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Original Research Article

Mucin Histochemical Study of the Colon in Normal and Malignant Lesions

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ABSTRACT

BACKGROUND: Mucins are complex carbohydrates secreted by different types of epithelial cells and glandular tissues of gastrointestinal tract. Mucins are mainly of two types- Neutral and Acidic.
OBJECTIVES: To know the mucin distribution of the colon in normal and malignant lesions and to find out difference in mucin distribution in normal and malignant lesions.
METHODOLOGY: The study was done on 30 specimens of normal and carcinoma colon collected from postmortem and surgically resected specimens in KIMSU and KHMRC hospital. Normal colonic specimens were used as controls with carcinoma of colon as test.
The type of study was observational, analytical and case control study.
Routine Hematoxylin / Eosin and special stains as PAS, PAS-Diastase, PAS-Phenyl Hydrazine, Alcian Blue PH -2.5 and 1, Aldehyde Fuchsin, combined AB-PAS and AF-AB were performed.
RESULTS: Results were tabulated according to color intensity into different grades ranging from + to ++++. Regarding mucin histochemistry of normal colon - "mixture" of mucins were observed with predominance of "neutral" and "sulphomucins". In colon carcinoma- "sialomucins" were seen predominant than neutral and sulphomucins.
CONCLUSION: Mucin histochemistry may provide a valuable and cost-effective tool for the diagnostic histopathology and for the researchers in histology.

KEY WORDS: Mucin, histochemistry, colon, carcinoma, special stains.

INTRODUCTION

Mucins are complex carbohydrates secreted by different types of epithelial cells and glandular tissues of gastrointestinal tract. There has been growing recognition in recent years that the demonstration of these substances is difficult, complex and affected by the types of mucins present. [1,2]

The term 'mucosubstances' is used, as recommended by Spicer, Leppi and Stoward [3] to denote all tissue components,

other than glycogen, rich in carbohydrates, which are present in connective tissue, or as secretion of certain epithelial structures. Connective tissue mucosubstances are called "mucopolysaccharides", while those secreted by epithelia are referred as "mucins". [4]

Mucins perform wide variety of functions like lubrication, protection against acids etc. The mucosubstances also contain immunoglobulin's, primarily of IgA type, lactoferrin which chelate the iron necessary for growth of some bacteria and lysosomes which destroy some of the bacteria. Hence they act as antibacterial and antiviral agents and have protective mechanism. [5] Mucosubstances are classified into two categories mainly.

A) Neutral mucins and B) Acidic mucins

Neutral mucins are slightly alkaline in nature and mainly help for reducing the pH and toxicity of substances. Acidic mucins are sub classified into weakly acidic and strongly acidic. [1,4,5] Weakly acidic mucins contain terminal carboxyl group and are called as carboxylated mucins or sialomucins. They contain chelating agents and have antibacterial and antiviral property. Strongly acidic mucins contain sulphate groups and are called as sulphomucins. They are thick, viscous and help for formation of protective coat for lubrication. [4,5]

Histochemistry is defined as any technique in which a chemical reaction is involved in coloring tissue, be it staining with dyes. The designation of a stain as special may be arbitrary but generally any stain other than H and E is regarded as Special stain. They are used in an attempt to identify cell and tissue components by virtue of their specific chemical reactions. [5]

Although the mucosubstances in the alimentary tract of rodents have been the subject of many chemical and histochemical studies by Spicer, [6] little is known about the character and chemical position or the cell of origin of the mucosubstances of different parts of the human alimentary tract under normal conditions and in various pathological conditions. With the development of new histochemical methods with special stains, specific chemical composition of mucosubstances is documented by various scientists. But on the other hand, there have been few studies of human alimentary tract mucosubstances (e.g. by Lev 1966, Lev & Spicer 1965, Gad 1968, Filipe 1969, Subbuswamy 1971 and Shah, Shrikhande 1989). [7]

In the present study, combination of special stains such as P.A.S. Diastase, P.A.S.- Phenyl hydrazine, Alcian Blue-P.A.S., Aldehyde fuchsin-Alcian blue were used to simultaneously assess the proportions of various mucin types in the epithelium of colon and rectum examined.

MATERIAL AND METHODS

The present study was conducted in the Department of Anatomy, Krishna Institute of Medical Sciences, University, Karad from May 2009 to June 2011.

The type of study was observational, analytical and case control study.

