues into the ampulla coli (Powers & Perpiñán, 2020). The several foldings that resemble spiral leaf-like are essential for microbial fermentation (Sohn and Couto, 2012; Ranjan and Das, 2021).

The cecum in chinchillas is placed slightly to the left side of abdominal cavity, it comprises two parts, the first part has folded-up look while the other part takes a tubular form (Perez *et al*., 2008; Kotze *et al.*, 2010; Stan, 2014). The cecum takes up approximately the total abdominal floor, relating to abdominal wall ventrally, to the portion of the sacculated colon, ileum, jejunum ,duodenum dorsally and to the portion of the smoothed colon Caudally, It has a link to the urinary bladder and is attached cranially to stomach and liver (Ranjan and Das, 2021).

The cecum, ileum, and ascending colon are unconnected in the chinchilla, and other hindgut fermenters Rodentia like nuetria (*Myocustor coypus*) (Perez et al., 2008), tuccu-tuccu (*Cetenomys pearsoni*) (Perez *et al.*, 2009) and capeybara (*Hydrochorus Hydrocheris*) (Vasquez *et al*., 2012). Cecum of Persian Squirrel is positioned in the right side of the abdominal cavity (Topografía, 2012).

The vermiform appendix, the blind end of caecum, appears a well-developed tissue (Perez *et al.*, 2007; Saleh, 2012). The vermiform

appendix is absent in most species but exists in specific mammals such as rabbits (Malla, 2003), and Cape Dume Mole-rat (Kotzé *et al.*, 2006).

The first part of the large intestine is usually the cecum, it is described as tube of blind termination among ascendant colon and ileum, through ileo cecal orifice is connected with the ileum and through the ceco-colic orifice connect with the colon (Ranjan and Das, 2021).

In the lagomorphs and rodents, the cecum had been split to three different morphological portions: ampulla ceci (cecal base), corpus ceci (cecal body) and cecal apex (Perez *et al.*, 2017).

2.1.2. Appendix of Cecum

Vermiform appendix is the distal portion of cecum end. It is a 5-inch, thick-walled, slim blind tube, and contains a large number of lymphoid aggregation and is assume as a structure related to the immune system mainly operating as beneficial bacterial safe house (Smith *et al.*, 2013). It also secretes bicarbonate ions into the cecum, acting as a fatty acid buffer for volatile fatty acids made by cecal fermentation (Yildiz *et al.*, 2001).

In all rabbits, the appendix is observed as a stretch of the enlarged cecum, and the variance in diameter, consistency, and color of the appendix in contrast to the cecum is definitely visible (Laurin *et al.*, 2011; Stan, 2014).

The rabbit has a well-developed vermiform appendix which its length is about 8.33±0.40 cm and its color is brighter if compared to the cecum (Smith *et al.*, 2013; Ranjan and Das, 2021). Distinct vermiformis appendix present morphologically in rabbits only that was stated by Malla (2003) and O'Malley (2008) whom mentioned that there were no other laboratory animals presented any obvious vermiform appendix.

In fact, numerous researchers assumed that the rabbits are convenient laboratory animal model used for studies related to immunity since its exceptional reply against various antigens (Besoluk *et al*., 2006).

In mammals, the appendix is stated in human, rabbits and certain rodent's species who participate a similar genetic origin and great lymphatic condensation (Smith *et al*., 2009). Therefore, it can be supposed that the immune role performed by the appendix of humans and rabbits is parallel to the immune roles performed by the final parts of cecum in other animals missing the vermiformis appendix (Stan, 2014).

2.1.3. Importance of Cecum

The absorption process taking place in the large intestine and cecum had been recognized in range of variety of mammals that includes the absorption of amino acids, water, carbohydrates and volatile fatty acids and electrolytes (Grant, 2014; Habeanu *et al*., 2022; Peretti and Mas, 2022).

The formation of acetic, butyric and propionic acids due to cellulose degradation by bacteria, results in delivering more than 40% of the animal's standard energy necessities (Huq *et al.*, 2021)**.**

For prolonged periods of time needed for microbial digestion of cellulose and the production of protein and vitamin B, the rabbit seems to have a highly developed mechanism that keeps digesta in the proximal colon and cecum (Davies & Davies, 2003).

All grass eating animals could be categorized as hindgut or foregut fermenters reliant on where the main site of microbial fermentation is placed , animals of hindgut fermenters could be more allocated into colon fermenters or cecum fermenters, anyway, Cecum fermenters are commonly smaller in size than the colon fermenters, cecum and colon fermenters were two additional types of hindgut fermenters, in the most

of small hindgut fermenters (below 10kgBW), the cecum is considered as primary or major fermentation compartment (Hume, 1989).

Rabbit digestive system is adjusted to a high dietetic fiber consumption which has the hindgut ferments (Herron, 2002).

Parker and McMillan (1976) proposed that the cecum is the chief place of fermentation in the rabbit since it occupies about 35% of the total bulk of the digestive tract. On the other hand, fermentation of nutritional fiber in rabbits had been proposed not to be restricted to the cecum as of short-chain fatty acids creation, which also has been noticed in the proximal colon (Gidenne and Perez, 2000).

An enlarged appendix develops in rabbits fed diets with high fermentable carbohydrates and low fiber; the later has been used as a proof that increased appendix secretory role was required to stabilize the products of increased carbohydrate fermentation (Davies and Davies, 2003).

Chinchillas and rabbit are considered a true herbivorus animals that have certain type of food digestion, referred to as posterior gut fermenters (Sakaguchi, 2003; Davies and Davis, 2003). Nevertheless, there are numerous recognizable variances in the intestinal anatomy of the animals that belonging to the lagomorph and rodent families, mostly in cecal morphology (Kotze *et al.*, 2006, 2010).

In guinea-pig, the structure of the mucosal wall of the cecum is mainly appreciated where significant fermentation process happens, primarily for the breakdown of cellulose, and population of bacteria that is required for this process (Koopman and Kenis, 1979). The internal structural variations of cecum was poorly structured in guinea pig in contrast to rabbits and vole (Snipes 1978) even though the guinea pig cecum has taeniae (Gabella, 2001).

2.1.4. Caecotrophy

It is interesting that the rabbits are representative of hindgut fermenter animal and true herbivores with a highly-developed cecum, Furthermore, rabbits have the caecotrophs nature (Irlbeck, 2001), that is through night, they unswervingly nourish on their personal soft feces.

This caecotrophic habit is a good source of vitamins B, minerals and proteins (Halls, 2008; Ranjan and Das, 2021).

The process of consumption of the soft feces resultant from the substances of cecum identified as caecotrophy, which is achieved by some cecum fermenters animals (comprising rabbits and ring tailed posum), the process of Caecotrophy is considered as a type of coprophagy (eating feaces) (Herron, 2002).

Cork & Foleiy (1997) briefed that the process of caecotrophy happens as the incident once the cecum substances were excluded in the form of soft feces (unique in both look and content), caecotrophy diminishes the necessity of small grass eating animals for commonly dietetic nitrogen and mostly essential amino acids.

Quesenberry and Carpenter (2011) stated that during the inactive time in the day, wild rabbits ingest soft pellets.

The digestive tract of a rabbit has thus modified to the quick consumption of large quantities of plants, as a prey kind, they rely on the rapid passage time of their unique digestive tract to preserve their body size and weight low, allowing them to be fast-moving and quick (Jenkins, 2001).

2.2. Histological properties of cecum

The microscopic structure of cecal wall of most mammals is constructed general from four layers which are tunica mucosa, tunica

submucosa, tunica muscularis, and tunica serosa (Bello, 2016; Muhson, 2022).

In rabbits, basis Ceci and Corpus Ceci have the same histo-architecture, but the microscopic structure of the vermiform appendix differed from that of the other two portions (Ranjan and Das, 2021).

The thickest portion of the cecum is ampulla ceci, in addition to thick muscular layer (Tunica muscularis), a parallel mucosal attendance has been described in segments of the rabbit cecum and in cecum of vole (Snips, 1979).

Corpus ceci: the body of cecum is definitely thinner than the region of ampulla. Each of tunica mucosa and tunica muscularis are reduced in thickness and the mucosa share an architecture compared to that in the ampulla area (Snipes, 1979).

The histological examination of the vermiformis appendix looked to be a lymphatic structure and considered, as has been stated by Saleh (2012), a portion of GALT in rabbits.

Overlying lymphatic nodules exist within the submucosa, tunica mucosa made a tree structure look like umbrella in its shaped, simple columnar epithelium with few goblet cells are lined these tree structures. The lamina propria shared structural similarities with the other intestinal segments, in the upper part of these structures, Crypts with many goblet cells were abundant (Ranjan and Das, 2021; Elseory *et al.*, 2023).

Follicle associated epithelium is lining the dome that has low columnar epithelium or cuboidal with many penetrated lymphocytes; however, goblet cells were missed, the whole nodule filled with lymphatic cells as well as few plasma cells (Ranjan and Das, 2021). Saleh (2012) stated parallel interpretations concerning the construction outline of lymphatic nodules, on the other hand, the vast lymphocyte

accumulation inside the nodules understood the immune response's role, chiefly in rabbits.

The intestines were unprotected against many possible antigens, pathogens, and microorganisms during the animal life (Brandtzaeg *et al*., 2008; Kanaya and Ohno, 2014). Pabst *et al.* (2007) stated that defense

against pathogenic factors on the one hand and keeping of compassion to harmless antigens on the other hand was talented.

The intestine has been found to include a multiplicity of lymphatic nodules, such as Peyer's patches, solitary lymphatic follicles, crypto patches, and lympho-glandular complexes in the large intestine (Cesta, 2006; Brandtzaeg *et al*., 2008; Ohno, 2016).

Every mucosal tissue has MALT on its surface, the most familiar examples of this tissue was GALT (Giuliano *et al*., 2002; Cesta, 2006). The major role of the MALT was to produce definite immune reactions or tolerance against antigenic infest surfaces of mucosa (Mowat, 2003; Kiyono and Fukuyama, 2004; Liebler-Tenorio and Pabst, 2006; Magalhaes *et al*., 2007). These tissues were more exactly termed concerning the sites where they were found, for example GALT (Brandtzaeg *et al*. 2008; Cesta, 2006). GALT is made up of aggregated as well as solitary lymphatic arrangements (Newberry & Lorenz, 2005; Cesta, 2006; Newberry, 2008).

The biggest immune organ of the body comprising about 70% of the body’s immunocytes is GALT. It is a highly-developed constituent of the mucosal immunity system, which plays a role in both the maturation of the postnatal immunity system and host defense against infections (Corr *et al.*, 2008; D’Inca *et al.*, 2010).

In contrast to other mammalian species, rabbits' GALT structure is remarkably well developed, generally, rabbit GALT contains lymph structures, comprising appendix, Payer´s patches and sacculus rotundus,

thus, previous investigations concerning the lymphatic structure in rabbits concentrated on these components (Lanning *et al.*, 2000; Halely, 2003; Cesta, 2006).

Notably, T-cell zones related to lymph nodes were similar to all MALT structure holding a range of antigen-presenting cells, such as dendritic cells (DCs) as well as the macrophage, and sometimes eosinophil and mast cells in the inter-follicular district (Neutra *et al.*, 2001; Hase *et al.*, 2005); Therefore, they have every kind of cell required to start an immune response; but all of these lymphoid constructions actively assemble foreign antigens straight forward from the surface of mucosa through a distinctive follicle-associated epithelium that comprises "microfold" or "membranous" (M) cells (Brandtzag and Pabst, 2004; Cesta, 2006).

Dome regions of GALT grow toward the gut lumens and are enveloped in epithelium rich in lymphocytes (Beyaz, 2004; Shaykhev and Bals, 2007).

The epithelium of the dome envelope the follicles of MALT, creating a thin selectively penetrable barrier between the intestinal substances and lymphatic tissues (Kraehenbuhl & Neutra, 2000).

The epithelium of dome consists of enterocytes, lymphocytes and M cells, that envelope the lymphatic follicle domes of gut-associated lymphatic tissue, but it occasionally also has enteroendocrine and goblet cells (Newberry, 2008). Typically, this epithelium was devoid of mucus secreting goblet cells and in some positions owns very few goblet cells. On other hand, this epithelium is invaded by great number of intra--epithelial lymphocytes and in some other places seemed as groups of lymphocytes particularly close to M cells which in turn forming pouches to enclose such lymph cells, FAE presented low columnar weakly stained

cells with light nuclei which were micro-fold cells that were noticed at all areas of FAE (Al-Haaik, 2017).

Moreover, M cell quantity in rabbits was larger if compared with other animals (Haley, 2003). M cells comprises about fifteen percent of covering epithelium cells in rabbits, while in humans and rat about 5–10 percent of epithelial cells were M cells (Haley, 2017).

2.3. M-cell Review

M cells are distinctive cell of intestine which is in charge for the immune detecting of bacteria in gut lumen (Hase *et al.,* 2009; Wang *et al*., 2011; Lelouard *et al.,* 2012; Kanaya *et al.,* 2018).

Specialized epithelial cells called M cells are crucial for the carriage of intra-luminal macromolecules and pathogenic factors from small intestinal lumen to the underlying mucosal lymphoid tissues, where the immune responses are initiated and antigen processing takes place (Man *et al*., 2004; Kyd and Cripps, 2008; Rouch *et al.,* 2016). Throughout the immunological response, there is variation between plasma cells which secrete definite immunoglobulin A (IgA) and B cells (Corr *et al.,* 2008). As a result, M cells serve as entries to the mucosal immunity whose task has been broken by several infest pathogenic factors (Neutra *et al.,* 2001; Corr *et al*., 2008; Zhou *et al*., 2021).

Follicular-associated epithelia that overlay mucosal lymphtic nodules comprises microfold cell (M cells) (Iwatsuki *et al.,* 2002), these cells pick up luminal antigens and move them to the antigen-presenting cells below via transcytosing them through the FAE (Sierro *et al*., 2000; Man *et al.,* 2004; Beyaz, 2004; Mabbott *et al.,* 2013; Khan *et al.,* 2017; Kimura, 2018; Dillon and Lo, 2019). The FAE is commonly composed of columnar epithelial cells , M cells, intra-epithelial lymph cells, and rarely mucus-producing goblet cells (Köhne *et al.,* 1996).

In 1965, J.F. Schmedtje initially discovered microfolded-cells within the appendix of rabbit, first known as lympho-epithelial cells, then they later were called M-cell. Scattered M-like cells have also occasionally been reported in the villous epithelium of rabbits (Iwatsuki *et al.*, 2002; Jepson *et al.*, 2004).

M cells surround the intraepithelial lymphocytes (IEL) underneath them by their intraepithelial invaginations and also are distinguished by their thin membrane-like apical cytoplasm (Nicoletti, 2000; Neutra *et al*., 2001; Miller *et al.*, 2007; Corr *et al*., 2008). Additionally, it has been demonstrated that M cells feature irregular microvilli, are shorter than the neighboring enterocytes, and comprise many mitochondria, vacuoles and vesicles within their cytoplasmic apex (Niedergang and Krahenbul, 2000; Neutra *et al.*, 2001; Brayden *et al*., 2005). Due to their unique morphology, M cells were appropriate to active endocytosis and/or transcytosis, their apical membrane has undeveloped microvilli and ill formed glycocalyx, with the absence of the stiff brush border, which makes it easier for these cells to admittance to lumen of intestine and give a ride to antigenic factors (Kimura, 2018).

Moreover, basolateral membrane of M-cell is intensely invaginated to create an enormous intra-epithelial pouches that are occupied by several lymphocytes and mononuclear phagocytes (Mantes *et al.*, 2000; Kucharzik *et al.*, 2000; Hathaway and Kraehenbuhl, 2000; Clark and Jepson, 2003; Zhong *et al.*, 2007; Magalhaes *et al*., 2007; Kimura, 2018).

In contrast to adjacent absorptive cells in FAE, apical membrane related to M -cells devoid of microvilli on their luminal but possesses short microstructures similar to folds (Ohno, 2016).

Lysosomes are few inside M cell than other epithelial cells (IEC) within intestine which exhibit minimal lysosome enzyme activity (Ohno, 2016).

M -cells might easily be recognized via light microscope in the epithelia of the appendix especially at dome area where a group of lymphocytes is engulfed. Furthermore, M cells at these locations are especially situated at the edges of the dome, rather than on domes' top (Gebert *et al.,* 1992).

Several interesting issues about M cells' renewal at a location close to FAE top and their elimination from the FAE sideline were elevated as they were infrequently existing in FAE apex (Miyazawa *et al.,* 2006). Scattered M-like cells have also occasionally been reported in the villous epithelium of rabbits (Borghesi *et al.*, 1999; Iwatsuki *et al*., 2002).

M cells express weak reaction against brush-border alkaline phosphatase compared to other cells of epithelium (Owen and Bhalla, 1983).

M cells show specific ultra-structural characteristics, in comparison with other adjacent columnar cells, they possess thin glycocalyx layer on their surfaces. Additionally, they possess less, and small bifurcated microvilli dissimilar to columnar cells and their nuclei generally situated basally lower to the basal invaginations that contain lymphocytes (Wu *et al*., 2001; Wang *et al*., 2005; Garinot *et al.*, 2007; Nochi *et al*., 2007; Lo *et al.,* 2012). An electron microscopy observation of the appendix revealed that cells also possesses many mitochondria and vesicular structures (Hathway & Kraehenbuhl, 2000; Magalhaes *et al.*, 2007). For a very long period, electron microscope was the famous method for identification of M cells depending on their morphologic features, such as the presence of pockets containing lymphocytes and the tiny microvilli, (Kanaya and Ohno, 2014).

The M-cell apical surface's thin brush border and insufficient enzymatic activity propose that they were not likely to be involved in absorption and digestion (Krahenbuhl & Neutra, 2000; Neutra *et al.*, 2001; Miller *et al*., 2007; Corr *et al.*, 2008).

M cell origin, differentiation process, and demise were still poorly clear; However, there were two different notions about how M -cell arise (Mach *et al*., 2005; Miyazawa *et al*., 2006).

About differentiation, some researchers have suggested two theories. One was that, like other epithelial cells, M cells developed and separated directly from stem cells in crypts (Gebert & Poselt, 1997; Leluard *et al.*, 2001; Gebert *et al*., 2004). Other researchers suggested that matured enterocytes altered to M cell under the effect of lymphocytes or some micro-organisms (Kerneis *et al.*, 1997; Borghesi *et al.*, 1999; Gebert *et al.*, 2004; Lelouard *et al*., 2001).

Clark and Jepson (2003) cited that arising and differentiation of M cell generally occurred in cryptal cells under activation of neighboring lymphoid tissue.

Recently, some researchers postulated that early phase in the life cycle of absorptive cell may represent M cell might represent non distinct cell brand (Dillon and Lo, 2019).

M cell apex comprises an intra-cellular vesicular transference system that enables trans-cellular movement and phagocytosis (Neutra & Kozlwski, 2006).

In phagocytosis and transcytosis, M cells were extremely dynamic, and thus can pick up luminal antigens and bacteria then transport them to dendritic cells in pockets of M -cell for begining an immune response (Kato and Owen, 2005; Corr *et al.*, 2008; Brandtzaeg *et al*., 2008; Kanaya and Ohno, 2014).

