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Study Some Immunological Factor (IL-17, INF-γ ,IL-8, and CA-12) as Predicator in The Diagnosis of Appendicitis

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Abstract:

Appendicitis is the most common surgical condition in all hospitals of the world, so it represents a terrible problem for the community because there was no accurate diagnosis for it, surgery might lead to many complications and the diagnostic methods for appendicitis were significantly not changed over in the past few decades ; therefore, the aims of our study were to identify different cytokine level (IL-17, INF-γ ,IL-8, and CA-125) in serum of patient with suspected appendicitis as predictor for its diagnosis.

The prospective case control study was carried out in Teaching Al-Sadder Hospital in Al- Najaf Government \Iraq, between December, 2018 to April, 2019. A total of (58)samples were enrolled in the study,40 from patients who were admitted to the emergency department with signs and symptoms of appendicitis)group I(&18 samples collected from apparently healthy control (AHC))group II(who were identical to Patients group in (age, gender ,habital,…etc) but not suffered from any symptom of appendicitis

Our result confirmed that all immune markers were significant at (P≤0.05) between the serum of group I and II for IL-17 test (median 210.1pg/ml &52.43pg/ml) respectively (P=0.004)when the cut of value(138.11 pg/ml) , INF-γ test(median36.9&21.04pg/ml)respectively(P=0.01)when the cut of value(46.53pg/ml),for IL-8 test (median 174pg/ml & 31.33pg/ml) respectively (P=0.0116) when the cut of value(81.74pg/ml) , and for CA-125 test (median 10.91u/ml & 6.57 u/ml) respectively (P=0.012) when the cut of value (11.31u/ml).

Our conclusion that the serum concentration of IL-17,IFN-γ, IL-8,and (CA-125)can be used as a predicator for the diagnosis of appendicitis, also The histopathological examination of all appendix specimens showed inflammation in all layers of the tissues characterized by dilatation of blood vessels with sharply increasing in the thickness of walls and its engorged with RBCs, infiltration of the inflammatory cells mainly neutrophils, and reactive hyperplasia.

***Key word**: Appendicitis,Interleukin-17 (IL-17), Interferon Gamma(IFN-γ) ,Interleukin-8 (IL-8), Cancer Antigen-125 (CA – 125).*

Introduction:

Appendicitis was one of the most common causes of inflammatory diseases in the gastrointestinal tract (**1**),the classical sequences of symptoms occurs in only 66% of patients, characterised by pain which started on the per-umbilical abdominal region, usually lasting for 4–6 h ,then the pain becomes somatic, steady, and more severe and aggravated by motion or cough due to the spreads of inflammation to the parietal peritoneal surfaces (2**)**,also another symptoms included loss of appetite, slight fever , diarrhoea or constipation, vomiting and nausea **(3)**.

The anatomical characters of the appendix which represented by small in diameter (blind tube, worm like structure) so in some cases intestinal contents might be trapped at the opening side and cause obstruction (**4** ;**5)**.there were various reasons of obstruction such as fecaliths ,lymphoid hyperplasia , benign or malignant tumours **(6)**, as well as infection like viral **(7)**, bacterial **(8;9;10)** , parasite **(11)** and fungal infection which might occur among immunosuppressed patients **(12),** also appendicitis were more common during the rainy season were the ratio of infection increased(13;14).

The general structure of the appendix are resembled to the rest of the large intestine, which had the same layers that included mucosa, sub-mucosa, muscular externa and serosa but different in that it's smaller in diameter and the mucosa contain many lymphatic follicles that was extend into the sub-mucosa(15).Lamina propria included the lymphoid follicles and another range of cells made the appendix as one part of the Gut Associated Lymphoid Tissue (GALT)(16), M cells were flattened epithelium cell without microvilli and the thick surfaces glycocalyx that present on the rest of mucosal epithelium which had a role in receiving antigen directly from the intestinal lumen instead of circulation (17),Another function could be digestion specially for cellulose with the aid of commensal gut flora that habitat the appendix (the safe house for the commensal gut flora) and could reintroduce it to the intestine in case of disease such as diarrhoea **(18).**