Sample size was 30 blocks of each normal and carcinoma colon.

The study was carried out on 30 specimens of normal and carcinoma colon collected from postmortems and surgically removed specimen from Krishna Hospital. Normal colonic specimens were used as controls with carcinoma colon as test.

The tissues were fixed in 10% formal saline with 2% calcium acetate and a pinch of phosphotungstic acid to help for preservation of mucins. The tissues were embedded in paraffin and blocks were prepared by histopathological technique and cut at 5-6 microns. Sections were stained with Hematoxylin and Eosin, and the following histochemical methods were

performed on paraffin- embedded sections for the characterization of different mucosubstances as PAS, PAS diastase, PAS- Phenyl hydrazine, Alcian blue(AB) – pH 1and 2.5, Aldehyde fuchsin(AF), combined AB-PAS and combined AF-AB.

1) P.A.S. -- Periodic acid Schiff reagent stains all carbohydrates including mucosubstances. Therefore mucosubstances are P.A.S. positive.
2) P.A.S. Diastase -- Diastase dissolves glycogen like carbohydrates, but mucin remains unaffected. This stain is used for confirmation of mucosubstances.
3) P.A.S. Phenyl hydrazine -- Phenyl hydrazine dissolves neutral mucosubstances only and hence to prove their presence.
4) Alcian blue -- This stain can be used at various pH levels.

a) AB pH 1 -- This stain is highly acidic and stains sulphomucins only.

b) AB pH 2.5 -- This stain is weakly acidic and stains both carboxylated and sulphomucins.

5) Aldehyde Fuchsin – This stain only stains sulphomucins and confirms their presence.
6) Combined AB-PAS – This staining procedure will stain all different types of mucin.

Neutral –Magenta, Carboxylated – Blue, Sulphated --Purple.

7) Combined AF-AB -- This staining procedure helps for differentiation and confirmation of carboxylated and sulphated mucins.

Carboxylated –Blue, Sulphated-- Purple

All the results obtained were tabulated according to color intensity into different grades ranging from + to ++++. [8,9,10]

OBSREVATIONS AND RESULTS

During the period of May 2009 to June 2011, thirty blocks of normal and carcinoma colon were collected. Normal colonic specimens were used as controls with carcinoma colon as test. Histological technique was preceded and staining with H and E and with special stains as PAS, PAS-Diastase, AB-pH 2.5 and 1, AF, combined AB-PAS, AF-AB, PAS-Phenyl hydrazine were carried out.

All the results were tabulated according to color intensity into different grades ranging from + to ++++. [8,9,10]

Colour index [8,9,10] :

1) ++++ : Very strong positive reaction.
2) +++ : Strong positive reaction
3) ++ : Moderate reaction
4) + : Weak reaction
5) - : Negative reaction

Table No.1 Histochemical reactions of normal colon.

Sr. No.	Stains used	Epithelium		Glands	
		Color intensity	Inference about mucosubstances	Color intensity	Inference about mucosubstances
1	H & E	Simple columnar epithelium, no villi, plenty of goblet cells.	Identified & confirmed	Small, tubular glands	Identified & confirmed
2	PAS	++++	PAS +ve substances +	++++	PAS +ve substances +
3	PAS-D	+++	No glycogen, mucosubstances +	++++	No glycogen, mucosubstances +
4	PAS-PH	++	Few neutral mucins	+/++	Few neutral Mucins
5	AB- pH 2.5	+++	Acidic mucosubstances	+++	Acidic mucosubstances
6	AB- pH 1	+++	Sulphated acidic mucosubstances	+++	Sulphated acidic mucosubstances
7	AB-PAS	+++ magenta +++ purple ++ blue	Neutral Sulphated Carboxylated	+++magenta +++ purple ++ blue	Neutral Sulphated Carboxylated
8	AF	+++	Sulphated pred.	++ to +++	Sulphated pred.
9	AF-AB	+++ purple ++ blue	Sulphated pred. Few carboxylated	+++ purple ++ blue	Sulphated pred. Few carboxylated

Inference of Table No 1:

Regarding mucin histochemistry of normal colon, "mixture" of mucosubstances were observed. In that predominantly "neutral mucin" and "sulphated acidic mucin" were observed and correlated with mucin histochemistry of carcinoma colon.

Table No. 2 Histochemical reactions of carcinoma colon.