There were positive and negative aspects to this kind of transportation release of antigen that triggers the production of a defense mechanism within the host which was beneficial. yet, various bacteria and viruses take use of the M cell path to enter the epithelium and reach target cells within the host (Fotopoulos *et al.*, 2002; Meyerholz *et al*., 2002; Helander *et al.*, 2003).

The inductive effect secreted factors from the organized lymphatic tissues on the differentiation of epithelium might be restrict M cells to these locations. With this pattern, there was less chance of microorganisms transient through the epithelial barrier since specialized phagocytes and antigen-presenting cells were exposed right away (DesRieux *et al.*, 2005; Tyrer *et al*., 2007; Wang *et al.*, 2014; Corr *et al.*, 2008).

Researchers were concerned about the roles that M cells play in mucosal immunity since of their significance for mucosal immunity regulation. For example, the antigens target delivery to M cells has been distinguished as an access to improve effectiveness of oral vaccine (Verbrugghe *et al.*, 2006; Devriendt *et al.*, 2012).

Recently, Zhu Yli *et al*. (2013), Rios *et al.* (2016), and Zhou *et al.*, (2021) cited that the number and size of M -cells in rabbit lymph tissue may be related to dietary fiber and starch levels intake.

2.4. Immunohistochemistry

Numerous markers for M cells have been determined by certain investigators; for example vimentin for rabbit (Jepson *et al*., 1993), cytokeratin "8" intended for rat (Rautenberg, *et al.*, 1996), lectin UEA-1 intended for mouse (Clark *et al.*, 1993) and cytokeratin "18" designed for

pigs (Gebert *et al.*, 1994). However, there were no universal kits for M cells available at the time (Kanaya *et al*., 2007; Hondo *et al.*, 2011).

Intestinal M cells of rabbit revealed to have vimentin intermediate filaments that moved from the cells' nucleus to the intra-epithelial pouches membrane (Fujimura & Iida 2001).

Because the protein component of the M cell cytoskeleton, it was not visible in other cells in the epithelium; for example lymphocytes and absorptive columnar cells, immunostaining of vimentin has been commonly familiar to investigate this cell in rabbits (Beyaz *et al.*, 2010; Zhu *et al.*, 2013).

The type of vimentin filaments is an intermediate that was commonly present in mesenchymal cells (Helfand *et al.*, 2005; Ivaska *et al.*, 2007), filaments of vimentin were a component of the cytoskeleton and also were involved in the firmness of cells and organelles stability in the cytoplasm.

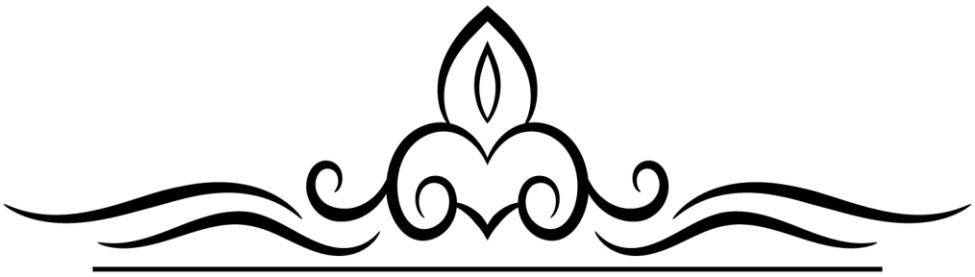
Glycoprotein 2 (GP2) was initially recognized as a glycosyl-phosphatidylinositol-anchored protein specially found in secretory particles of acinar cells of the pancreas (Terahara *et al*., 2008; Hase *et al*., 2009).

In M cells, GP2 was the identified protein on the cell surface that functions as a transcytotic receptor (Hase *et al*., 2009; Khan *et al.*, 2017; Kimura, 2018).

Additionally, according to the GP2 expression, newly formed M cells were categorized into distinct sub-types, Matured M cells with an excessive uptake capability which known as GP2-high cells and immature M cell which known as GP2-low cells (GP2-negative cells) (Kimura *et al*., 2015; Kimura, 2018).

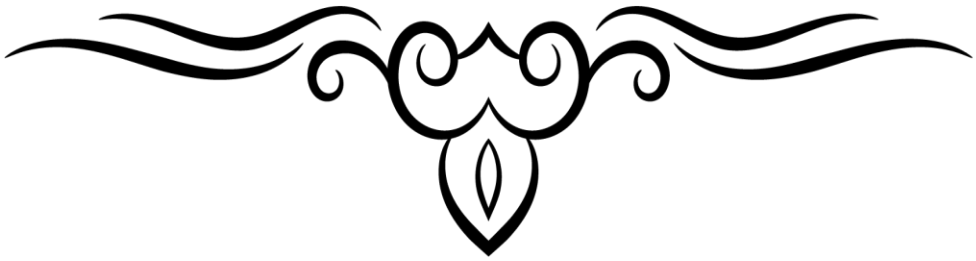
M-cell had been recognized by immunostaining for microvillar proteins actin and villin that were discovered by lack of stain because of their irregular tiny microvilli (Kanaya *et al*., 2007).

Typically, the absorptive cells related with thick glycocalyx that miss in M-cells and was exchanged by a tinny glycocalyx that was thought to support more access to antigen in the intestinal lumen (Hathaway and Kraehenbuhl, 2000). Some enterocyte apical surface glycoproteins were lacking from M-cells, these comprise sucrase-isomaltase activity and alkaline phosphatase, which were distinctive to enterocytes brush border, and have both been used as M-cell negative indicators (Gebert *et al.*, 1996).



Chapter Three

Materials and Methods



Materials and Methods

3.1. Materials and devices used in the study

3.1.1. The Instruments

No.	Instruments name	Type	Origin
1-	Oven	ELECTRO-MAG m420 BP	Germany
2-	Water bath	Electrothermal BG7311	England
3-	Microtome	WES WOX optic model 1090A	Italy
4-	Refrigerator (4) ºC	Arçelik	Turkey
5-	Light microscope	Olympus Dm-CBAD	Japan
6-	Digital camera	Usb 2.0 scope image cam	China
7-	Hot plate	Lab -smith	India
8-	Electronic balance	EK-I-EW-I	japan

3.1.2. The Chemical Materials

1. Distilled water
2. Formalin 37%
3. Ethyl alcohol
4. Xylene
5. Paraffin wax
6. Harris hematoxylin and eosin stain prepared according to Appendix (1)
7. Masson s trichrome stain prepared according to Appendix (2)
8. Combined PAS- Alcian blue technique prepared according to Appendix (3)
9. Vimentin Polyclonal Antibody / Elabscience
10. GP2 Polyclonal Antibody / Elabscience

3.2. study design

Fifteen (15) adult indigenous rabbits and same number of adult hamsters (regardless to sex) were conducted in this study.

15 **Rabbit**

15 **Hamster**

Animals were **euthanized** by intra-cardiac injection of over dose of sodium pentobarbital (100 mg/kg) after anesthesia by chloroform inhalation

↓

the cecum was harvested ,location and relations were studied in situ and photographed

↓

- Ceci of two species of animals were dissected away from the abdomen and the entire lengths/cm of cecum and relative lengths of each segment of cecum (apex, body and base) were measured using measuring tape.

↓

2 Representative specimens of one cm cut from each segment of ceci

↓

Fixation : 10% neutral buffered formalin for 48-72 hrs.

↓ Washing with running tab water

Routine histological processing method

↓ Rotary microtome

5µm paraffin sections

Using charged slides

• H&E stain	• MTS stains	•PAS-AB$_{ph2.5}$ stain	IHC
Histomorphometrical study	Demonstration of C.T. and smooth muscle fibers	Demonstration of goblet cell	Demonstration of M-cell

3.3Animals

This study employed 15 adult rabbit (*Oryctolagus cuniculus*) and 15 adult hamster (*Mesocricetus auratus*) regardless of gender. All animals were reserved under the lab. environments of 25˚C temp. as well as 12 hours per day and 12 hours at night and permitted free access of tap water and food. The animals were bought from a local market of Mosul city. The ceci of animals were harvested and studied microscopically.

3.3.1.Preparation of animals

All experimental animals were set in study after approvment of the scientific committee / Coll. Vet. Med. Mosul University (Ref no. UM.VET.2023.087; issue date: 20/8/2023). Overdose of sodium pentobarbital (100 mg/ kg bw) was injected intracardiac to euthanize the animals (Underwood, 2013). By using a surgical scissor , the abdominal cavity was opened, ceci were observed and the location and relation in situ were studied.

3.4. Morphological approach

Ceci of the two species of animals were removed from the abdomen and washed with saline solution to achieve morphological study, the shape, color, location and relations of cecum in situ. The ceci of both types of animals were harvested. After that entire lengths/cm of cecum and relative lengths of each segment of cecum (base, body and apex(appendix)) were measured using a measuring tape.

3.5. Histological approach

Specimens of 1 cm were taken from each portion of the cecum: the base (*bulbous ampulla coli*), the body (*corpus ceci*) and the apex

(*appendix vermiformis*)) for both species of animals. All specimens were rinsed gently with normal saline to empty the content of the intestine and immersed in 10% neutral buffer formalin directly for 48 hours (Survarna *et al*., 2019). Through a series of ethanol (70%; 80%; 90% & 100%) (two changes for each) for dehydration, then with xylene cleared for ½ hour, then the Specimens were infiltrated by 58ºC melting point paraffin wax on 58 – 60 ºC in oven then embedded in paraffin wax to gain paraffin blocks. Using a rotary microtome, 5µm paraffin sections were obtained (Histo-line – Italy) (Culling *et al*., 1985).

3.6. Histological and Histochemical procedures

3.6.1. Histological structures

Common microsopic structures were examined with Harris Hematoxylin & eosin stain as well as Masson Trichrome stain **(Appendices-1&2)** (Luna, 1968, Survarna *et al*., 2019). Sections from all segments of the cecum of rabbit and hamster were stained by using Harris H&E stain used for common microscopic purposes which are:

a. To make sure that the preserved slices were undamaged as well as the epithelial cells because of their importance for the investigation of the expression of M-cell later (Zaghair, 2014).

b. To achieve measuring for the following parameters of the cecal wall in all pieces of cecum :

- Tunica mucosa thickness, submucosa thickness, muscularis thickness /µm.
- cecal folds Height / µm.
- Percentage of goblet cell in the epithelium%.
- Number and area of Auerbach's nerve plexuses/ µm².

c. To study the existence of the followings; lymphoid follicles, auerbach's and meissner nerve plexus in various segments of the cecum in both rabbits and hamsters.

d. Massons Trichrome stain was conducted for demonstration of muscle and collagen fibers.

3.7. Carbohydrate Histochemical Study

3.7.1. Combined PAS-Alcian blue $_{pH2.5}$ procedure

This method was applied to distinguish acidic mucin from neutral mucin in goblet cells in cecal segments of rabbit and hamster (Culling *et al*., 1985) **(Appendix-3**).

3. 8. M- cell identification using immunohistochemistry

Anti –vimentin antibody (**Appendix-5**) and anti GP2 antibody (**Appendix-6**) were used as primary antibody for identification of M-cells and its manifestation, distribution and concentration in varies segments of the cecum (base , body and apex) in two species of animals. Paraffin sections at 5 μm were prepared to achieve IHC technique. The histological sections were treated conferring to formula stated by the supplier the staining set. Primary antibody information was listed in **(Appendix-4**) (Hase *et al.*, 2009; Zhu *et al.*, 2013).

3.9. Procedure for coating slides with gelatin for IHC sections

To prevent tissue sections from becoming lost while staining and excessive washing processes on histological slides, adhesive materials

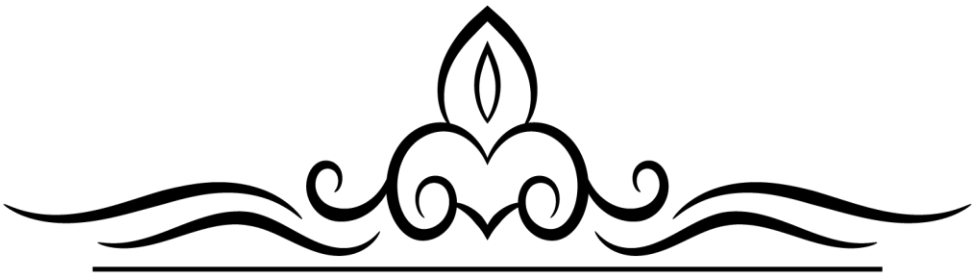
(+ve charged slides) were be applied on these slides. This adhering solution was prepared according to Kiernan (1999) (**Appendix-4**).

3.10. Micromorphometric measurements and Photograph

All micromorphometric measurements were achieved by using digital tube microscope camera (OMAX 18MP) china, which is provided with image processing software (Toup view). All lenses of Olympus microscope (CX21) were calibrated to the software of the camera by using stage micrometer to ensure the accuracy of the micromorphometric measurements.

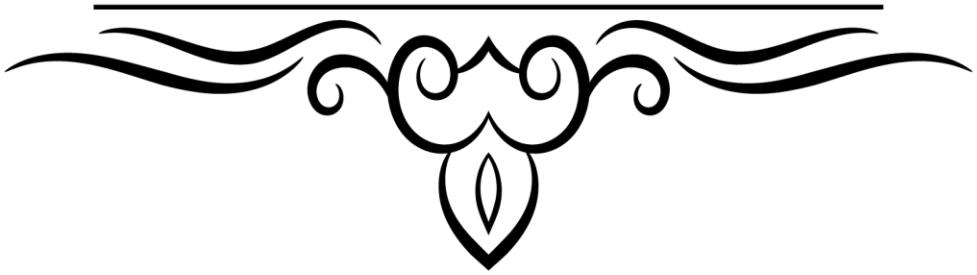
3.11. Statistical Analysis

Sigma stat V13.2 / SYSTAT (computer program) was conducted to accomplish the micromorphometrical investigation. The Data were offered as means±standard error and the comparison between parameters of both species of animals was achieved by T. test and Chi square (X^2) with significance level was set as $P \leq 0.05$ (Petrie and Watson, 2013).



Chapter Four

Results



Results

4.1. Macroscopic Findings

Grossly, three main segments which were recognized in the cecum of rabbit: the base, the body and the appendix vermiformis**,** while the cecum of hamster can be divided into: the base, the body and the apex (Figure 1).

Generally, the cecum of rabbit was haustrated, it employed approximately the whole ventral portion of the abdominal cavity in contrast to the cecum of hamster that was situated on the median plane's left side, this manner is known as a general division in most rodents (Figure 2).

The first part of the large intestine usually is the cecum. It described as tube of closed end that connects ascending colon to the ileum (Figure 3).

4.2. Macroscopic Measurements

Table 1: show the macromorphometric measurements of cecum in rabbit and hamster /cm

Variables/cm	Rabbit	Hamster
Entire length of cecum	43.12±1.23 *	4.14±0.18
Length of apex	9.25±0.75 *	0.52±0.06
Relative length of apex	21.36± 1.18 *	12.42±1.4

*** meaning there was a significant variance between rabbit and hamster at p≤0.05**

The macro morphometric measurements of the length of cecum and apex showed that there was a significant variance in the length of the

entire length of cecum and length of apex as well as the relative length of apex between the rabbit and hamster at $p \leq 0.05$ as showed in (Table 1) (Figure 4).

4.3. Histological Findings

4.3.1. General description of cecal wall

A microscopic examination of the cecal wall of the three portions (base, body and apex) of rabbit and hamster revealed that their structure is comprised of mucosa, submucosa, muscularis, and serosa, which were the four primary tunicae (Figure 5). Tunica mucosa displayed a large folds and simple columnar epithelium lining it with abundant goblet cells among them. Goblet cells appeared dispersed between enterocytes of the folds, they had a cylinder-shape with broad free apical portions and the nuclei was basally located, their cytoplasm seemed clear before staining with hematoxyline and eosin stain. The core of the folds constructed from loose connective tissue infiltrated with lymphocytes and reticular cells.

Surface epithelium was down with many crypts that lined by one layer of columnar cells in addition to goblet cells. There were several intra epithelium lymphocytes infiltered with surface epithelium.

The Lamina Properia (LP) is composed of connective tissue which was filled with many crypts (intestinal glands).

Between the LP and submucosal layers there was a tinny fiber of smooth muscle named as muscularis mucosa.

A thick layer of areolar connective tissue named tunica submucosa is richly provided with many blood vessels (Figure 5).

Also, there are two layers of smooth muscle bundles that created the tunica muscularis which are an inner thick circular layer and an outer

thin longitudinal muscular layer. Between the inner and outer layer which provides motor innervation of both layers of the muscular layer. Auerbach's nerve plexuses were inspected microscopically and appeared different in size, it had a round or elongated shape. These nerve plexuses exposed two types of cells when used hematoxyline and eosin stain, which were glial cells and neurons. Basophilic light cytoplasm with deep blue stained nuclei and one or two distinguished nucleoli were the most features of these neurons, whereas basophilic deep cytoplasm appeared in some neurons. The location of nuclei of most neurons was centrally but for some neuron the location of nuclei was eccentrically. The glial cells seemed with different sizes, it had a dark color with elongated oval shape (Figure 6).

The outer most layer of the cecal wall named tunica serosa, which consist of tinny connective tissue layer enclosed by a one row of mesothelial cells, this layer was rich with nerves and blood vessels.

4.3.2. Apex of cecum in rabbit and hamster

4.3.2.1. Rabbit Appendix

The surface epithelium of appendix was thrown with leaf-like folds or villi-like folds with narrow base and wide apical portion, and simple columnar epithelium with great number of goblet cells lining these folds. The core of these folds constricted from loose connective tissue infiltered with large number of lymphocytes. Each fold is filled with cross sections of intestinal glands or crypts (Figure 7). The surface of these folds exhibit downward invagination which represent crypts.

Three types of cells were known in its mucosa which were enterocytes, goblet cells, and microfolded cells (M-cell). A thick lamina propria with many well-developed lymphoid follicles was present around the entire

perimeter of its wall. The core of these follicles was invested with connective tissue fibers and contained large number of lymphocyte in addition to reticular cells, plasma cell, mast cell, and macrophage (Figure 8). These follicles are composed of germinal center which is pale and located in the center of the follicle and dark peripheral zone (Figure 9). The lymphoid follicle appeared to be encapsulated by a connective tissue.

Among these folds there was a dome shape structure which is constricted from aggregation of large number of lymphoid follicles (lymph nodule). These nodules were covered with a special kind of epithelium which was termed dome epithelium (follicle associated epithelium). This type of epithelium consists of enterocytes with numerous modified cells called M-cell (microfolded cells)(Figure 10). This epithelium looked different from adjacent epithelium lining the mucosal folds which are thrown with numerous goblet cells but not M-cells. The goblet cells in the dome epithelium were absent but in certain positions there were a few goblet cells. On other hand, enormous quantity of intra-epithelial lymphocytes occupied the epithelium covering domes and in certain sites seemed as bunches of lymphocytes specifically that close to M cells that form pouches for enclosing such bunches, neither folds epithelium.

The M-cell is characterized by its large size in comparison with columnar cells and exhibit pocket that housed several lymphocytes.

The lamina propria in the dome shape was very scant while it appeared clearly within the core of mucosal fold (Figure 11).

Muscularis mucosa appeared as a very thin interrupted sheet of smooth muscle fibers separated lamina propria from underlying submucosal layer (Figure 12).