Appendix obstruction lead to the accumulation of appendix secretions would lead to enlargement and pain **,** impaired of blood and lymphatic flow would cause local edema **(3)**,so that appendicular epithelium failed to act as a barrier against commensal bacteria which result in bacterial invasion into the sub-mucosal layers (19(that lead to the initiation of a fast immune response, which involves the activation of Pathogen Recognaition Receptor)PRR) by microbial pathogens and their products (8).also the M cells of the appendix express Major histocompatibility complex (MHC)class II antigen and have deep invagination in the basolateral plasma membrane which were filled with a clusters of B cells, T cells, and macrophages(17,16).

all the previous factors lead to secretion of pro-inflammatory cytokines and antimicrobial peptides (AMP),the expression of AMP stimulated different types of TLRs and NOD-2 depending on the costive agents(20).Then activation of immune defense mechanisms and locally interleukins and chemokine were released to recruit T cells, monocytes, and natural killer cells (19;21)**.**depending on the costive agents that cause naive T cell activation , these cells can be differentiate into one of three effector helper T cell Th1,Th2 &Th17,**t**he differentiation of naive T cells into Th1 cells induced by Interleukin-12 , whereas interleukin-4 induce the differentiation of naive T cells into Th2 cells, While a combination of transforming growth factor β (TGF-β) and interleukin-6 will induced the differentiation of naive T cells into Th17 cells **(22)**.

This study is aimed to Investigate and compare the level of some immunological factor (IL-17, IFN-γ , IL-8, & CA-125 in peripheral blood of patients with appendicitis and apparently healthy control, and identified the significant levels to be used as a diagnostic test for appendicitis, and the Histopathological evaluations of the inflamed appendix to confirm Appendicitis.

Methods

The present study was carried out on(58)samples, which divided in to two groups: group I , included (40) patients who wear suffered from Loss of appetite, constipation , nausea, vomiting and abdominal pain which migrate to the right iliac Fossa , to elucidate the presence of appendicitis , samples were collected one hour before the operation from patients who were attending to Teaching Al-Sadder Hospital in Al-Najaf Government \Iraq, between December, 2018 to April, 2019 ,& (18) samples collected from apparently healthy control (AHC)(group II),that were identical to Patients group but not suffered from any symptom of appendicitis.

A. Serological test

Three ml of blood were collected from both group(I & II) ,then added to serum gel tube (without anticoagulant) ,samples should be allowed to clot for 2 hours at room temperature or overnight at 2-8℃ before centrifugation for 15 min at 1000×rpm at 2-8℃, then collect the supernatant to carry out the assay. Blood collection tubes should be disposable and be non-endotoxin. Finally each serum samples should be divided into four small (200 µl) aliquots and keep at deep freeze (-20°C) until used. All the immune markers (IL-17,IFN-γ,IL-8 &CA-125) were measured in the serum samples by using Enzyme Linked Immuno Sorbent Assay (ELISA(techniques**.**

B. Histopathological Examination:

All Appendix specimens were collected from patients group I at the end of the operations in order for histopathological evaluations of inflamed appendix ,it involved tissue processing which included many steps starting from Fixation by formalin 10% for (3-4)days with Changing the formalin 10% after (12hrs, 24hrs, and 48hrs), then all specimens should cutting in cross and longitudinal section preparing for manually tissue processing, which involved many steps:

First step: dehydration by increasing the concentration of ethyl alcohol (70,80,90 &100%) every 2hrs for each concentration.

Second step: included clearing by Xylene (two times for 2hrs).

Third step: involved infiltration in warm paraffin at 56˚c .

After that all specimens would transport to embedding tissues in paraffin block. In order for making a tissue sectioning by using microtome instrument (Histo-line Laboratory /Italia), all tissues sectioned cut at 5 µm thickness, then put in water bath (FALC /Italia) after that transport on slide .

Finally all slides stained with (Haematoxylin and eosin stain) which involve many steps :

1- Xylene deparafinization (two times for 15 min).
2- Dehydration by decreasing concentrations of ethanol 100%, 90%, 80%, 70% for 5 min for each concentration.
3- Immersion in Haematoxylin stain for 10 min.
4- Discoloration of excess stain by acid alcohol (99 mi of 70% ethanol and 1ml HCl) one dipping.
5- Dipping in eosin stain for 5 min.
6- Increase concentration of ethyl alcohol for dehydration; 70%, 80%, 90%, and 100% every 5 min to each concentration.
7- Mounting with D.P.X. and covered with cover slide .