Sr. No.	Stains used	Epithelium		Glands	
		Colour intensity	Inference about mucosubstances	Colour intensity	Inference about mucosubstances
1	H & E	No villi, dysplastic epithelium & tumor invading, muscle layer	Adenocarcinoma of colon	Malignant glands with nuclear feature of anaplasia	Adenocarcinoma of colon
2	PAS	+	Few PAS +ve substances present.	-/+	Trace/ Few PAS+ve substances
3	PAS- D	++	No glycogen, mucosubstances +	++	No glycogen, mucosubstances +
4	PAS-PH	++	Few neutral mucins	+/++	Few neutral mucin
5	AB- pH 2.5	+++	Acidic mucosubstances	++++	Acidic mucosubstances
6	AB-pH1	+ to ++	Few Sulphated	+ to ++	Few Sulphated
7	AB-PAS	magenta ++ blue +++ purple +	Few Neutral Carboxylated Few sulphated	magenta ++ blue ++++ purple +	Few Neutral Carboxylated pred. Few Sulphated
8	AF	+	Few Sulphated	+	Few Sulphated
9	AF-AB	blue +++ purple +	Carboxylated pred. Few sulphated	blue +++ to ++++ purple -/+	Carboxylated pred. Few Sulphated

Inference of Table No 2:

Regarding mucin histochemistry of carcinoma colon, "mixture of mucosubstances" were observed, but in contrast to normal colon "carboxylated acidic mucins" were seen predominantly than neutral and sulphated acidic mucins.

Table No.3 Mucin histochemistry of normal colon and carcinoma colon.

Sr. No.	Stains used	Inference about mucosubstances	
		Normal colon	**Carcinoma colorectum**
1	H & E	Simple columnar epithelium, no villi, plenty of goblet cells. Identified and confirmed	Adenocarcinoma identified and confirmed
2	PAS	Neutral mucin present	Neutral mucin present
3	PAS- Diastase	No glycogen	No glycogen
4	PAS-PH	Moderate neutral mucins	Few neutral mucins
5	AB- pH 2.5	Acidic mucins present	Acidic mucins present
6	AB-PH 1	Sulphated mucins predominant	Few sulphated mucins
7	AB-PAS	Mixture of mucins-- neutral and acidic, sulphated predominant	Mixture of mucins --neutral and acidic, carboxylated predominant
8	AF	Predominant sulphated	Few sulphated
9	AF-AB	Sulphated predominant with few carboxylated mucins.	Carboxylated predominant and few sulphated with reduction in neutral mucins.

Inference of Table No 3:

Regarding comparison of mucin histochemistry in normal and carcinoma colon, mixture of mucosubstances were found in both; they were neutral and acidic mucosubstances.

In acidic mucosubstances reversal of mucin pattern was observed. In present study predominant sulphated acidic mucins (Sulphomucins) were seen in normal colon, whereas loss of sulphation with predominant carboxylated (Sialomucin) was seen in carcinoma colon.

DISCUSSION

Mucin histochemistry of the normal colon:

In the present study, H & E and special stains were used for mucin histochemistry of 30 specimens of normal colon. Mucosa of the large intestine showed predominance of neutral and sulphated mucins. Mucin histochemical stains like PAS, AB-pH 2.5, 1 and AB-PAS gave strong reaction leading to conclusion of neutral and acidic sulphated mucins. Sulphated mucins again confirmed by strong reaction with AF and AF-AB. (Table no. 1) The results were correlated with carcinoma colon. (Table No.3)

These observations were correlated with Shah & Shrikhande, [11] Subbuswamy SG, [12] Gad A, [8] Sheahan DG & Jerwis HR, [13] Filipe MI, [14,15] Filipe MI and Bancroft ME, [2] Ganga GM. [16] (Table No 4)

Hematoxylin and eosin stain used for identification and confirmation of normal colon, reveled simple columnar epithelium with absence of villi and presence of plenty of goblet cells. The glands are simple, tubular and regular and some with luminal secretions. (Photomicrograph No.1).

Table No. 4 Comparative study of mucin histochemistry of normal colon.