Tunica submucosa appeared as thin layer of vascularized areolar connective tissue, and lymphocytes. There was ganglionic nerve plexus in the submucosa which is characterized by its pale basophilic cytoplasm and prominent nucleus and this is defined as meissner plexus (Figure 13).

Tunica muscularis constricted from two layer the circular inner layer and the longitudinal outer layer, between the two layers, there were a numerous auerbach's nerve plexus (my enteric) of different sizes most of them are surrounded with connective tissue fibers (Figure 6).

The outermost layer, known as the tunica serosa, that was consist of a one layer of mesothelium covering a tinny layer of loose collagen fibers.

4.3.2.2. Hamster apex

The wall of cecum apex in hamster has histological structure different from that of rabbit where the mucosa of this part is thrown with many folds of different sizes and there is no lymphoid follicles nor related follicle associated epithelium.

Generally, the wall of cecum apex has the same layer of tunicae of rabbit but the surface epithelium is thrown with large number of crypts (intestinal glands) which are lined by enterocyte and very large number of goblet cells (Figure 15).

Lamina properia is composed of loose connective tissue infiltrated with large number of aggregated lymphocytes. Neither follicles, nor the dome epithelium were present.

Small aggregation of lymphocytes in lamina propria appeared near the folds or within the core of folds with non-significant shape that do not reach to the level of lymphoid follicle (Figure 15).

The thickness of tunica mucosa in hamster apex is less than that of rabbit appendix (Figure 16)(Table 2).

The muscularis mucosa in the apex of hamster appeared well defined and constricted from numerous layers of smooth muscle fibers. It appeared continuous and thicker than that of rabbit (Figure 12))(Table 2). The crypts were numerous and deep, also simple columnar epithelium with goblet cells lining it. Obvious different levels of mitotic figures were well noticed in the crypts especially found within the basal part of crypts (Figure 17).

Muscularis mucosa which is a continuous bundle of smooth muscle fibers, also appeared relatively thicker than that of rabbit and extends within the core of folds (Figure 12)(Table 2).

The submucosa of apex of hamster is well developed (Figure 18) and occupied with large number of blood vessels and aggregation of lymphocytes, plasma cell and well-defined connective tissue more than that of rabbit, also the submucosa has large number of nerve cells.

Meissner nerve plexus is found in few numbers compared with that of rabbit and is located mainly in the upper zone of submucosa (Figure 13).

The tunica muscularis in the hamster appears thicker than that of rabbit and constricted of two layer the outer layer which is thin and the inner layer which is thick (Figure 19))(Table 2).

Massive number of auerbach (myenteric) nerve plexuses are observed between inner and outer layers of tunica muscularis and some blood vessels are invested the inner thick layer. The density and sizes of auerbach's nerve plexuses in the hamster appeared more than that of rabbit (Figure 6).

Tunica serosa is a very slim coat of collagen fibers with mesothelial cells (Figure 19).

4.3.3. Body of cecum in rabbit and hamster

4.3.3.1. Body of cecum in rabbit

The wall of cecum body in rabbit constructed from four main tunicae of gastrointestinal tract (GIT). A simple columnar absorptive cells and some intermingled goblet cells lining the surface epithelium, and the intestinal crypts. The folds of mucosa in this part are very small. The intestinal crypts are short and extend to the level of muscularis mucosa and the crypts in this part of cecum are very few (Figure 20).

The lamina propria is constructed from a connective tissue and contains large number of lymphocytes, Plasma cell , eosinophil, mast cell and blood vessels (Figure 21). In some regions there is aggregation of lymphocytes in the lamina propria but they do not reach to the level of lymphoid follicle. Some of lymphocytes appeared to be infiltered within the epithelium.

The dome shape is absent in this part of the cecum so that the follicle associated epithelium is absent also and there are few M- cell in the epithelium and few lymphocytes infiltered within the epithelium.

Muscularis mucosa appeared well developed in this part of cecum and consist of several smooth muscle fibers and isolated the lamina propria from submucosa (Figure 22).

Tunica muscularis consists of two layer of smooth muscle fibers, inner circular layer which seemed thicker than the outer longitudinal one. Notably, tunica muscularis appears thicker than that of appendix of rabbit (Figure 24))(Table 3).

. Between the two layers of tunica muscularis, there were aggregation of neurons which were auerbach's nerve plexuses (myenteric) The wall of the body of cecum in rabbit contain large number of myenteric plexus.

4.3.3.2. Body in hamster

The general histological structure of wall of the body of the cecum in hamster is similar to that of rabbit except that the mucosal folds are slightly larger than that of rabbit and the crypts of lieberkuhn are deeper and numerous than those in rabbit (Figure 20). Large number of mitotic figures are noticed within the deep part of crypts (Figure 25). The lining epithelium contains a great number of goblet cells in comparison to that of rabbit.

The mucosa of body in hamster cecum is characterized by the presence of longitudinal fold called plica circularis where each one of these plicae constructed from lamina propria and submucosa (Figure 26).

The lamina propria constructed from a loose connective tissue which contains aggregation of lymphocytes besides the existence of large blood vessels (Figure 23).

The lymphoid aggregation appear more condensed and more numerous in the wall of cecal body in hamster more than that of rabbit (Figure 27).

Muscularis mucosa seems as a tinny sheet of smooth muscle fibers that separates mucosa from submucosal layer and involved in the formation of plica circularis together with the tunica muscularis (the inner layer) (Figure 26).

Tunica submucosa is constructed from a connective tissue rich with big blood vessels and several meissner nerve plexus which are mostly in the upper part of tunica submucosa which was scant in rabbit (Figure 23).

Tunica muscularis consist of two layers, inner thicker than the outer layer with numerous and large Auerbach's (myenteric) nerve plexus noticed among the two layers. Tunica serosa is a slim layer of connective tissue with mesothelial cells (Figure24).

4.3.4. Base of cecum in rabbit and hamster

4.3.4.1. Base in rabbit

The common histological construction of the base of cecum in rabbit has analogous structure of the body except that mucosal folds of the base appeared as a small elevations in the mucosa and few number of crypts were found in the lamina propria with aggregation of lymphocytes.

Muscularis mucosa appeared very clear and separate the lamina propria from submucosa.

Submucosa is constructed from connective tissue with small blood vessels. In tunica muscularis, the inner layer is thicker than the outer (Figure 28).

4.3.4.2. Base in hamster

In comparison with that of rabbit, the mucosal folds of the base of hamster appeared higher than that of rabbit and well-developed. Lamina propria contain numerous sections of crypts.

Submucosa contains large blood vessels in comparison with that of rabbit (Figure 28).

4.4. Micromorphometric Findings

Table 2: showing the micromorphometric measurements of appendix of rabbit cecum and apex of hamster/ µm

Apex

Variables	Rabbit	Hamster
T. mucosa thickness	1334.53±34.64 *	270.73±59.97
T. submucosa thickness	159.69±11.65	421.18±51.01 *
T. muscularis thickness	32.78±2.11	104.86±5.91 *
M. mucosa thickness	9.67±0.64	24.87±2.04 *
fold length	734.41±13.38 *	645.54±64.74

* meaning there was a significant variance between rabbit and hamster at $p \leq 0.05$

The results of Table 2 show the micromorphometric measurements of the layers of apex in cecum of rabbit and hamster. The results show that there was a significant variance at $p \leq 0.05$ in all layers of the cecal wall of apex as well as the fold length between rabbit and hamster.

Table 3: showing the micromorphometric measurements of body of cecum in rabbit and hamster/ µm

Body

Variables	Rabbit	Hamster
T. mucosa	131.01±10.53	167.07±6.14
T. submucosa	116.71±5.91	266.49±25.61 *
T. muscularis	117.09±4.54 *	84.89±4.56
M. mucosa	6.78±0.49	16.71±0.95 *
fold length	95.73±8.35	457.23±37.31 *

* meaning there was a significant variance between rabbit and hamster at p ≤0.05

The results of Table 3 show the micromorphometric measurements of the layers of body in the cecum of rabbit and hamster. Tunica mucosa thickness revealed no significant variance at p≤0.05 between rabbit and hamster while the thickness of tunica submucosa, muscularis mucosa and muscularis show significant difference between the two species of animals at p≤0.05. The length of the folds in the hamster appeared about four folds longer than that of rabbit with a significant variance at p≤0.05.

Table 4: showing the histometrical measurements of base of cecum in rabbit and hamster/ µm

Base

Variables	Rabbit	Hamster
T. mucosa	125.79±8.402	157.23±6.53
T. submucosa	109.57±12.19	420.5±4.73 *
T. muscularis	119.2±6.30	145.22±9.03 *
M. mucosa	5.58±0.40	17.57±0.73 *
fold length	93.39±4.603	601.96±15.63 *

* meaning there was a significant variance between rabbit and hamster at $p \leq 0.05$

The results of Table 4 show the micromorphometric measurements of the layers of base in cecum of rabbit and hamster. Tunica mucosa thickness shows no significant variance at $p \leq 0.05$ between rabbit and hamster, while the thickness of tunica submucosa, muscularis mucosa, and muscularis show significant difference between the two species of animal at $p \leq 0.05$. The fold length in hamster appeared about sixth folds longer than that of rabbit with a significant variance at $p \leq 0.05$.

Table 5: showing the mean number of auerbach's nerve plexuses/ section in rabbit and hamster cecum

part	No. of auerbach's plexuses/ section /µm	
	Rabbit	**Hamster**
Apex	12.5±0.8	19.5±1.75 *
Body	5.4±0.5	14.1±0.8 *
Base	5.4±0.5	19.2±1.3 *

* meaning there was a significant variance between rabbit and hamster at p≤0.05

Table 6: showing the mean area of each Auerbach's nerve plexuses/ mµ² in rabbit and hamster cecum

part	Mean area of each Auerbach's plexus/ section / mµ²	
	Rabbit	**Hamster**
Apex	570.78±32.4	1030.83±149.3 *
Body	552.23±51.1	726.35±51.5 *
Base	803.2±71.3	1163.54±73.7 *

* meaning there was a significant variance between rabbit and hamster at p≤0.05

The micromorphometric measurements of auerbach's nerve plexuses as shown in Tables 5 and 6 exposed that there was a significant variance in the number and the mean area of auerbach's nerve plexus for the three segments of the cecum between rabbit and hamster. Anyway; all parameters in hamster were higher than those in rabbit.

4.5. Carbohydrate Histochemistry

4.5.3. Apex of cecum

The histochemical results of goblet cells in the wall of the appendix in rabbit reveal that all goblet cells give a positive reaction to PAS stain indicating the presence of neutral glycoprotein or mucin. Anyway, the goblet cells in the surface epithelium give a strong reaction to PAS stain whereas crypts goblet cells give moderate reaction to PAS stain. Similar results were found in the apex of cecum in hamster except that both goblet cells of surface gave a strong reaction to PAS stain. Furthermore, the luminal surface epithelium in both species of animals gave positive reaction to PAS stain (Figure 31).

4.5.2. Body of cecum

The goblet cells of cecal body wall in rabbit and hamster give strong positive reaction to PAS as well as the luminal surface of epithelium. Some goblet cells give positive reaction to both PAS stain and Alcian blue, the result indicate that the most of mucin in the wall of body of cecum in both species was neutral mucin or glycoprotein (Figure 30).

4.5.1. Base of cecum

The histochemical findings of the base of cecum in rabbit and hamster revealed that most of goblet cells within the surface epithelium as well as the luminal surface of epithelium give a strong positive reaction to PAS stain which indicated the presence of neutral mucin. Some of goblet cells in the surface epithelium appeared dark magenta which indicate the presence of mixed (neutral and acidic) mucin in these cells, whereas most of goblet cells in the crypts (intestinal glands) give a positive reaction to each of PAS stain and Alcian blue indicating the

existence of mixed and acidic mucin in the goblet cells of crypts (Figure 29) .

Table 7: showing the percentage of goblet cell in the cecum of rabbit and hamster

% of goblet cell		
part	**Rabbit**	**Hamster**
Apex	73.21 *	57.83
Body	8.31	18.33 *
Base	27.58	48.83 *

* meaning there was a significant variance between rabbit and hamster at p≤0.05

Table 7: shows the percentage of distribution of goblet cell in the three segments of cecum in rabbit and hamster and the results show that the higher concentration or percentage of goblet cell was found in the apex of rabbit while the least percentage was in the body of rabbit.

Statistically, a notable variance was noticed in the percentage of goblet cell in the three segments of cecum between rabbit and hamster at p≤0.05.

4.6. Immunohistochemical Findings

Table 8: showing the percentage of M - cell in the epithelium of cecum in rabbit and hamster

% of M – cell		
part	**Rabbit**	**Hamster**
Apex	11 *	4
Body	2	4 *
Base	6	6

* meaning there was a significant variance between rabbit and hamster at $p \leq 0.05$

As shown in Table 8, the highest percentage of M cell was found in the appendix of rabbit while the least percentage was appeared in the body of rabbit. There was a variation in the percentage of M cell in the apex and body between rabbit and hamster while its percentage in the base was similar in the cecum of both animals. on the other hand, the M cell that found in the dome epithelium especially in rabbit were distributed.

Table 9: showing the distribution percentage of M_ cell in FAE in rabbit

% of M _cell distribution in FAE of rabbit	
Dome apex	5%
Dome mid-edge	20%
Dome base	75%

Immunohistochemical expression of M cell showed strong reaction against GP2 (brown color) which appeared in the cytoplasm and on the apical and lateral cell membrane of M cell (mature M cell) in the dome epithelium especially in the appendix of rabbit(Figures 32,33). In other segments of the cecum as well as in the cecal epithelium of hamster, the manifestation of M-cell appeared in the epithelium lining small aggregations of lymphocytes was mild, While the manifestation of M cell was a mild or weak against vimentin, the highest number of M cell was found in the appendix of rabbit while the least number was found in the body of rabbit (Table 8).

Concerning FAE of dome shape in the cecum of rabbit, the most number of M cell was concentrated in the basal part of this epithelium whereas least number expressed in the apex of the FAE (Table 9).

Notably, it was noticed that goblet cells had also moderate reaction against both vimentin and GP2 which appeared on the apical cell membrane only (Figure 34).

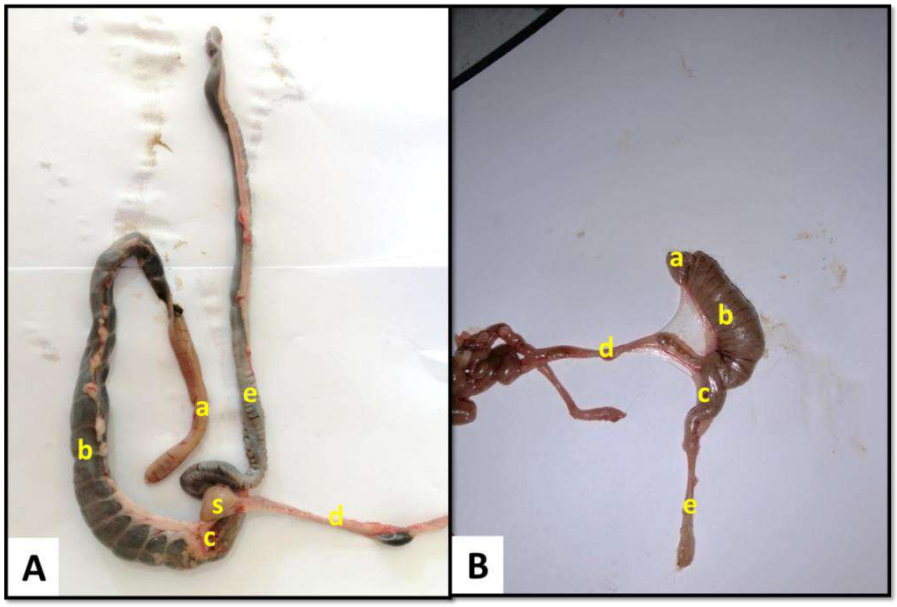


Figure 1. Shows the portions of cecum in rabbit(A) and hamster(B). (a) appendix in rabbit(A), apex in hamster(B) , (b) body, (c) base, (d) ileum, (e) colon, (s) sacculus

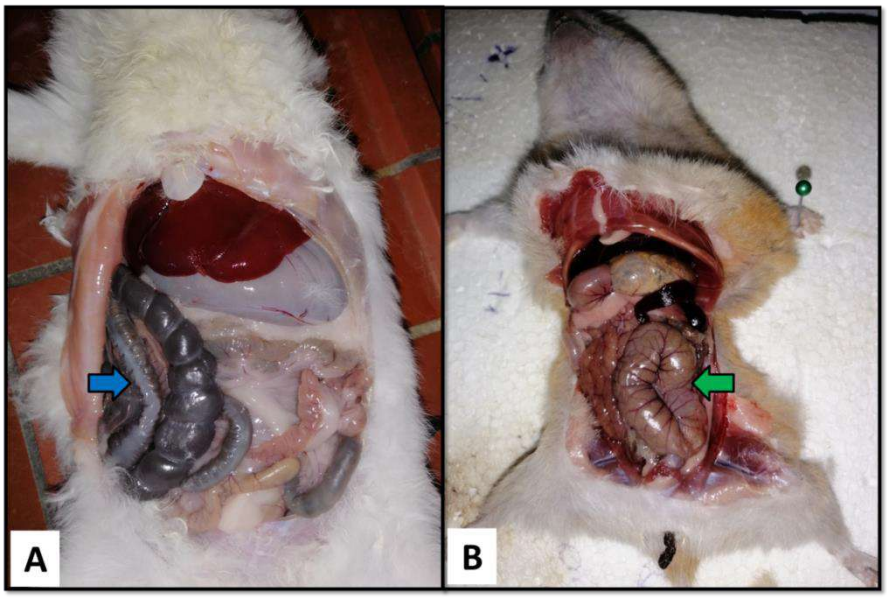


Figure 2. Shows the location of cecum in rabbit and hamster. It showed cecum of rabbit(A) on the right side (blue arrow) and cecum of hamster(B) on the left side (green arrow).