Finally examined under light microscope (10x).

Statistical Analysis

Data of ELISA techniques were statistically analysed by using Graph pad prisim program version 2015, to find out the median , and made figure that compare between values of patient and AHC depending on the cut off value of AHC. P value were measured by using Mann Whitney test ,when $p \leq 0.05$ & $p \leq 0.01$ were considered statistically significant(19).

Results

The serological result demonstrated that there were significant differences between group I and II as in table (1-1) for IL-17 level in the serum (P=0.004) appendicitis group (median 210.1;range 20.53 -981.08 pg/ml) and AHC)median 52.43; range 13.09-138.11pg/ml), at P=0.001.The cut off value of IL-17 was (138.11 pg/ml).

Also there were significant differences (P=0.01) in the serum levels of IFN-γ in the appendicitis group (median 36.9;range 18.36 -996.47pg/ml) and AHC)median 21.04; range 18.77- 46.53pg/ml) , when P<0.05.The cut off value of IFN-γ was (46.53pg/ml).

The CXCL2 test there were significant differences (p=0.0116) in the serum levels of IL-8 in the appendicitis group(median 174;range 14.21-1996.3pg/ml) and AHC)median 31.33; range 12.59-81.74 pg/ml),when P<0.05.The cut off value of IL-8 was (81.74pg/ml), also our result show that there were significant differences (P=0.012) in the serum levels of CA-125 in the appendicitis group (median 10.91;range 4.6 -119.09 u/ml) and AHC)median 6.57; range 4.14-11.31u/ml),at P≤0.05.The cut off value of CA-125 was (11.31u/ml), for all marker patients with values greater than the cut off value were considered to have appendicitis as in figure (1-1).

Table (1-1): Determination of Serum Cytokine Level in Appendicitis and Control Group

Serum cytokine	Appendicitis group (n=40)		Control group (AHC) (n=18)		Mann Whitney test	
	Median	Range	Median	Range	P value	significant
IL-17 pg/ml	210.1	20.53-981.08	52.43	13.09-138.11	0.004	0.001
IFN-γ pg/ml	36.9	18.36 -996.47	21.04	18.77 - 46.53	0.01	0.05
IL-8 pg/ml	174	14.21-1996.3	31.33	12.59- 81.74	0.0116	0.05
CA-125 u/ml	10.91	4.6 -119.09	6.57	4.14-11.31	0.012	0.05

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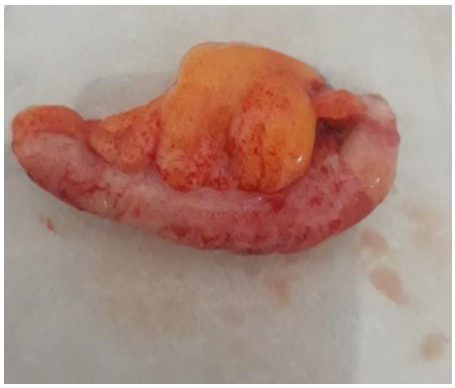


Figure (1): Comparison between Serum IL-17,IFN-γ,IL-8 &CA-125 of Patients and Apparently Healthy Controls, P ≤ 0.05.

Redline represented the cut off value of IL-17,IFN-γ,IL-8 &CA-125 (cut off mean that results below this value were considered "negative" and those above were considered "positive.")& * represent the p value.

While the results of the examination of the histopathological change which include gross examination and microscopic examination of all appendix tissue from group I.The gross examination of the Appendix samples were included the causes of the obstruction (fecalith ,stone, and warm) and macroscopic features of the serosal as in figure (1-2).

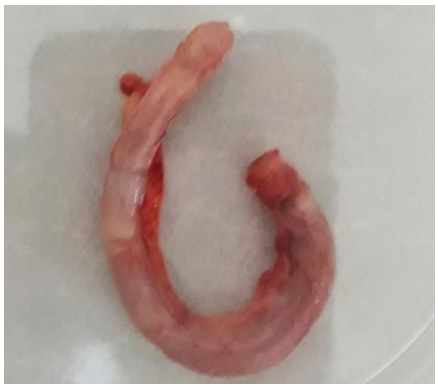


Figure (2) The Macroscopically Feature of Appendix from Patients with Appendicitis. Both pictures showed serosal covering looks hyperaemic, severely inflamed, distended, enlarged and severely congested. The serosa is edematous and loses its glistening appearance. The inflammatory process could extended into the cecum or involve the meso-appendix.