Sr.No.	Study	Inference / Observations
1	Shah & Shrikhande [11]	Normal colonic goblet cells predominantly secrete sulphomucin with small amount of sialomucin
2	Subbuswamy SG [12]	Colon predominantly produces acidic mucin. Small intestine produces predominantly neutral mucin.
3	Gad A [8]	Mixture of both acid mucosubstances in colon and predominantly non-sulphated in rectum.
4	Filipe MI [14,15]	Most of the crypts of normal colon and rectum occupied by sulphomucins.
5	Ganga GM [16]	In normal adult, large intestinal mucosa showed predominance of sulphomucin.
6	Present study	Normal colonic and rectal mucosa showed predominance of neutral and sulphated (acidic) mucins.

Regarding special stains-- PAS stain is used to assess presence of neutral mucosubstances. PAS stain for normal colon gave magenta color with strong reactivity suggestive of presence of neutral mucin. (Photomicrograph No.3). Epithelium and glands, both gave moderate to strong reactivity.

PAS-diastase stain gave magenta color in epithelium and glands suggestive of presence of PAS positive mucins and no glycogen. Normal colon in this gave moderate to strong positivity. (Photomicrograph No.5) Diastase digestion followed by PAS was utilized to distinguish the diastase-resistant mucosubstances from glycogen.

PAS-phenyl hydrazine stain gave mild to moderate reaction in 30 specimens of normal colon s/o presence of few neutral substances. Phenyl hydrazine blocks the subsequent reaction with Schiff reagent, so the staining is faint for neutral mucins. (Photomicrograph No.7)

The acidic mucins in all 30 specimens of normal colon were assessed by Alcian blue pH 2.5 stain. The AB-pH 2.5 stain gave moderate to strong reactivity as blue color. (Photomicrograph No.9)

Alcian blue pH 2.5 gave result for acidic mucins which are non-sulphated, for confirmation and assessment of sulphated mucins, alcian blue pH 1 was carried out.

AB-pH 1 in all 30 specimens gave moderate to strong reactivity as blue color suggestive of presence of sulphomucin in normal colon. (Photomicrograph No.11)

Combined stains as AB-PAS were carried out to assess neutral and acidic mucins. In AB-PAS stain mixture of colors seen, with predominance of purple and magenta color suggestive of presence of sulphated mucins and neutral mucins respectively. The carboxylated /sialomucin were few as blue colour and appeared as weak reaction. (Photomicrograph No.13) Mixtures of neutral and acidic (sulphated) mucins were observed with AB-PAS staining.

Furthermore, sulphated mucins were assessed by aldehyde fuchsin stain. The aldehyde fuchsin gave moderate to strong reaction as purple color. This confirmed presence of sulphomucin in normal colon in our study. (Photomicrograph No.15).

Combined stain AF-AB was done, as it was reliable means to separate sulphated from carboxylated mucins. Aldehyde fuchsin-alcian blue pH 2.5 gave strong reaction with purple colour and weak reaction as blue color suggestive of predominance of sulphomucins with small amount of sialomucin/ carboxylated mucins in normal colon (Photomicrograph No.17).

Mucin histochemistry of the carcinoma colon:

In colorectal carcinoma, regarding mucin histochemistry reversal of mucin

patterns was observed. Predominance of acidic mucins with carboxylated mucins was observed in contrast to normal colon, which had predominance of neutral and sulphated mucins.(Table No.2)

These observations were correlated with various workers as Shah & Shrikhande, [11] Subbuswamy SG, [12] Gad A, [8] Filipe MI [14] and Ganga GM. [16] (Table No.5)

Table No.5 Comparative study of mucin histochemistry of colonic carcinoma.

Sr.No.	Study	Inference / Observations
1	Shah & Shrikhande [11]	Increase in sialomucin and marked decrease in sulphomucins (reversal of mucin secretion pattern)
2	Subbuswamy SG [12]	Carcinoma of colon contained very little secretion with very scanty or no evidence of sulphation.
3	Gad A [8]	Sulphomucins were increased in large polyp and well differentiated ca, where as sialomucin predominantly in moderately and undifferentiated carcinomas.
4	Filipe MI [14,15]	Increase in non-sulphated acid mucin with decrease or absence of sulphated mucin in 60-90% cases according to differentiation of tumor.
5	Ganga GM [16]	Well differentiated adenocarcinoma showed increased amount of sialomucin with small amount of sulphomucins and traces of neutral mucin.
6	Present study	Carcinoma of colon showed predominance of carboxylated mucins (sialomucin) with small amount of sulphomucins and traces of neutral mucin.