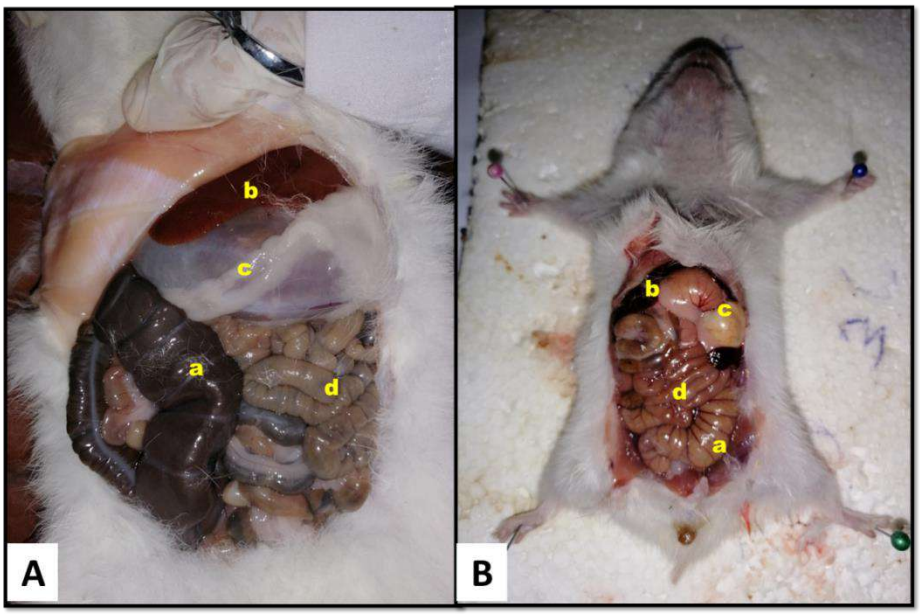


Figure 3. Shows the relation of cecum with the adjacent organs in rabbit(A) and hamster(B). (a) cecum, (b) liver, (c) stomach, (d) small intestine

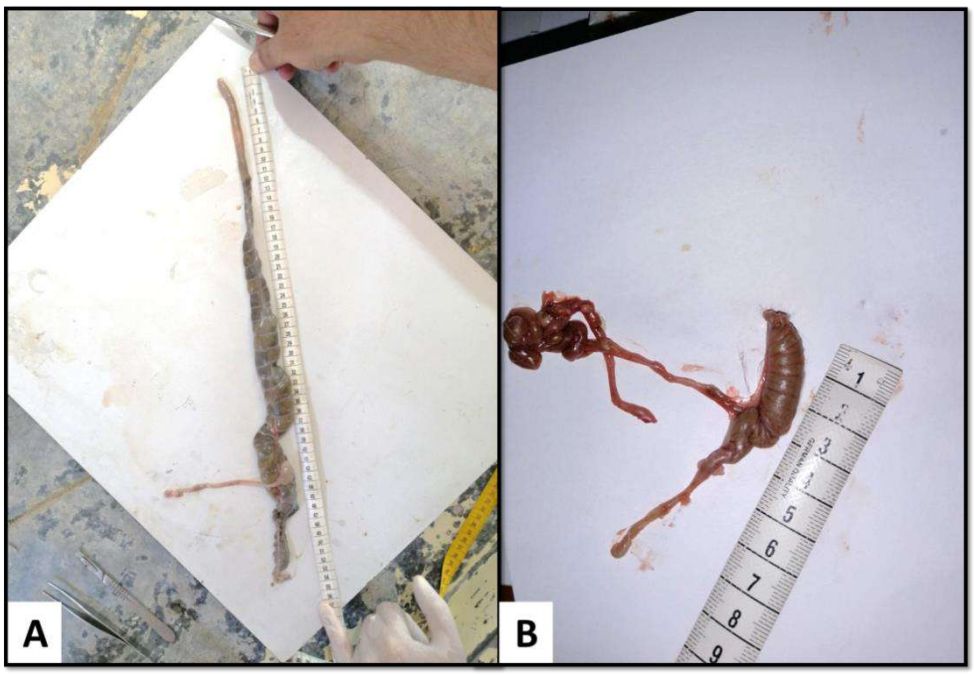


Figure 4. Macromorphometric measurements of cecum in rabbit(A) and hamster(B)

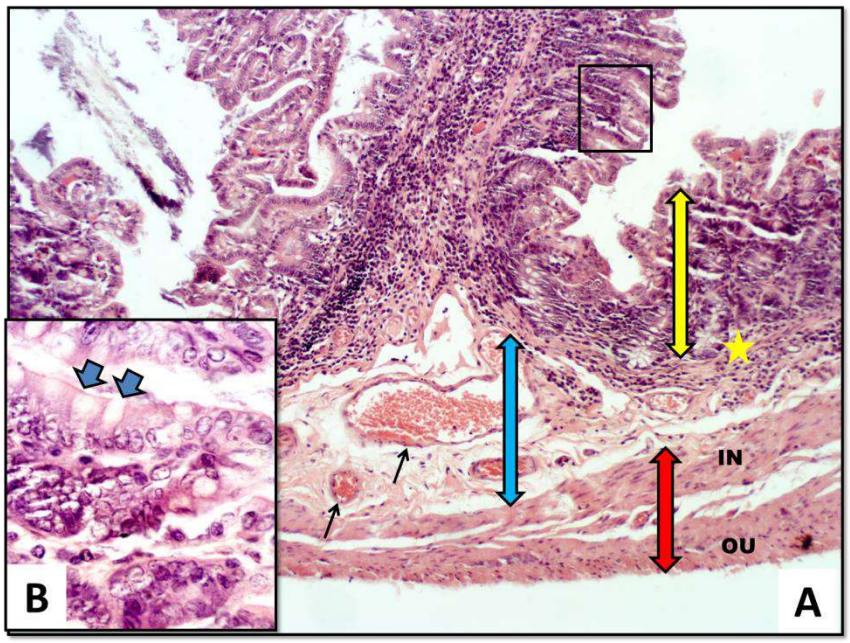


Figure 5. Histological section of apex of hamster cecum shows the four layers, goblet cells (blue arrows) and blood vessels (black arrows). Tunica mucosa (yellow double heads arrow), Muscularis mucosa (yellow star), Tunica submucosa (blue double heads arrow) and Tunica muscularis (red double heads arrow), inner layer (IN) and outer layer (OU) of tunica muscularis. X100 (A) and X400 (B). H&E

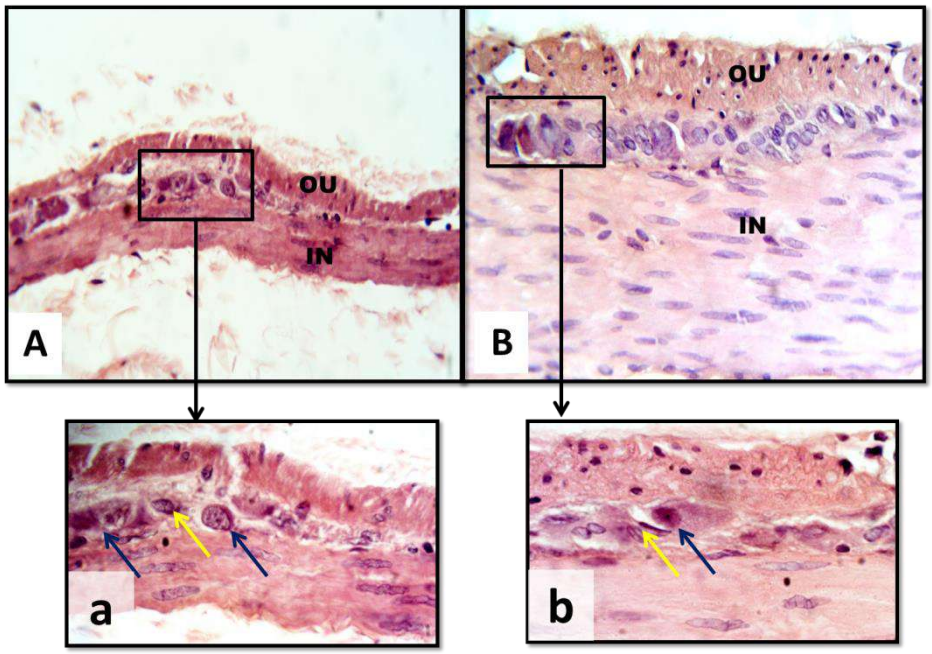


Figure 6. Histological sections show the auerbach's nerve plexus in the appendix of rabbit(A) and apex of hamster(B). Neuron (blue arrows), glial cell (yellow arrows). X400 (A & B) and X1000 (a & b). H&E

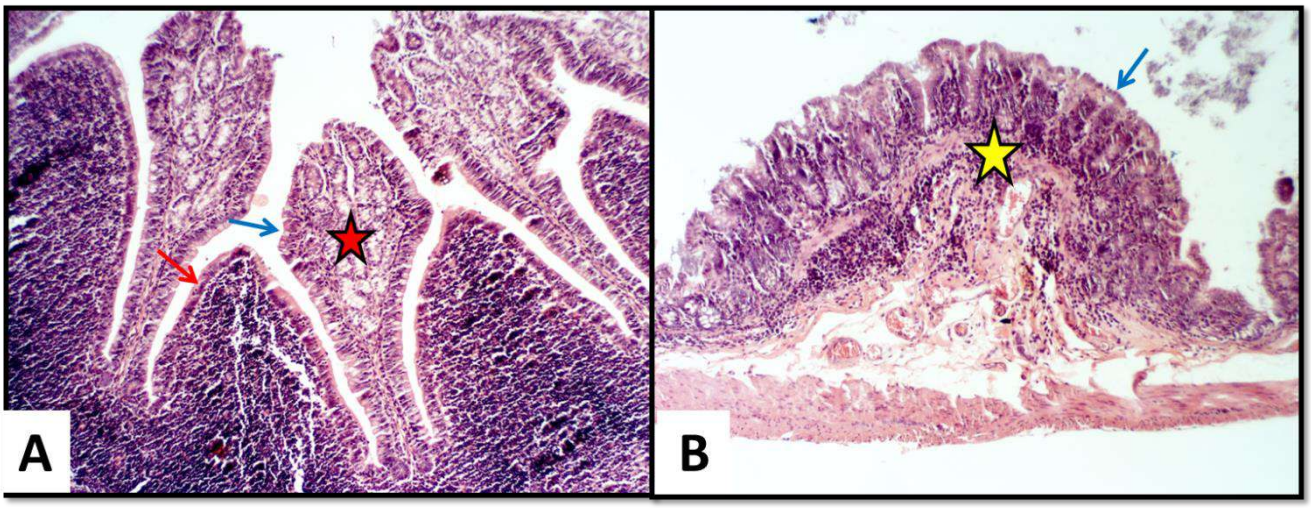


Figure 7. Histological sections show the leaf-like folds of appendix (red star) in rabbit(A), folds of apex (yellow star) in hamster(B), follicle associated epithelium (red arrow) and simple columnar epithelium (blue arrows). X100. H&E

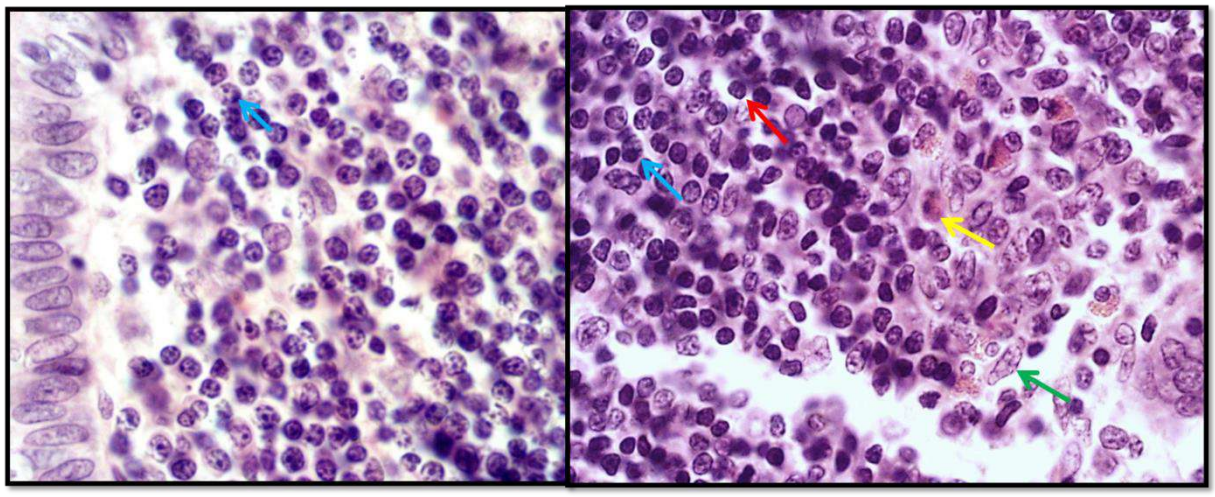


Figure 8. Histological sections of the appendix in rabbit show the cells present within the dome. Lymphocytes (red arrow), reticular cell (green arrow), plasma cell (blue arrows) and mast cell (yellow arrow). X1000. H&E

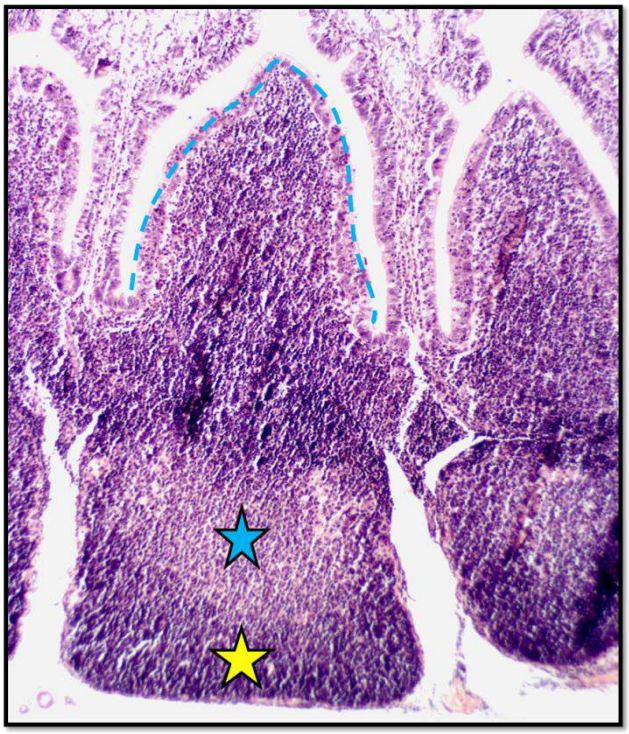


Figure 9. Histological section of appendix in rabbit shows the dome epithelium (blue dotted line), germinal center (blue star) and peripheral zone (yellow star). X100. H&E

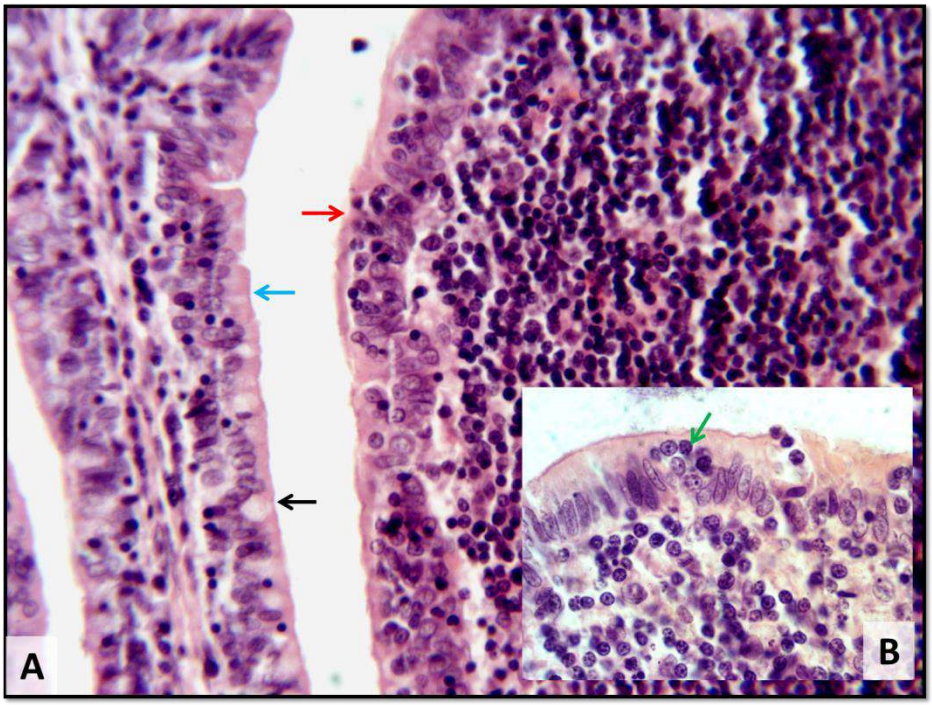


Figure 10. Histological section in the appendix of rabbit shows the M cell (green arrow), follicle associated epithelium (red arrow), simple columnar epithelium (blue arrow), goblet cell (black arrow). X400 (A) and X1000 (B). H&E

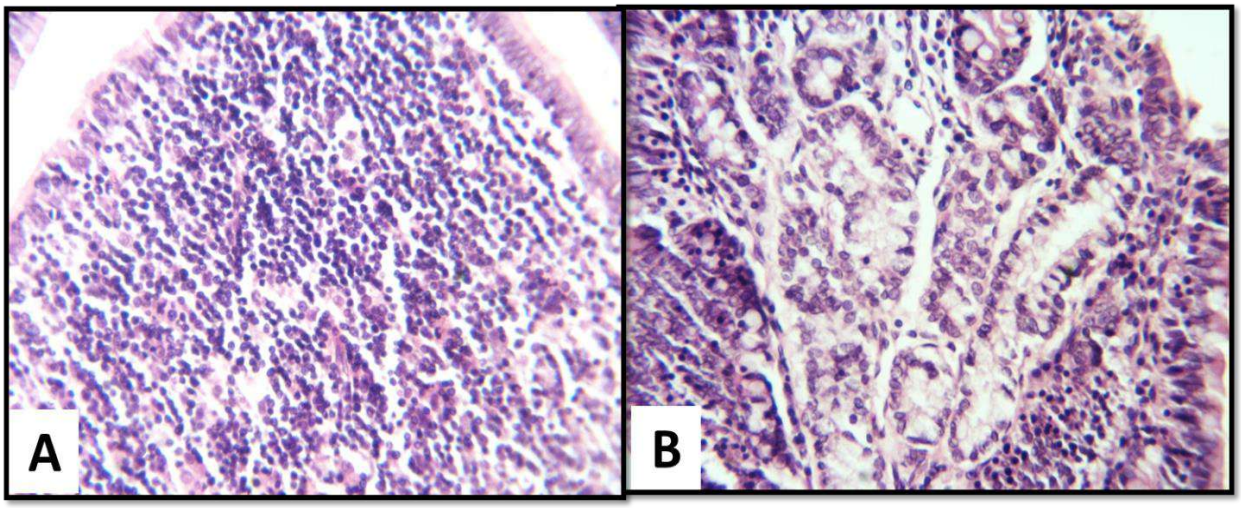


Figure 11. Histological sections of the appendix in rabbit show the lamina propria. Dome (A) and fold (B). X400. H&E

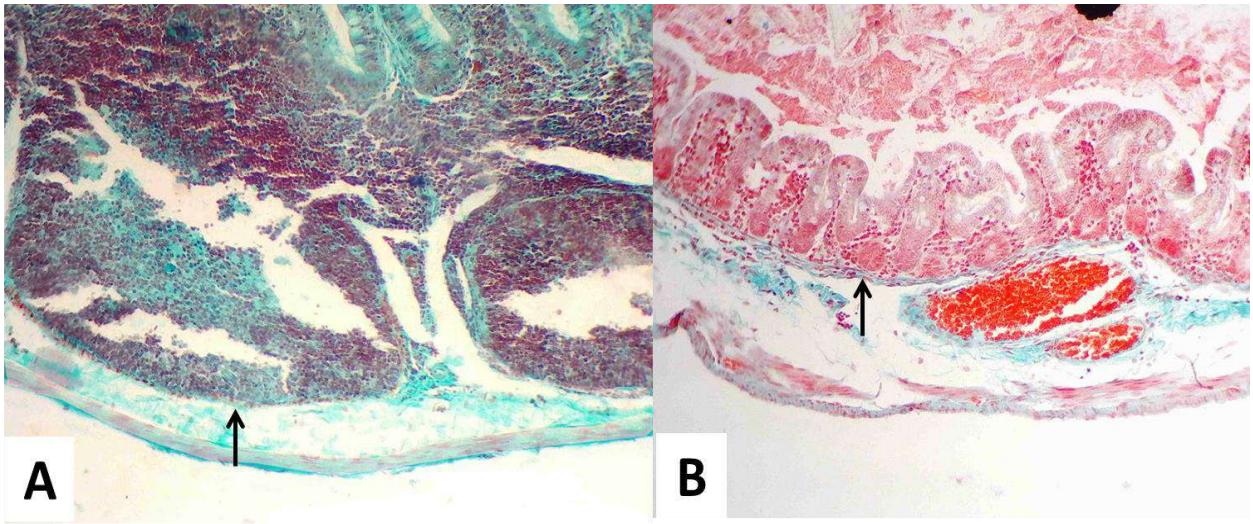


Figure 12. Histological sections show the muscularis mucosa (black arrow) of the appendix in rabbit (A) and apex in hamster (B). X100. Masson Trichrome stain

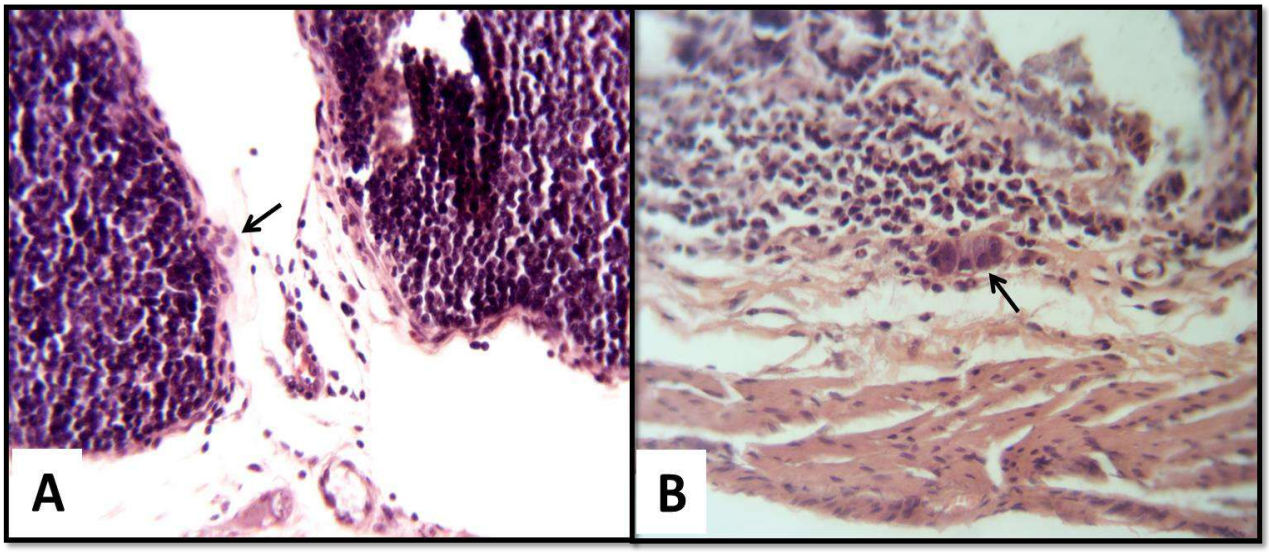


Figure 13. Histological sections show the meissner nerve plexus (black arrow) in submucosa of appendix in rabbit (A) and apex in hamster (B). X400. H&E

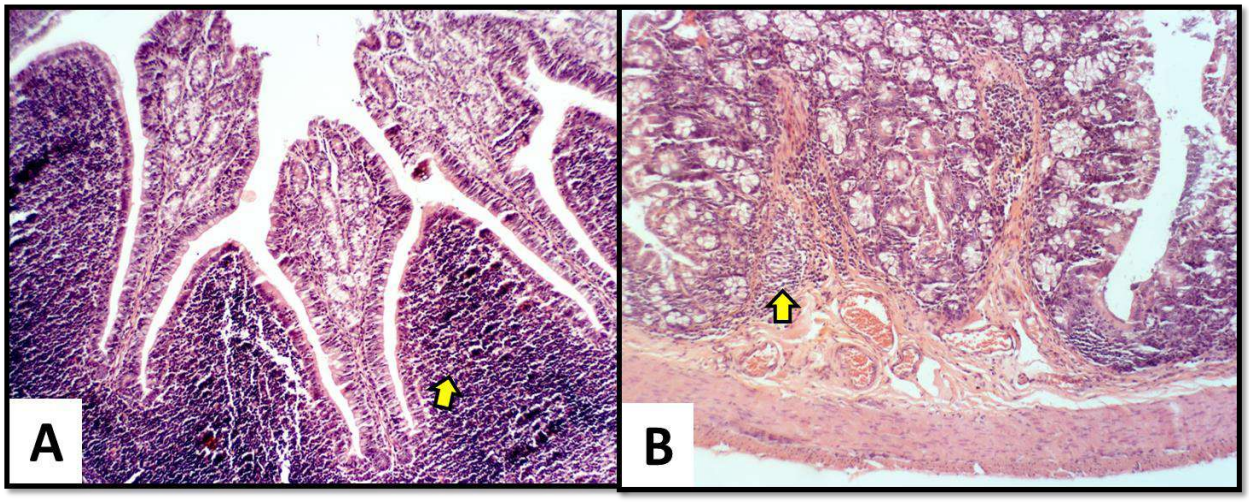


Figure 14. Histological sections in the appendix of rabbit(A) and apex of hamster(B) show aggregations of lymphocytes (yellow arrow). X100. H&E

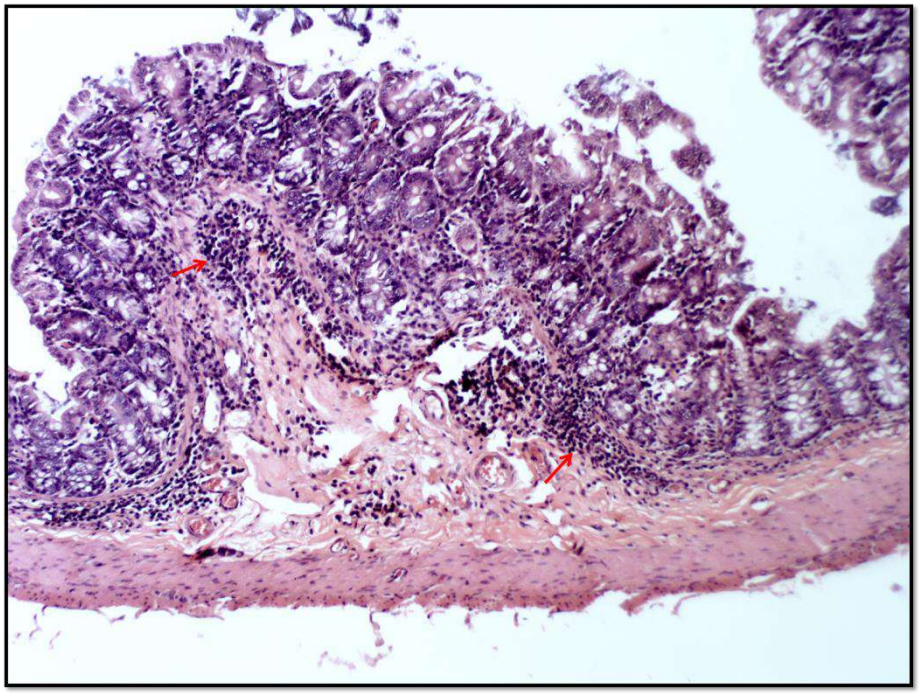


Figure 15. Histological section in hamster apex shows Small aggregation of lymphocytes. X100. H&E

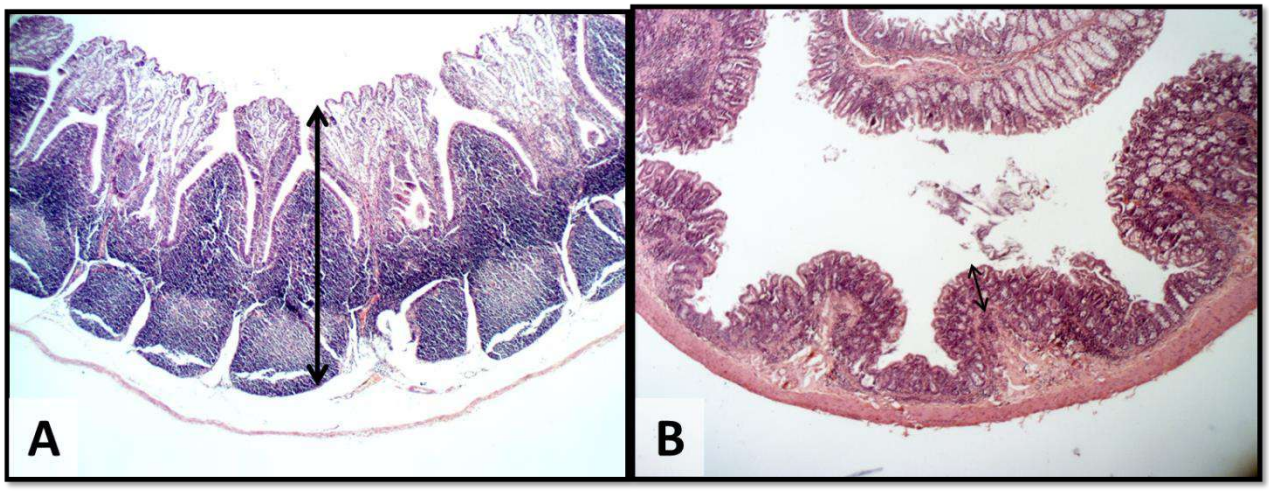


Figure 16. Histological sections of the appendix of rabbit(A) and apex of hamster(B) show tunica mucosa thickness. X40. H&E

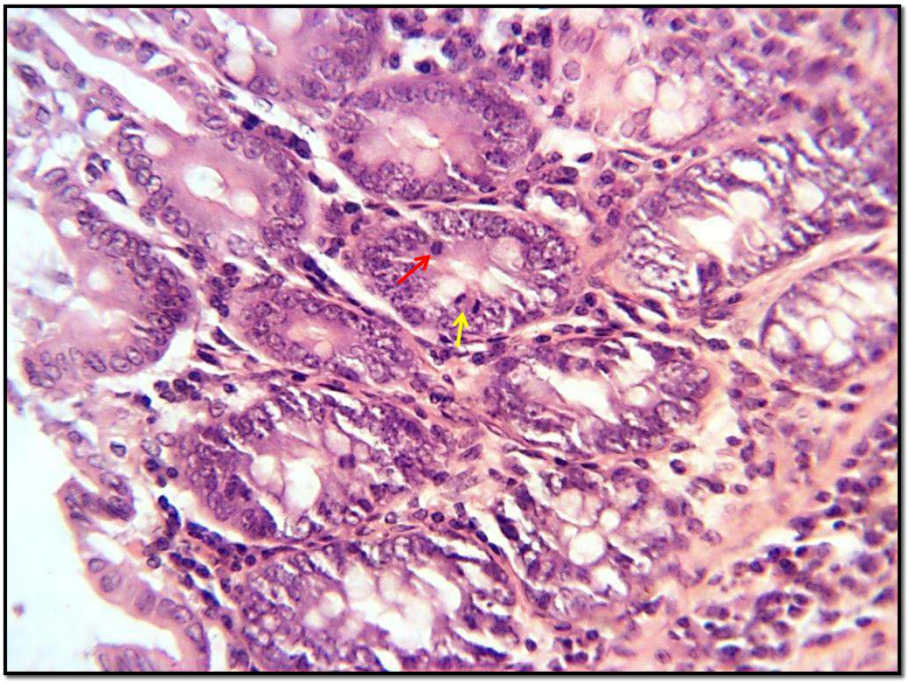


Figure 17. Histological section of hamster apex shows the mitotic figures. Prophase (red arrow) and Anaphase (yellow arrow).

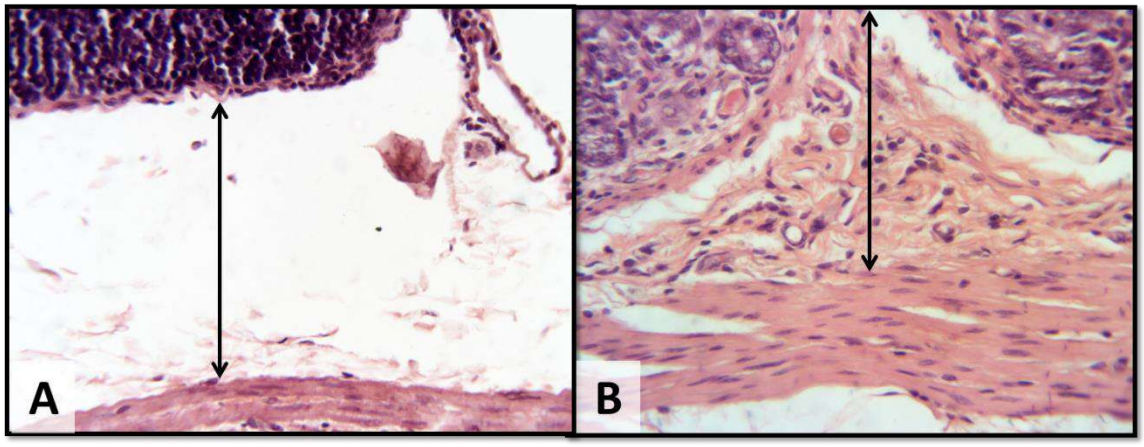


Figure 18. Histological sections of the appendix of rabbit(A) and apex of hamster(B) show the tunica submucosa. X400. H&E

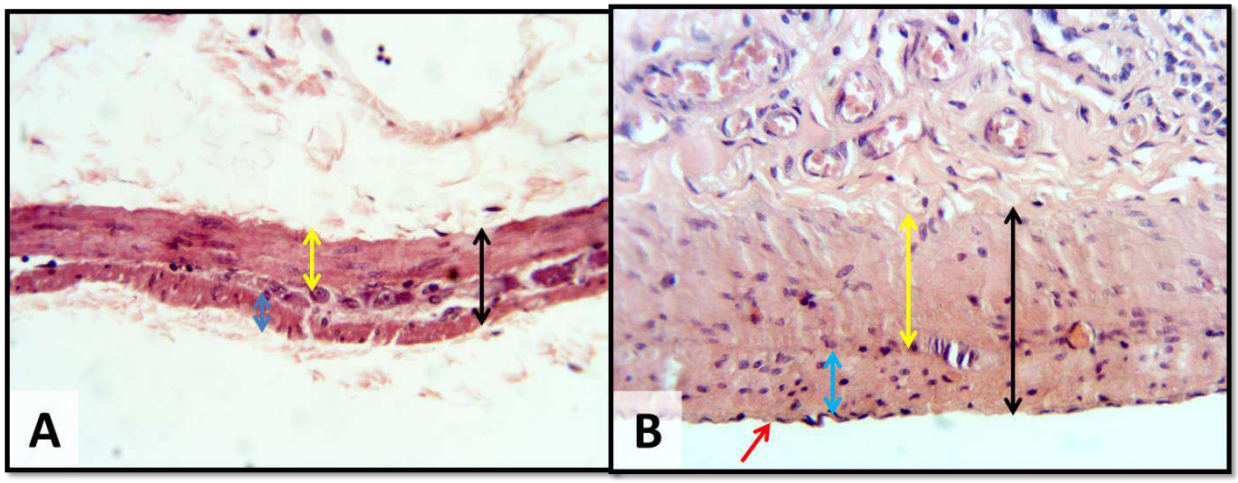


Figure 19. Histological sections of the appendix of rabbit (A) and apex of hamster show the thickness of tunica muscularis(black double head arrow), outer layer (blue double head arrow), inner layer (yellow double head arrow) and tunica serosa (red arrow). X400. H&E

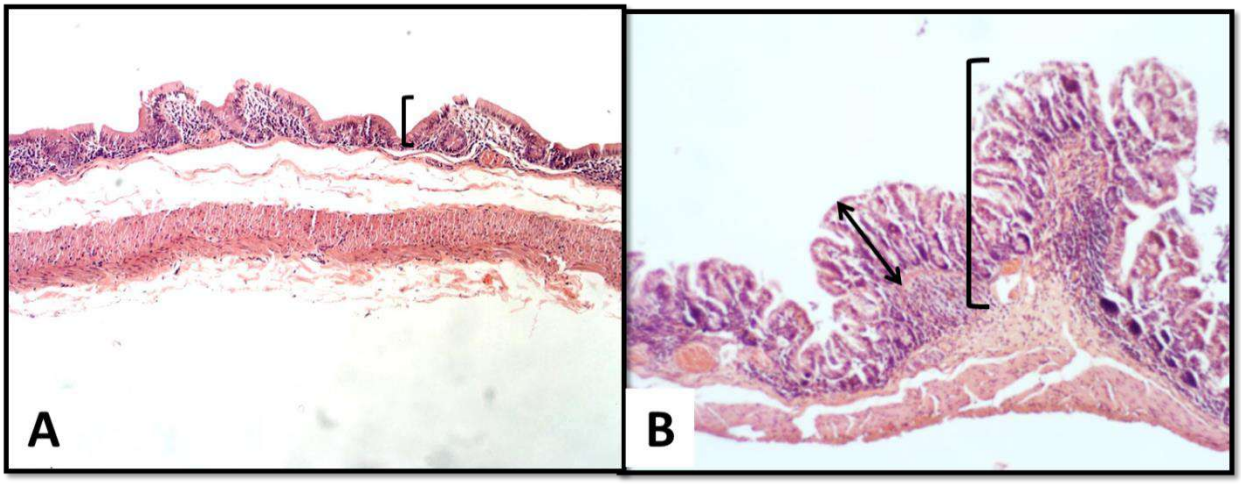


Figure 20. Histological sections of cecal body in rabbit(A) and hamster(B) show the folds (black left average arrow) and depth of crypts (black double heads arrow). X100. H&E

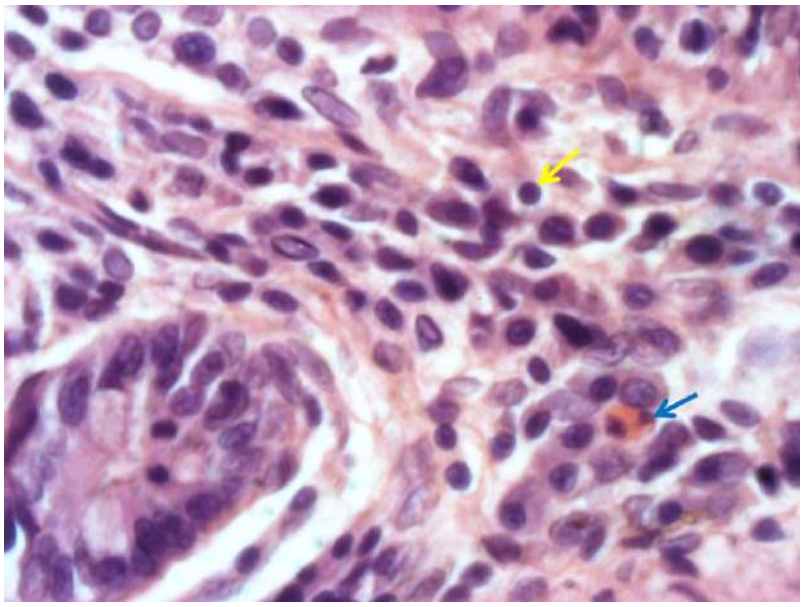


Figure 21. Histological section of cecal body in hamster shows the lamina propria cells. Lymphocyte (yellow arrow) and mast cell (blue arrow). X1000. H&E