In the present study, H and E and special stains were used for mucin histochemistry of 30 specimens of surgically resected colonic carcinomas.

Hematoxylin and eosin were used to identify and confirm the diagnosis of adenocarcinoma and further categorized into mucinous and non-mucinous adenocarcinoma. We had 4 specimens of mucinous adenocarcinoma and rest was non-mucinous adenocarcinoma. Adenocarcinoma arranged in tubular, glandular, fashion with invading into muscular layer having nuclear anaplasia. (Photomicrograph No.2).

Mucinous adenocarcinoma showed lakes of mucin pools and tumor cells float in it. Regarding special stains, PAS stain is used to assess presence of neutral mucosubstances. PAS stain for colorectal carcinoma gave mild reaction as focal magenta staining suggestive of presence of few neutral mucosubstances to reduced neutral mucosubstances as compared to normal colon. (Photomicrograph No.4).

PAS- diastase used for confirmation of mucosubstances as diastase dissolves glycogen like carbohydrates but mucins remain unaffected. This gave mild to moderate reaction as magenta color confirms presence of few PAS positive mucins. (Photomicrograph No.6).

PAS-phenyl hydrazine in colon cancers used to prove the presence of neutral mucosubstances, as phenyl hydrazine dissolves neutral mucosubstances only. PAS phenyl hydrazine gave mild to moderate reaction as magenta color confirms few neutral mucosubstances. (Photomicrograph No.8).

For acidic mucin, in all colonic tumors AB-pH 2.5 was carried out, gave strong reaction as blue colour. (Photomicrograph No.10). This is suggestive of presence of both acidic mucins i.e. carboxylated as well as sulphated.

To differentiate between the two, carboxylated (weakly acidic) and sulphated (strong acidic) -- alcian blue pH1 was carried out. AB-pH1 showed mild reaction

as faint blue colour suggestive of few sulphated mucins. (Photomicrograph No.12).

Combined stains as AB-PAS were done to assess all different types of mucin mainly neutral and acidic. AB-PAS stain gave strong reaction with blue and weak reaction as magenta and purple color suggestive of predominantly carboxylated mucin with few amount of neutral mucin with traces of sulphomucin (Photomicrograph No.14).

Aldehyde fuchsin stains only sulphomucins and confirms their presence. AF gave weak reactions as faint purple color suggestive of presence of few sulphated mucins and compared to normal shows reduced or loss of sulphation (Photomicrograph No.16).Only 4 cases of colorectal carcinoma show strong reaction to AF as they were mucinous carcinomas.

AF-AB technique helps to differentiate and confirm carboxylated and sulphated mucins. AF-AB on all 30 specimens of carcinoma colon and rectum gave strong reaction as blue color and weak reaction as purple color suggestive of predominance of carboxylated mucins and traces or few sulphated mucins. (Photomicrograph No.18).

SUMMARY AND CONCLUSIONS

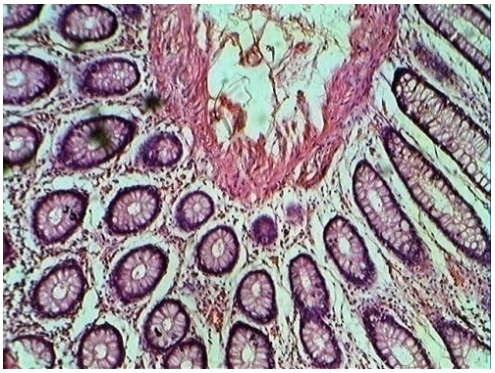
- In the present study, routine and special histochemical methods were applied for comparison of mucins of normal and colorectal carcinoma. H &E used as routine stain for the identification of tissue and diagnosis of colorectal carcinoma.
- Special stains as PAS and PAS-diastase, PAS-phenyl hydrazine were used for confirmation of neutral mucins. Alcian blue pH 2.5 was used to assess acidic mucin. Further categorization of acidic mucins into sulpho and sialomucins was carried out by Alcian blue pH 1 and aldehyde fuchsin.
- Combined stains such as AB-PAS, AF-AB, PAS-phenyl hydrazine were used to differentiate between neural and acidic mucins. The varied heterogenecity of acidic mucins was indicated by a mixture of sulpho and sialomucins.
- In the present study, the mucin histochemistry of normal adult colon revealed mixture of mucosubstances as neutral and acidic mucins with predominance of sulphomucins. Carcinoma of colon and rectum showed predominantly acidic mucins with traces of neutral mucin. In the acidic mucins, predominantly sialomucins were observed as compared to normal colon and rectum.
- In colonic carcinoma, predominantly loss of sulphation was observed i.e. in 26 specimens, whereas 4 cases of mucinous carcinoma showed sulphomucin predominance. Loss of sulphation in colorectal tumors may be due to reaction to neoplasia by various extracellular and intracellular factors as proposed and hypothesized by various scientists. The present study is in concordance with various other studies by Shah & Shrikhande, [11] Subbuswamy SG, [12] Filipe MI [14] and Ganga GM [16] etc.
- Due to recent advances in diagnostic modalities such as IHC (Immunohistochemistry), the mucin histochemistry lags behind as a diagnostic modality now a day. But it is the mucin histochemistry which has opened many doors for the researchers to study tumors' genesis at a molecular level. Recent histochemical advances in