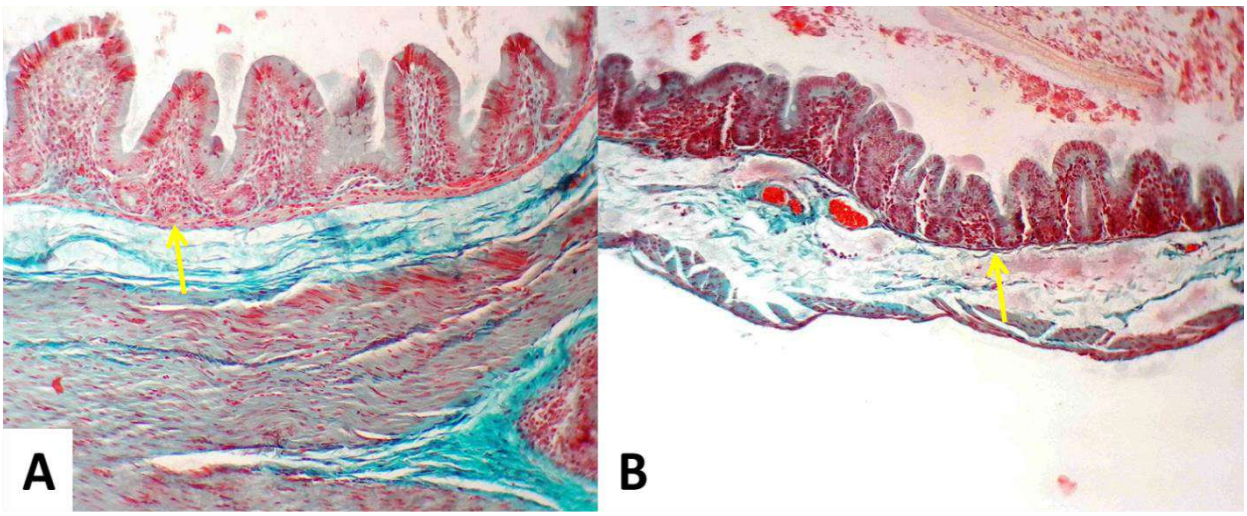


Figure 22. Histological section of cecal body in rabbit(A) and hamster(B) show muscularis mucosa (yellow arrow). X100. Masson Trichrome stain

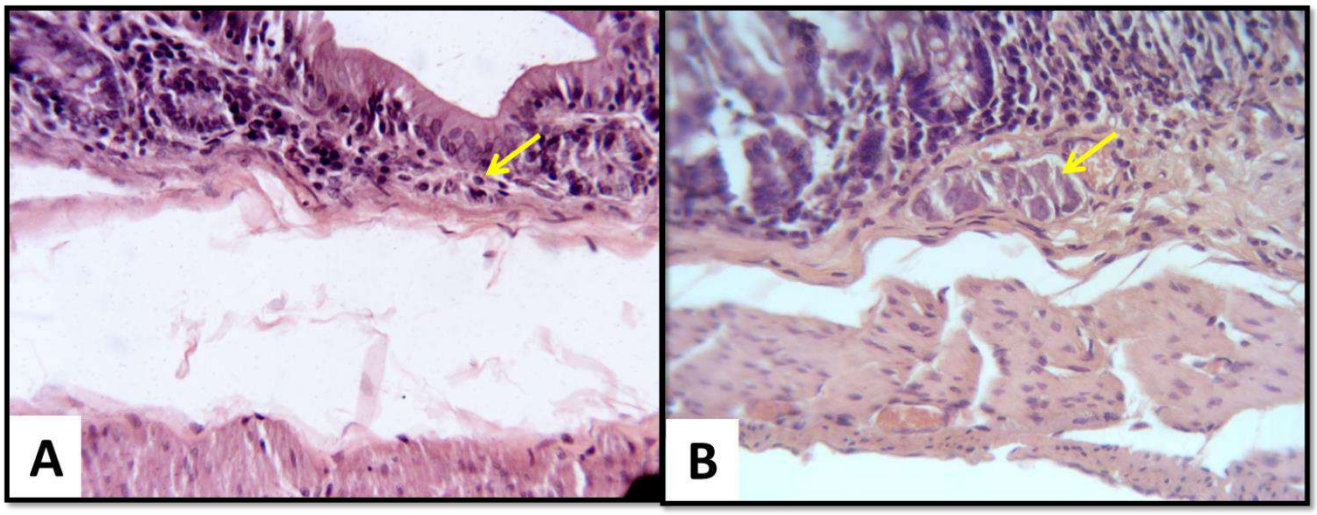


Figure 23. Histological sections show the tunica submucosa of cecal body in rabbit(A) and hamster(B). Meissner nerve plexus (yellow arrow). X400. H&E

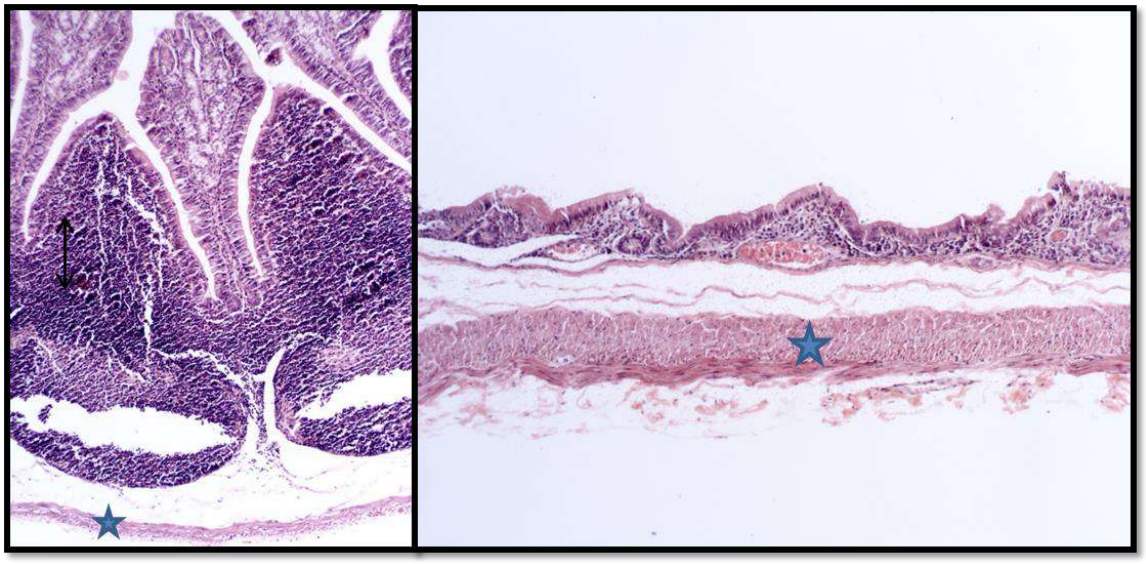


Figure 24. Histological section of rabbit cecum shows the thickness of tunica muscularis (blue star) in the appendix (A) and in the body (B). X100. H&E

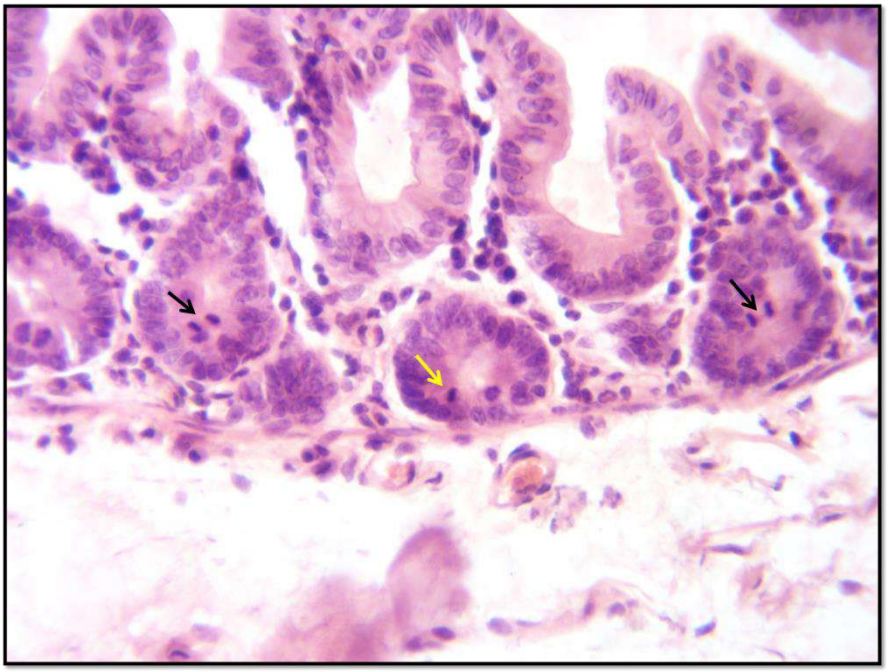


Figure 25. Histological section of cecal body in hamster shows the mitotic figures. Anaphase (black arrows) and Prophase (yellow arrow). X400. H&E

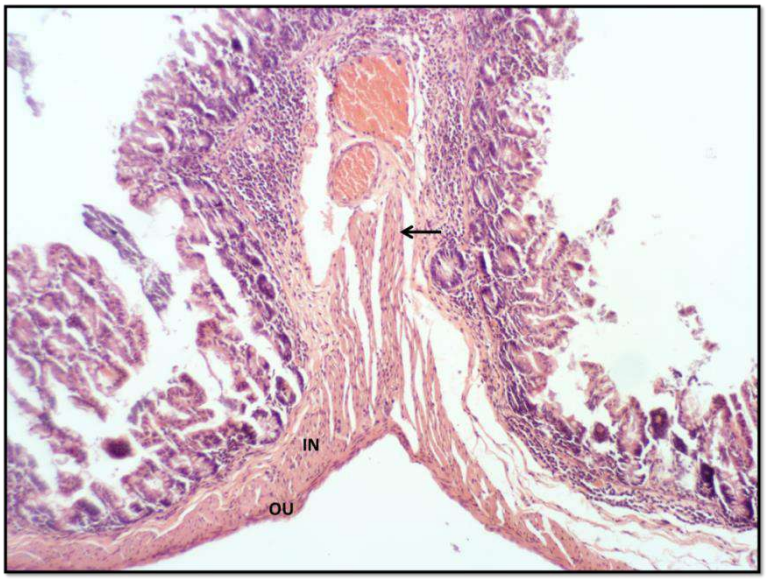


Figure 26. Histological section of cecal body in hamster shows the plica circularis (black arrow), IN (inner layer) and OU (outer layer) of tunica

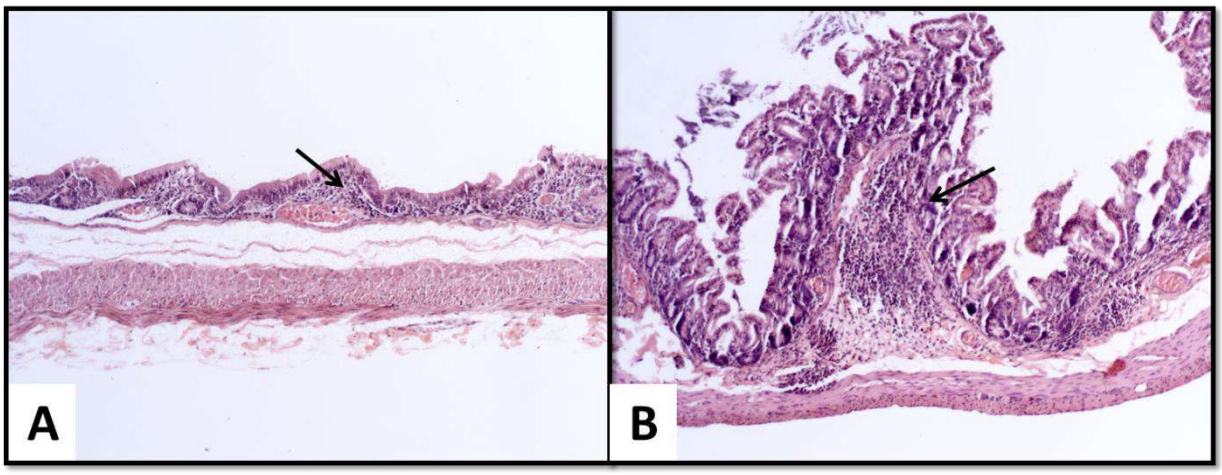


Figure 27. Histological section shows aggregation of lymphocytes (black arrow) in the lamina propria of cecal body in rabbit(A) and hamster(B). X100. H&E

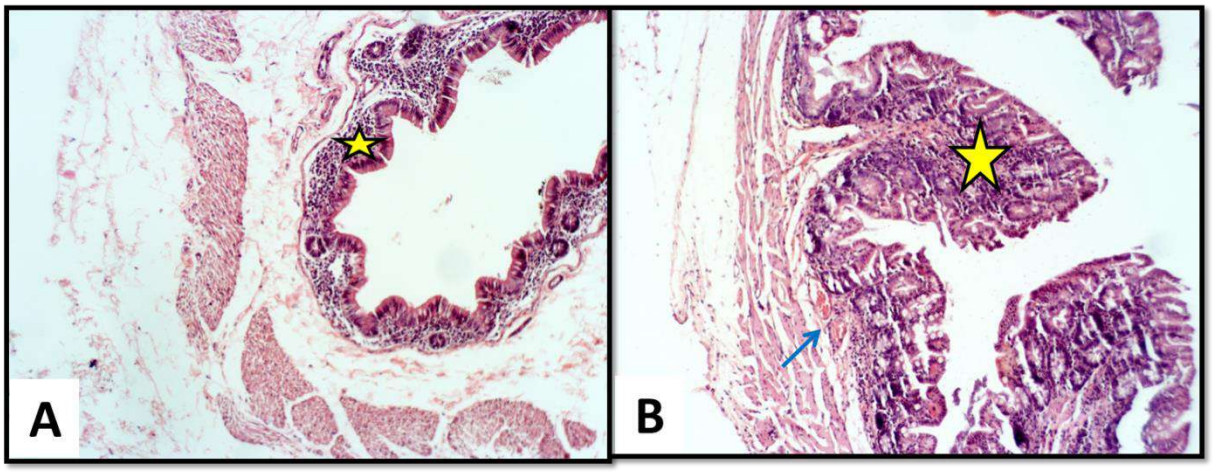


Figure 28. Histological sections of cecal base in rabbit(A) and hamster(B) show the folds (yellow star) and blood vessels (blue arrow). X100. H&E

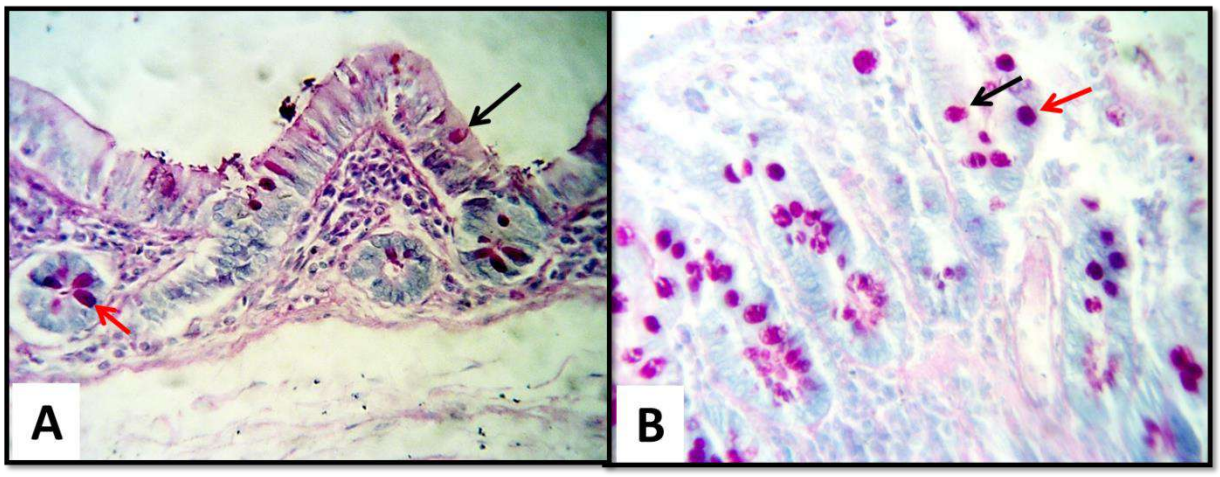


Figure 29. Histological sections show cecal base in rabbit(A) and hamster(B). It show some mixed mucin (red arrow) and neutral mucin (black arrow). X400. PAS-AB$_{PH2.5}$

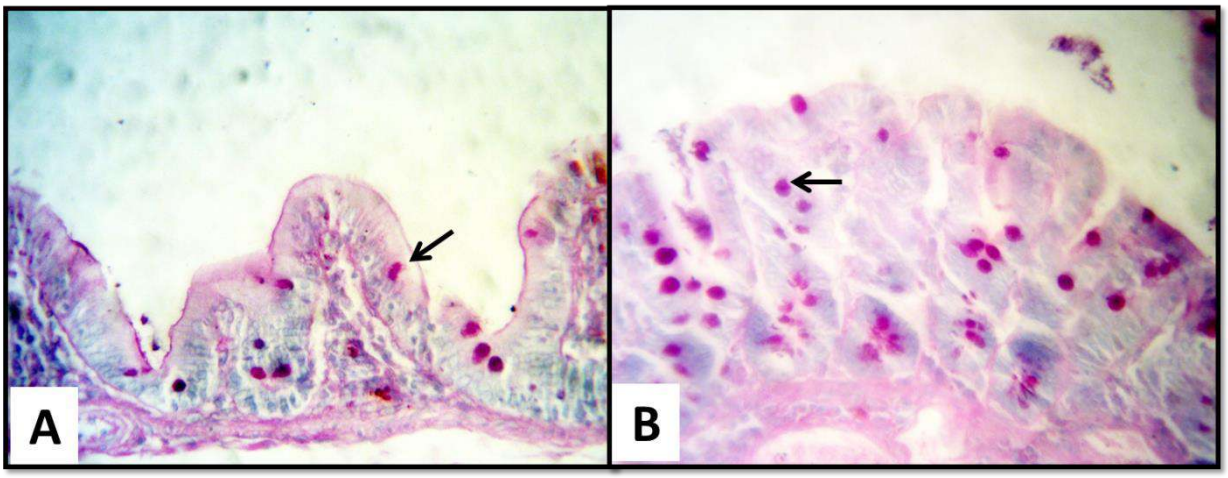


Figure 30. Histological sections show cecal body in rabbit(A) and hamster(B). It show neutral mucin (black arrow). X400. PAS-AB $_{PH2.5}$

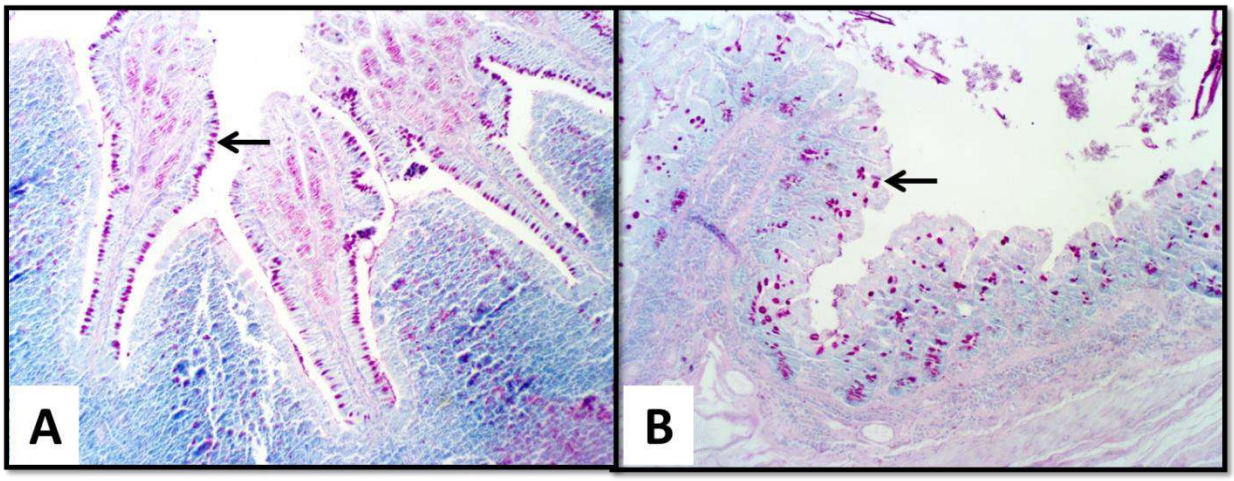


Figure 31. Histological sections show cecal apex in rabbit(A) and hamster(B). It show many neutral mucin (black arrow). X100. PAS-AB $_{PH2.5}$

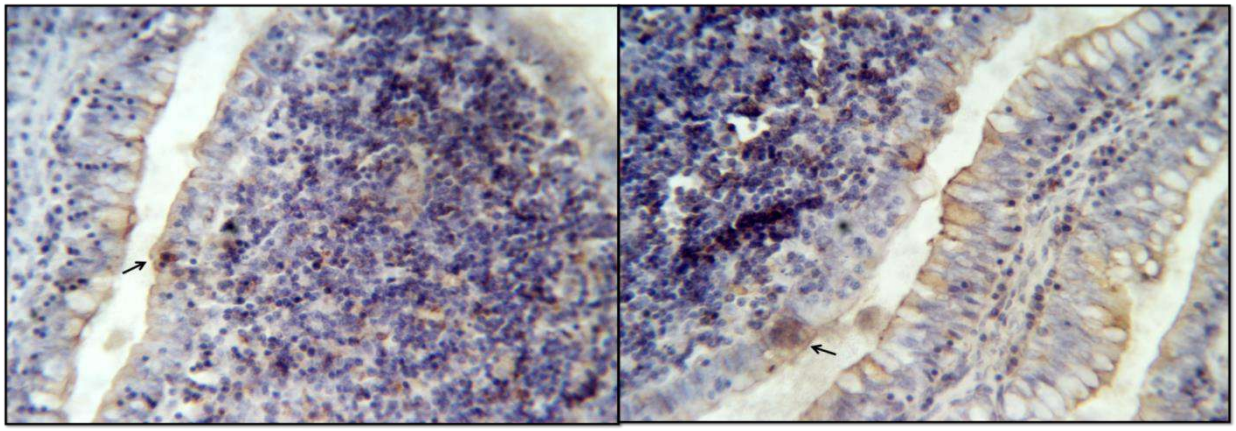


Figure 32. Histological section shows immunohistochemical staining with GP2 marker to the sections of appendix in rabbit. It showed of M-cell (black arrow). X400. GP2

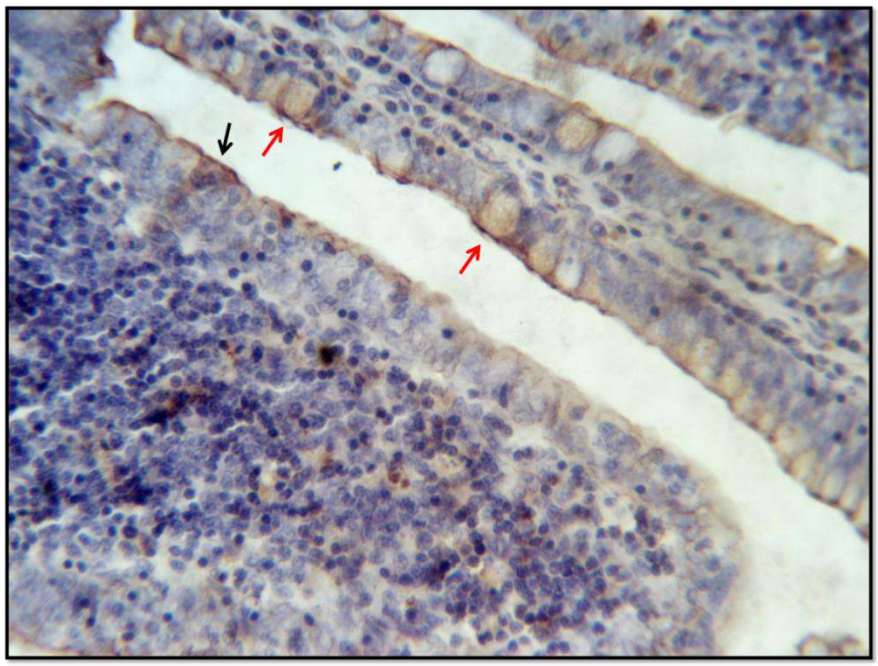
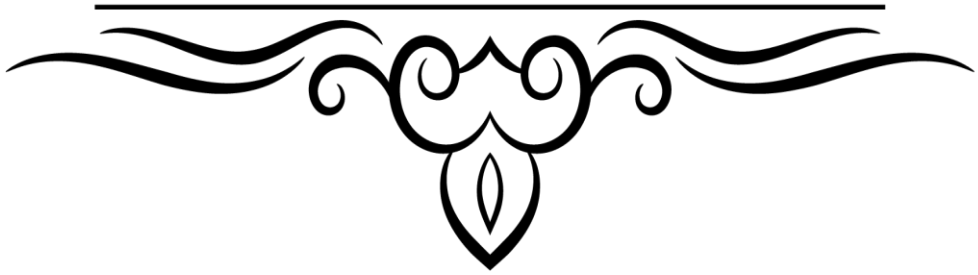


Figure 33. Histological section of appendix in rabbit shows immunohistochemical staining with GP2 marker. It showed goblet cell (red arrow) and M-cell (black arrow. X400. GP2

Chapter Five

Discussion



Discussion

5.1. Macroscopic Study

The cecum is the major site of fermentation in many mammalian species as well as acting as a main immune organ because of its content of lymphoid follicles. There was species variation in the structure of cecum among mammals so that, the study of its structure in different species is becomes very important.

Our study showed that the cecum of rabbit appeared to be divided macroscopically into three main parts the base, the body, and appendix vermiformis, while the cecum of hamster can be split into the base, the body and the apex. These divisions were mentioned by Saleh (2012) in rabbit and Perez *et al.* (2007 & 2008) in the hamster.

Generally, the rabbit had haustrated cecum and often employed the whole ventral portion of the abdominal cavity in contrast to the hamster where the cecum is situated left to the median plane, and the later arrangement was recognized as a public feature in most rodents as reported by Stan (2014). This arrangement was recognized as a typical trait of numerous rodents (Perez *et al*., 2008; Kotze *et al*., 2010).

In the current study, the caecum of rabbit was a tubular structure about 43.12 cm in length which was closer to the finding of (Ranjan and Das, 2021) who reported that the length of entire cecum in rabbit was 49.75 cm. While in hamster it was about 4.14 cm in length, this difference in the length between the rabbit and hamster might be related to the animal size and the function of cecum where the main site of fermentation in rabbit is the cecum so its size reflect its function.

The length of appendix in rabbit was 9.25 cm and it had a brighter color compared to other portions of cecum since the ingesta were not noticeable over the vastly lymphoid wall, this finding was in agreement

with that of Ranjan and Das (2021) who mentioned that the vermiform appendix was well advanced and its length was approximately 8.33 cm. Physiologically, in rabbits, the vermiform appendix was contributory in the excretion of bicarbonate to buffer the acidity of the cecum and in the excretion of water to form semifluid cecal materials (Davies & Davies, 2003; Kohles, 2014).

5.2. Microscopic Study

Generally, current study revealed that the histological construction of the cecal wall in rabbits appeared well developed in comparison with that in hamsters and this may be attributed to functional properties of the cecum especially the appendix in rabbit.

Microscopic inspection results revealed that the cecal wall in the three portions (base, body and apex) in both animals has the same identified four intestinal tunicae , but the folds of appendix in rabbit were distinguished by having broad apices and small bases (leaf-like folds) and this feature was also reported by Ranjan and Das (2021) who mentioned that the folds of mucosa seemed as a long leaf-like folds of various sizes and a well-developed lymphoid follicle alongside the entire perimeter of its wall was filled the lamina properia. These findings come in consistent with those of Quesenberry and Carpenter (2011) and Stan (2014) in chinchilla and rabbit.

In the current study, a simple columnar epithelium lined these folds with large number of goblet cells whose count was significantly more in appendix than the other portions of cecum in rabbit because of the large and tall leaf-like folds of appendix. It is interesting to note that the appendix's morphological and histological construction differed from other portions of the rabbit's cecum by had well-developed lymphoid tissue which possesses several aggregation of lymphoid follicles, each of

it comprises four distinct areas: a dome area, a germinal midpoint, a coronal area, and a broad inter follicular area among adjacent follicles, a specialized follicle associated epithelium also bounded the follicular areas and these findings was also reported by Beyaz *et al*. (2010). Similar findings on the architectural layout of lymphoid follicles were also stated by Saleh (2012). In mammals, the appendix was stated in certain rodents, rabbits and humans who participate a similar origin and elevated lymph cells concentration (Smith *et al.,* 2009).

The FAE was present in the appendix and absent in the rest parts of the cecum because there were no lymphatic follicles underneath the epithelium in base and body of cecum, in addition to its immune function and this made the epithelium lining the folds appeared different from the adjacent epithelium lining the dome shape structure as mentioned by Rouch *et al.* (2016), Al-Haaik and Al-Saffar (2017) and Kanaya *et al*. (2018).

Among these folds there was a dome shape structures which was constructed from aggregation of large number of lymphocytes and these nodules were covered with special type of epithelium which was called FAE. This type of epithelium consist of simple columnar epithelium with numerous modified cells called M-cell (microfolded cells), same findings was also reported by Magalhaes *et al.* (2007), Zhong *et al.* (2007) and Kimura (2018). Usually, the goblet cells were absent in this epithelium but in certain sites had single goblet cells, this may be due to the FAE has an immune function and does not need mucous cells like simple columnar epithelium which has an absorptive function, These findings was in agreement with that of Newberry (2008) Ermund *et al.* (2013) and Al-Haaik (2017).

The M-cell was characterized by its large size in comparison with columnar cells and exhibited pocket that housed several lymphocytes

which explain the participation of these cells in the immunological reactions development against antigens through transporting pathogen factor to antigen-presenting cells below M cells via trans-epithelial transportation, this finding was in agreement with that of Beyaz (2004), Man *et al.* (2004),Magalhaes *et al.* (2007) and Shaykhev & Bals (2007). The current study displayed that M cells number decreases or disappears towards the apex of the dome shape structure and this fact was confirmed by Jepson *et al.* (1993), whose mentioned that the lack of M cells towards the dome apex was poorly understood and it was believed that the process of differentiation of M cells happens in dome base. The process of migration of M cells towards the apex takes time, so, the apoptosis occurs before the M cells reach the apex of the dome.

The mucosal epithelium of the cecal wall has some mucous cells only, this denote that most of the epithelial cells may perhaps comprise absorptive cells, that are useful for absorption of volatile fatty acids and electrolytes through the mucosa of cecum as reported by Meredith (2006). The mucosa of the hamster within its three portions are abundant with intestinal glands (crypts) that was rich with many goblet cells and obvious different levels of mitotic figures were well noticed in the crypts of cecum of hamster and this due to the nature of the hamster's diet which depends on dry grains and needs more mucus to moisturize it, that was secreted from the mucous cells existing in abundance in the crypts of the lieberkuhn and the meissner nerve plexus control its secretion while the rabbit feeds on green grass so it needs less number of goblet cells to be moistened with mucus, but rather needs a fermentation process, similar results was reported by Smith *et al.* (2020).

In this study, the tunica mucosa mean thickness of the appendix in the rabbit was higher compared with the apex in the hamster, this might be due to the presence of large leaf-like folds in the appendix in addition

to presence of lymphoid follicles. Whereas the tunica submucosa mean thickness of in hamster was greater and further developed than that present in the rabbit might be attributed to the presence of large sizes blood vessels, also meissner nerve plexus was noticed in the submucosa of hamster and this might be related to its function that include controlling the secretion of goblet cells of mucin for lubrication of dry food, these results come in consistency with Amiry *et al*. (2019).

In the cecum, especially in rabbit, the tunica muscularis was thin, which even could not generate wall contraction, as a result proposing that in the cecum, the ingesta will possibly be mildly mixed to initiate fermentation that occur inside the cecum and its mean thickness was more in hamster than in rabbit and had a well-developed with an interesting large size auerbach's nerve plexus in the cecum of hamster compared with that of rabbit. This might be associated with the nature of the hamster's food, as it feeds on grains and does not need the fermentation process like rabbit which feeds on green grass but rather, it needs contractions to push the food towards the colon ; thus, this function is related to auerbach's nerve plexus, these findings was in agreement with the observations of Amiry *et al*. (2019) and Smith *et al*, (2020).

5.3. Carbohydrate Histochemistry

In vertebrates, the gastrointestinal excretion contains an amount of glycoprotiens that can vary depending on the type of cell, their locations, age, gender, species and pathological condition (Pedini *et al.,* 2001; Liquori *et al.,* 2002; Choi *et al.,* 2003; Schumacher *et al.,* 2004), since alterations in mucosal conformation and mucin histochemical composition are correlated with the etiology of a number of neoplastic and inflammatory gastrointestinal conditions, they received increasing attention.

Recent microscopic inspection has shown the existence of goblet cells between other mucosal epithelial cells of the folds and the latter is characterized by its cylindrical shape with wide apical border and basally situated nuclei. After staining with H&E, it seemed with clear cytoplasm, however, with periodic acid Schiff stain, it took magenta color and with Masson's Trichrome stain, it took a pale green color, this finding was in agreement with the observations of Al-Haaik and Al-Saffar (2017).

The appendix exhibit maximum quantity of goblet cells in comparison with body and base in rabbit and this was due to the presence of leaf -like folds with large sizes.

The carbohydrate histochemical findings revealed that most of surface epithelium goblet cells in the of base, body and apex in both species of animals give positive reaction to PAS stain indicating the presence of neutral mucin in these cells, these results come in consistency with AL-Samawy *et al*. (2019) in camels, whereas most of goblet cells in the crypts give positive reaction to each of PAS and $AB_{ph2.5}$ indicating the presence of mixed materials of neutral and acidic mucin in both species of animals.

Generally, these reactions of PAS and $AB_{ph2.5}$ were mentioned by (Al-Saffar and Al-Haaik, 2016) whom cited that most of surface epithelium goblet cells in the colon contain neutral mucin while those of crypts contain acidic mucin. A comparative histochemical analysis of thirteen different mammalian species' mucin (rabbits, guinea pigs, rats and voles) identified a similar mucin histochemical profile (Schumacher *et al*., 2004) which is in accordance with the findings of white Italian rabbits from New Zealand and studies conducted by Zanuzi *et al*. (2010) as well as Desantis *et al*. (2011).

5.4. Immunohistochemistry

The role of the M cells has been formerly established (Jang *et al*., 2004) considering antigen sampling to be critical to start of immune responses specific to gastrointestinal environmental antigens (Renfeng *et al*., 2015).

Actually, there were various markers used for expression of M-cell as lectin UEA-1 , claudin 4, Glycoprotein 2, vimentine and cytokeratin (Mabbott *et al*., 2013). Actually, in our study two markers (vimentine and GP2) were used, anyway, the GP2 marker give the best reaction, therefore the results of vimentine was excluded from our study.

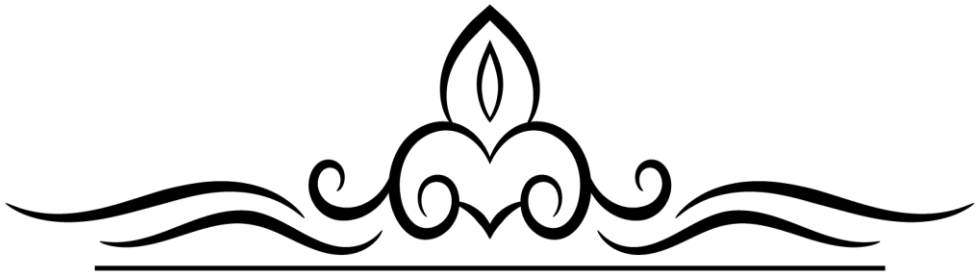
Currently, the Immunohistochemical procedure showed that GP2 gave a strong reaction with M-cell which appeared with brown color (apical and basolateral membrane) compared with vimentin which showed moderate reaction.

Notably, in our study, the highest existence of M-cell was in the lowest part on the edges of the dome while in the apex it was almost absent and this is may be due to that M cell arises from stem cells of intestinal crypts. This finding was in agreement with the observations of Lelouard *et al.* (2001), Clark & Jepson (2003), and Mach *et al.* (2005) in addition to its life cycle was so short.

In the current study, concerning mature M cells, GP2 immuno-expression was seen in each of cytoplasmic areas nearby IEL and perinuclear cytoplasm whereas the perinuclear cytoplasm reaction was seen in immature M cells. Immune reactions were not detected throughout the cells' apical cytoplasm, this result was in agreement with the observations of Beyaz *et al*. (2010).

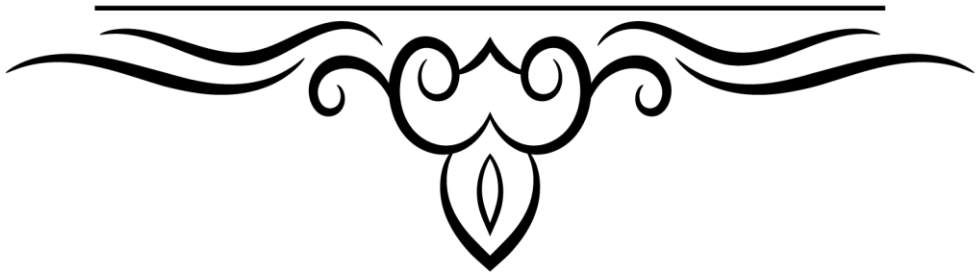
Prims *et al.* (2017), in his study on pigs, stated that young M cell, due to adjacent connection with lymphocytes in margin related to FAE, is differentiated firstly into mature M cell, and far along into absorptive

columnar cell close the apex of dome. Other scholars stated that pig's Peyer's patches M cells, were the same as M cells of the mouse (Sierro *et al.*, 2000) and rabbit (Takeushi& Gonda, 2004), afterward developed into enterocyte and then in the apex, suffer from apoptosis, then expelled to lumen. In the current study, depending on existence of developed M cells with a tinny cytoplasm, that border intra-epithelial lymphocytes with their pouches in FAE in the margin of the area of dome, and demonstration of young M cells missing IEL pouches in the FAE close the crypts by GP2 immuno-histochemical staining, the views proposing that M cells arise from crypts has been favored. Additionally, according to our observation of numerous GP2-positive M cells in the apex FAE of some dome areas in the appendix thus, our study does not prove that M cells are differentiate into enterocytes and are expelled into the apex lumen these results come in consistency with (Miyazawa *et al.*, 2006; Renfeng *et al.*, 2015).