identifying different classes of mucosubstances give more tools which may be usefully applied to solve some kind of diagnostic difficulties.

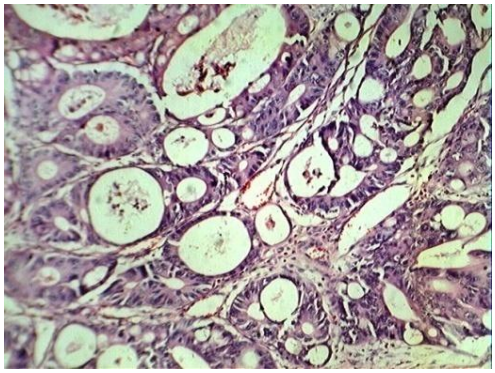
- Mucin histochemistry can effectively determine the mucin profile of colorectal adenocarcinoma. Mucin content and type of mucin present can be regarded as important prognostic indicator and early diagnosis may help in reducing mortality regarding colorectal tumors.

TO CONCLUDE

- Mucin histochemistry may provide a valuable and cost-effective tool for the diagnostic histopathology and for the researchers in histology.

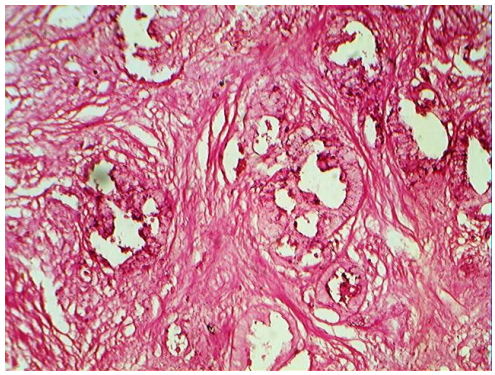


Photomicrograph 1 (H&E 10X)

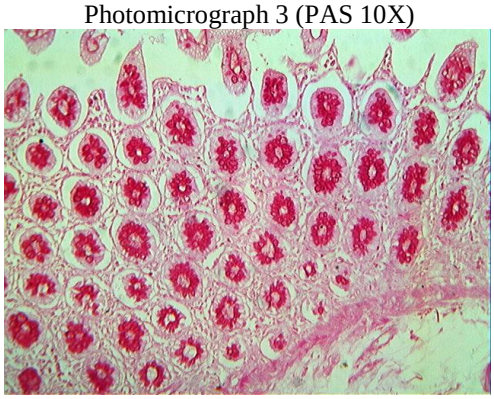


Photomicrograph 2 (H&E 10X)

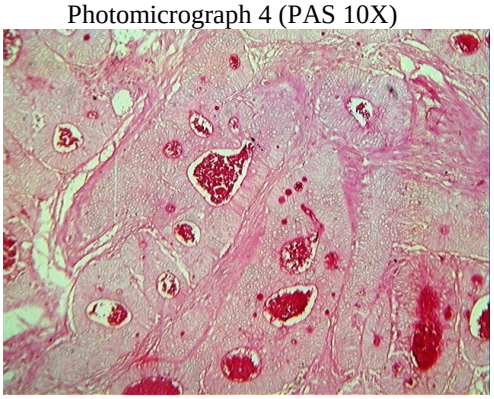
Photomicrograph 3 (PAS 10X)



Photomicrograph 4 (PAS 10X)



Photomicrograph 5 (PAS-D 10X)

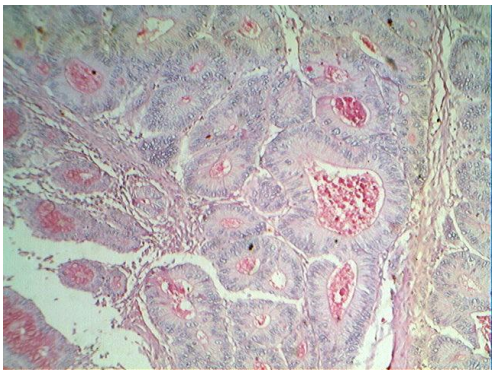


Photomicrograph 6 (PAS-D 10X)

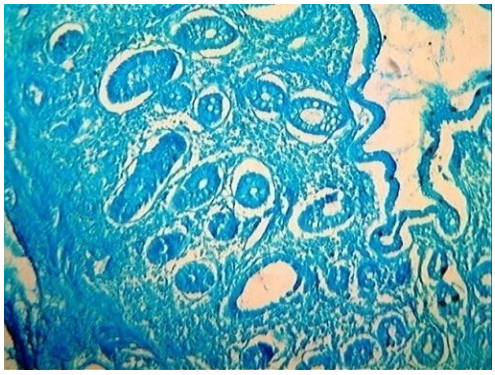
NORMAL COLON

CARCINOMA COLON

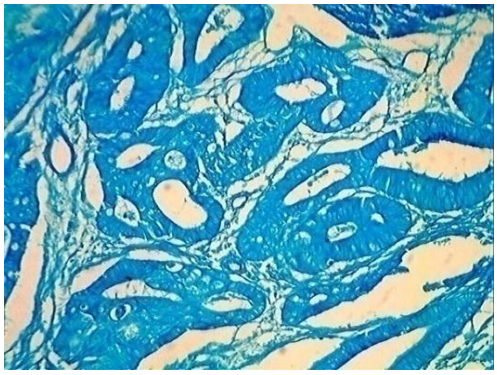
Photomicrograph 7 (PAS-PH 10X)



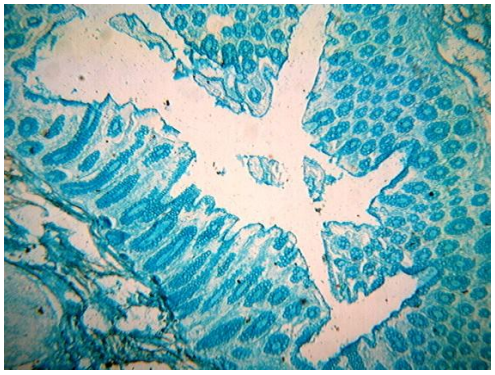
Photomicrograph 8 (PAS-PH 10X)



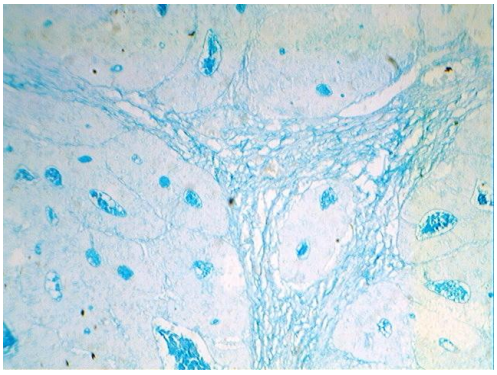
Photomicrograph 9 (AB PH 2.5, 10X)



Photomicrograph 10 (AB PH 2.5, 10X)



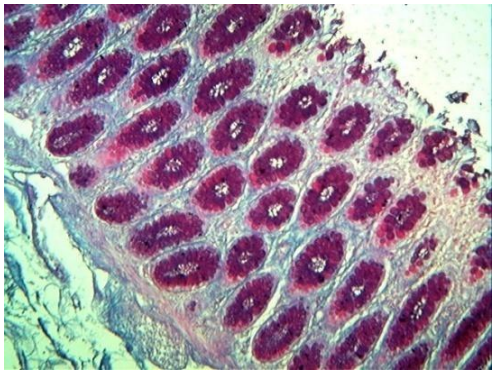
Photomicrograph 11 (AB PH 1, 10X)