Chapter Six

Conclusions and Recommendations



Chapter Six

Conclusions and Recommendations

6.1 . Conclusions

1. The results showed that the location of the cecum is on the left side in hamsters and on the right side in rabbits.
2. The fermentation process occurs largely in the cecum of rabbits and less in hamsters.
3. There are differences in the thickness of the cecal wall between its three parts and also between the two animals.
4. Nerve plexuses are more developed and their number is greater in hamsters than in rabbits and this is due to the nature of the hamsters' diet.
5. M cell is located in the basal part of the dome, and there are two types of epithelium in the appendix of rabbit.

6.2. Recommendations

1. Conducting a carbohydrate histochemical and histomorphological, study in the cecum among different types of mammals and birds.
2. conduct ultrastructure study about follicular associated epithelium and determine types of mature and immature M cells.
3. Immunohistochemistry study of follicles and study of types of lymphocytes present within the follicles in the appendix.
4. Histological, chemical, immunological and ultrastructure study of the nerve plexuses and its cells include cajal cells that associated with it.

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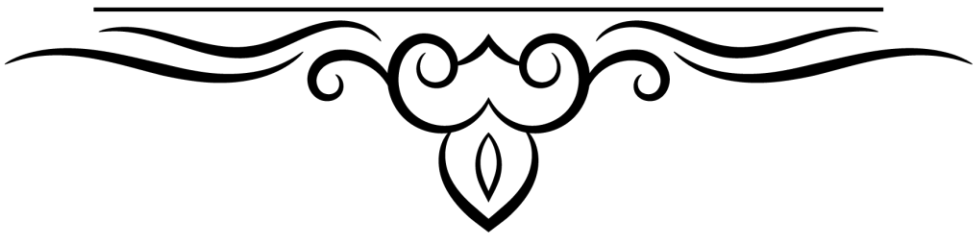

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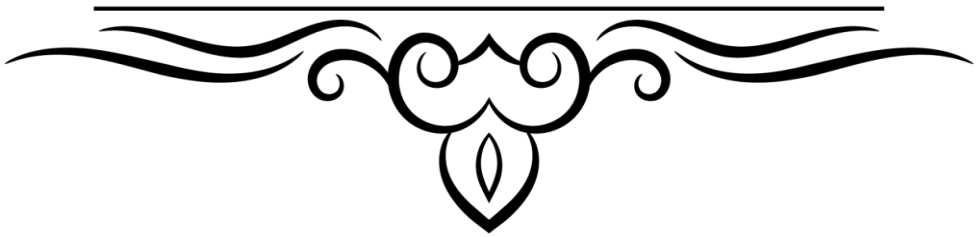
Appendices



Appendix 1: Hematoxylin and eosin (H&E) stain

Solutions

Hematoxylin Solution (Harris):

Potassium or ammonium (alum……...….... 10 g

Distilled water ……..……... 500 ml

Heat to dissolve. Add 25 ml of 10% alcoholic hematoxylin solution and heat to boil for 1 minute. Remove from heat and slowly add 1.25 g of mercuric oxide (red). Heat to the solution and until it becomes dark purple color. Cool the solution in cold water bath and add 10 ml of glacial acetic acid (concentrated). Filter before use.

Eosin Y Stock Solution (1%):

Eosin Y ……... 1 g

Distilled water …… 20 ml

95% Ethanol……. 80 ml

Mix to dissolve and store at room temperature.

Eosin Y Working Solution (0.25%):

Eosin Y stock solution …… 25 ml

80% Ethanol …… 75 ml

Glacial acetic acid (concentrated) ……...0.5 ml

Mix well and store at room temperature.

1% Acid Alcohol Solution (for differentiation):

Hydrochloric acid …….. 1 ml

70% ethanol ……... 100 ml

Mix well.

Procedure:

1. Deparaffinize sections, 2 changes of xylene, 10 minutes each.
2. Re-hydrate in 2 changes of absolute alcohol, 5 minutes each.
3. 95% alcohol for 2 minutes and 70% alcohol for 2 minutes.
4. Wash briefly in distilled water.
5. Stain in Harris hematoxylin solution for 8-10 minutes.
6. Wash in running tap water for 15 minutes.
7. Differentiate in 1% acid alcohol for 30 seconds.
8. Wash running tap water for 1 minute.
9. Rinse in 95% alcohol, 10 dips.
10. Counterstain in eosin Y solution for 30 seconds to 1 minute.
11. Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each.
12. Clear in 2 changes of xylene, 5 minutes each.
13. Mount with xylene based mounting medium.

Results: nuclei ….blue

Cytoplasm ….pink

Appendix 2: Preparation of Masson's Trichrome stain:

A. Bouin's solution

Picric acid (saturated) ---------- 75 ml

Formaldehyde (37-40%) ---------- 25 ml

Glacial acetic acid ---------- 5 ml

B. Weigert's iron haematoxylin solution

Stock Solution A:

Hematoxylin ---------- 1 g

95% Alcohol ---------- 100 ml

Stock Solution B:

29% Ferric chloride in water --------- 4 ml

Distilled water ---------- 95 ml

Hydrochloric acid, concentrated ----- 1ml

Weigert's Iron Hematoxylin Working Solution:

Mix equal parts of stock solution A and B. This working solution is stable for 3 months (no good after 4 months)

C. Biebrich scarlet acid fuchsine solution:

1- Biebrich scarlet aqueous solution 1%.....90 ml
2- Acid fuchsine aqueous solution 1%.....10 ml
3- Glacial acetic acid…….……..…1 ml

D. Phosphotungstic –acid solution:

1- Phosphotungstic acid……..…….…… 5 gm
2- Distilled water……...……...….100 ml

E. Light green solution:

1- Light geen……..……2.5 gm
2- Glacial acetic acid…….……..…..…….……2 ml
3- Distilled water……..…..……..…….100 ml

F. 1% Glacial acetic acid:

1- Glacial acetic acid……..……...…..1 ml
2- Distilled water……..……...……100 ml

Procedure:

1. Deparaffinize and rehydrate through 100% alcohol, 95% alcohol 70% alcohol.
2. Wash in distilled water.
3. For Formalin fixed tissue, re-fix in Bouin's solution for 1 hour at 56 C to improve staining quality although this step is not absolutely necessary.
4. Rinse running tap water for 5-10 minutes to remove the yellow color.
3. Stain in Weigert's iron hematoxylin working solution for 10 minutes.
4. Rinse in running warm tap water for 10 minutes.
5. Wash in distilled water.
6. Stain in Biebrich scarlet-acid fuchsin solution for 10-15 minutes. Solution can be saved for future use.
7. Wash in distilled water.
8. Differentiate in phosphomolybdic acid solution for 10-15 minutes or until collagen is not red.
9. Transfer sections directly (without rinse) to aniline blue solution and stain for 5-10 minutes. Rinse briefly in distilled water and differentiate in 1% acetic acid solution for 2-5 minutes.
10. Wash in distilled water.
11. Dehydrate very quickly through 95% ethyl alcohol, absolute ethyl alcohol (these step will wipe off Biebrich scarlet-acid fuchsin staining) and clear in xylene.
12. Mount with resinous mounting medium.

Results:

Collagen ---------- green

Nuclei ---------- black

Muscle, cytoplasm, keratin ---------- red

Appendix 3: Combined PAS/ Alcian blue technique:

Solutions

0.5% Periodic Acid Solution:

Periodic acid ……. 0.5 g
Distilled water……..… 100 ml

Schiff Reagent:

Basic fuchsin…….....1.0 gm.
Sodium metabisulfite ……..…1.8 gm.
Distilled water……100 ml
Hydrochloric acid……..…… 1.0 ml

Procedure

1. Deparaffinize and hydrate to distilled water.
2. Stain with Alcian blue 15 mins
3. Wash well in running tap water 2 mins
4. Rinse in distilled water
5. Treat with periodic acid 5 mins
6. Wash well in distilled water
7. Stain with Schiff's reagent 10 mins
8. Wash well in running tap water 5 mins
9. Stain nuclei with haematoxylin 1 min
10. Wash in running tap water 2 mins
11. Differentiate with acid alcohol
12. Wash and blue nuclei in Scott’s tap water
13. Wash in water
14. Dehydrate, clear and mount

Results

- acidic mucins ..... blue
- neutral mucins ..... magenta

- mixtures of above ..... blue/purple
- nuclei ..... deep blue

Appendix 4: Procedure for coating slides with gelatin for IHC sections

Reagents Required

* Gelatin-coating solution: 1 L deionized H2O, ,

* 5 g gelatin

* 0.5 g chromium potassium sulfate dodecahydrate CrK(SO4)2 · 12H2O

Procedure

1- Prepare the gelatin-coating solution by dissolving 5 g of gelatin in 1 L of heated, deionized H2O (temperature should not exceed 45 °C).

2- After the gelatin has dissolved; add 0.5 g of chromium potassium sulfate dodecahydrate. Chromium potassium sulfate dodecahydrate will positively charge the slides allowing them to attract negatively charged tissue sections.

3- Filter this solution and store at 2-8 °C until use. It is recommended that this solution be filtered again immediately before use (adjust to room temperature before filtration).

4- Place the histological slides into metal racks.

Note: The slides should be cleaned by washing them in soapy water and rinsing them thoroughly, first in tap water and finally in absolute ethanol.

5- Dip the racks containing the slides 3 to 5 times (~5 seconds each) into the gelatin-coating solution.

6- Remove the racks containing the slides and let them drain. Blot excess solution from the racks onto filter paper (gently tap the racks against the filter paper for better drainage).

7- Place the racks containing the slides on the lab bench and cover them with paper towels to protect them from dust. (The slides were dried in vertical position in a dust free location.

8- Dry at room temperature for 48 hours.

9- Dried slides can be put back into the boxes that they arrived in and stored at room temperature until use. Slides intended for cryostat sections can be stored at -20 °C.

Appendix 5 :

Vimentin Polyclonal Antibody / Elabscience

Catalog Number:E-AB-93320

Description

Reactivity Human,Mouse,Rat

Immunogen Recombinant fusion protein of human Vimentin

Host Rabbit

Isotype IgG

Purification Affinity purification

Conjugation Unconjugated

Formulation PBS with 0.05% proclin300,50% glycerol,pH7.3.

Applications Recommended Dilution

WB 1:500-1:2000

IHC 1:50-1:100

IF 1:50-1:200

2-step plus Poly-HRP Anti Mouse/Rabbit IgG Detection System (with DAB solution)

Sample dyeing

1. Dewax and hydrate the paraffin section.

2. Make thermal repair or digestion treatment to antigen of the tissue section if necessary according to antigen/antibody situation.

3. Incubate with E-IR-R217C (3% H_2O_2) for 10 min to eliminate endogenous peroxidase activity. Wash with PBS or TBS, 2 min×3 times.

4. Add E-IR-R217A (Normal Goat Blocking Buffer (Ready-to-Use)), incubate at 37°C for 30 min. Shake off any excess liquid.

5. Add primary antibody (From Mouse or Rabbit) with proper dilution ratio, incubate at 20~37°C for 1~2h or at 4°C overnight (then rewarm at 37°C for 30 min). Wash with PBS or TBS, 2 min×3 times. Dry the section with absorbent paper.

6. Add E-IR-R217B (Polyperoxidase-anti-Mouse/Rabbit IgG), incubate at room temperature or 37°C for 20 min. Wash with PBS or TBS, 2 min×3 times.

7. Add 1 drop (approximately 50 μL) of E-IR-R217D (DAB Concentrate) into each 1 mL of E-IR-R217E (DAB Substrate), mix fully and the mixed reagent is the DAB Working Solution. Prepare fresh solution before use and the prepared solution should be stored in the dark. Fresh prepared DAB Working Solution is valid within 4 hours and the unused solution must be abandoned

8. Take control of the DAB coloration period, the color of tan or brownish yellow is the positive signal. Avoid of excessive reaction.

9. Wash the section with deionized water terminate the chromogenic reaction, then operate the procedures of counterstaining, dehydrating, transparentizing and sealing

Appendix 6 :

GP2 Polyclonal Antibody / Elabscience

Catalog Number:E-AB-90418

Reactivity Mouse, Rat

Immunogen Recombinant fusion protein of human GP2

Host Rabbit

Isotype IgG

Purification Affinity purification

Conjugation Unconjugated

Formulation PBS with 0. 02% sodium azide, 50% glycerol,pH7.3.

2-step plus Poly-HRP Anti Mouse/Rabbit IgG Detection System (with DAB solution)

Sample dyeing

1. Dewax and hydrate the paraffin section.

2. Make thermal repair or digestion treatment to antigen of the tissue section if necessary according to antigen/antibody situation.

3. Incubate with E-IR-R217C (3% H_2O_2) for 10 min to eliminate endogenous peroxidase activity. Wash with PBS or TBS, 2 min×3 times.

4. Add E-IR-R217A (Normal Goat Blocking Buffer (Ready-to-Use)), incubate at 37°C for 30 min. Shake off any excess liquid.

5. Add primary antibody (From Mouse or Rabbit) with proper dilution ratio, incubate at 20~37°C for 1~2h or at 4°C overnight (then rewarm at 37°C for 30 min). Wash with PBS or TBS, 2 min×3 times. Dry the section with absorbent paper.

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7. Add 1 drop (approximately 50 μL) of E-IR-R217D (DAB Concentrate) into each 1 mL of E-IR-R217E (DAB Substrate), mix fully and the mixed reagent is the DAB Working Solution. Prepare fresh solution before use and the prepared solution should be stored in the dark. Fresh prepared DAB Working Solution is valid within 4 hours and the unused solution must be abandoned

8. Take control of the DAB coloration period, the color of tan or brownish yellow is the positive signal. Avoid of excessive reaction.

9. Wash the section with deionized water terminate the chromogenic reaction, then operate the procedures of counterstaining, dehydrating, transparentizing and sealing

لوحظ وجود نوعين من الضفائر العصبية المعوية في الأعور في الأرانب والهامستر، الأول، ضفائر مايسنر العصبية والتي كانت صغيرة وتقع في الطبقة تحت المخاطية المجاورة للطبقة العضلية الدائرية الداخلية للغلالة العضلية وقد لوحظت في اعور الهامستر أكثر من الأرنب. توجد الضفيرة العصبية لأورباخ الثانية بين طبقتي الغلالة العضلية تحتوي على خلايا عصبية وخلايا دبقية. توجد ضفائر أورباخ في جميع أجزاء الأعور وكانت مميزة وكبيرة ومتعددة في الهامستر.

ظهرت الخلايا الكأسية كخلايا كبيرة شاحبة او بدون لون مع ملون الهيماتوكسيلين والايوسين وبلون أحمر مع تقنية حمض شيف الدوري وبلون أزرق مع ملون الاليشيان الازرق. لوحظ أكبر عدد من الخلايا الكأسية في اعور كلا الحيوانين بأجزائها الثلاثة في الزائدة الدودية للأرانب وأقل عدد سجل في جسم اعور الأرنب. كشف ملف الكربوهيدرات في الأعور عن هيمنة المخاط الحمضي في الخبايا بينما كان المخاط المتعادل هو السائد في الطيات والظهارة السطحية.

الخلاصة

أجريت الدراسة الحالية لمعرفة الصفات الشكلية النسيجية والكيميائية النسيجية والكيميائية النسيجية المناعية لتركيب الأعور في سلالات الأرنب المحلي والهامستر السوري. ولتحقيق هذا الهدف تم جمع عينات من الأعور بأجزائه الثلاثة (القاعدة والجسم والقمة) من 15 أرنباً بالغاً ونفس العدد من الهامستر. تم إجراء الصبغة النسيجية الروتينية الهيماتوكسيلين والايوسين ، وملون ماسون ثلاثي الألوان، وملون حمض شيف الدوري وتقنيات الألشيان الأزرق بالإضافة إلى عدة الكيمياء النسيجية المناعية الخاصة (GP2وVimentin) لتحديد خلايا M cells لتحقيق الأهداف الحالية.

أظهرت الفحوصات المجهرية أن جدار الأعور في الأجزاء الثلاثة (القاعدة والجسم والقمة) في كلا الحيوانين له نفس الغلالات المعوية الأربعة المعروفة ولكن تميزت طيات الزائدة الدودية للأرنب بقواعدها الضيقة وقممها الواسعة. كانت هذه الطيات مبطنة بظهارة عمودية بسيطة وخلايا كأسية وفيرة فيما بينها والتي تتميز بشكل أسطواني مع أجزاء قمية حرة واسعة ونواة ذات موقع قاعدي، وظهر السيتوبلازم الخاص بها شاحبًا بعد صبغها بملون الهيماتوكسيلين والأيوسين. كما امتلكت الزائدة الدودية للأرنب نسيجًا لمفاويًا متطورًا يحتوي على العديد من الجريبات اللمفاوية المتجمعة، ويحتوي كل منها على أربعة مواقع مختلفة: منطقة القبة، والمركز الجرثومي، والمنطقة الإكليلية، ومنطقة واسعة بين الجريبات المجاورة. حيث ظهرت مناطق القبة محاطة بظهارة متخصصة مرافقة للجريبات ، يتكون هذا النوع من الظهارة من خلايا عمودية بسيطة اضافة الى خلايا تسمى M-cell (خلايا ذات طيات دقيقة). تميزت هذه الظهارة بخلوها من الخلايا الكأسية ولكن في بعض المواقع تحتوي على خلايا كأسية انفرادية .

اختلفت خلايا M عن الخلايا المعوية بحجمها الكبير ونواتها الكبيرة الجانبية الشاحبة وجيبوها التي احتوت على العديد من الخلايا الليمفاوية مما يوضح مشاركة هذه الخلايا في تطور الاستجابة المناعية .

أظهر استخدام GP2 وجود الخلايا M باللون البني في السايتوبلازم وكذلك في غشاء الخلية القمي والقاعدي الجانبي.

دراسة نسيجية شكلية قياسية وكيميائية نسيجية مناعية مقارنة للأعور في الارنب(*Oryctolagus cuniculus*) والهامستر(*Mesocricetus auratus*) البالغين

رسالة تقدمت بها

دعاء سعد امين النعيمي

إلى
مجلس كلية الطب البيطري في جامعة الموصل
وهي جزء من متطلبات نيل شهادة الماجستير
في اختصاص الطب البيطري / التشريح البيطري

بإشراف
الأستاذ المساعد الدكتور
عمار غانم الحائك

صفر / 1446هـ **أب / 2024م**

جامعة الموصل
كلية الطب البيطري

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رسالة ماجستير

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