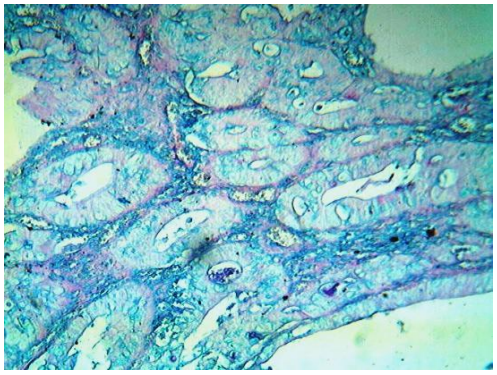
Photomicrograph 12 (AB PH 1, 10X)

NORMAL COLON

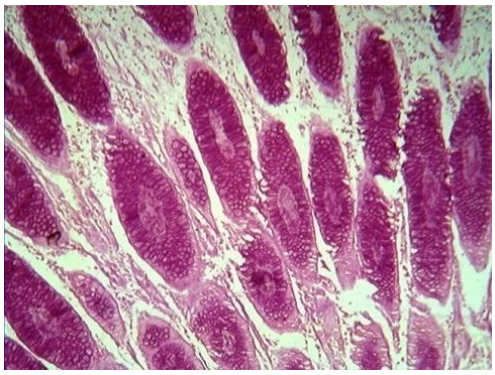
CARCINOMA COLON



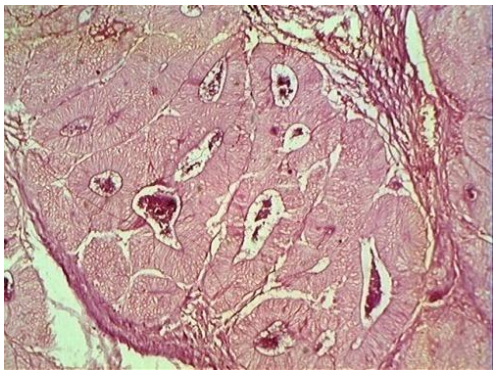
Photomicrograph 13 (AB-PAS, 10X)



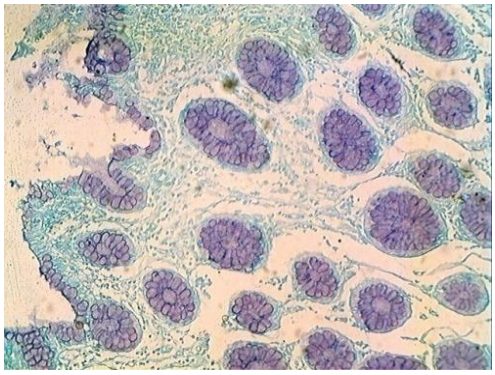
Photomicrograph 14 (AB-PAS, 10X)



Photomicrograph 15 (AF, 10X)

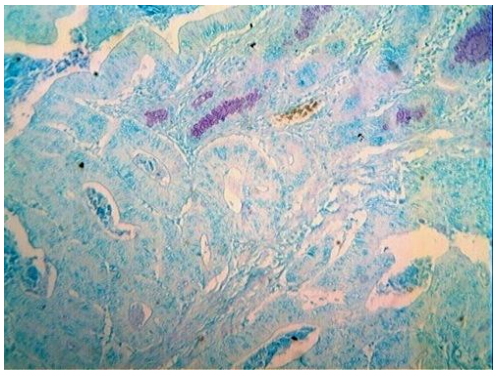


Photomicrograph 16(AF, 10X)



Photomicrograph 17 (AF-AB, 10X)

NORMAL COLON



Photomicrograph 18 (AF-AB, 10X)

CARCINOMA COLON

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ABBREVIATIONS

AB	–	Alcian Blue stain
AB pH1	–	Alcian Blue pH1
AB pH 2.5	–	Alcian Blue Ph 2.5
AF	–	Aldehyde Fuchsin
AF-AB	–	Aldehyde Fuchsin- Alcian Blue
Ca	–	Carcinoma
DPX	–	Distrene Dibutyl phthalate xylene
GIT	–	Gastrointestinal tract
H & E	–	Hematoxylin and Eosin

IgA	–	Immunoglobulin A.
i.e.	–	that is
PAS	–	Periodic Acid Schiff
PAS-D	–	Periodic Acid Schiff- Diastase
PAS-PH	–	Periodic Acid Schiff-Phenyl hydrazine

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