The microscopic examination in the presented study showed that both mucosa and submucosa layers of the appendix has been disappeared with completely blockage of the lumen by fat droplets, fibrous connective tissues and thick-walled blood vessels (chronic obliterative appendicitis) as in figure(1-3). All samples showed reactive hyperplasia as in figure (1-4), infiltration with acute inflammatory cells **as** in figure (1-5),(1-6),while in figure (1-7)our detection that the submucosal area are greatly dilated associated with sharply increasing in the thickness of blood vessel walls and its engorged with RBCs as well, as in figure (1-8).

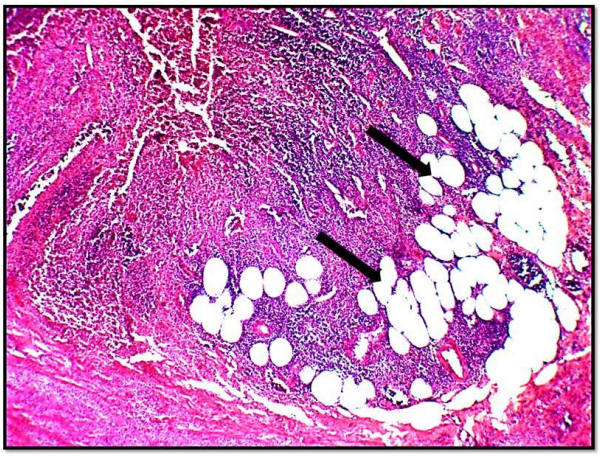
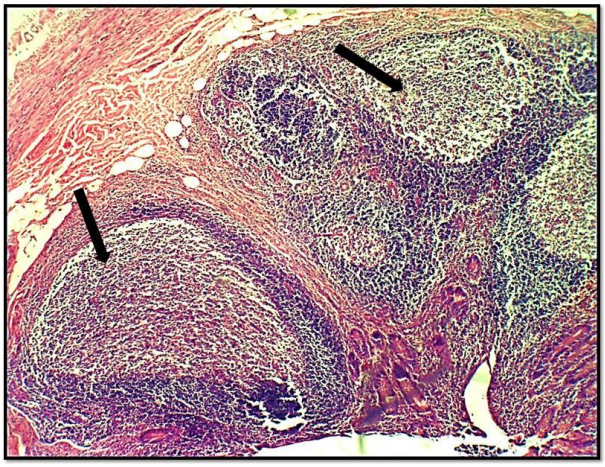
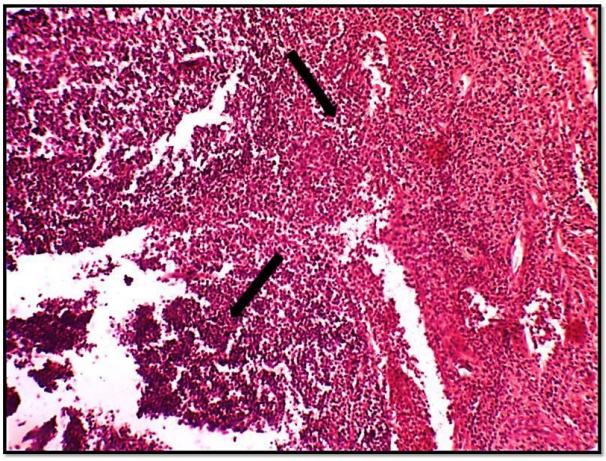


Figure (3) Representative Histopathological Section of Appendix.H&E stained from the section of appendix shows both mucosa and submucosa of the appendix has been disappeared with completely blockage to the lumen of the appendix by fat droplets, fibrous connective tissues and thick-walled blood vessels(chronic obliterative appendicitis).Image was captured using the light microscope at 10X magnified areas.



Figure(4)Representative Histopathological Section of Appendix.H&E stained from the section of appendix ,the pointer indicated to lymphoid follicles are more active and larger than normal (reactive hyperplasia or hyperplastic changes). Image was captured using the light microscope at 10X magnified areas.



Figure(5) Representative Histopathological Section of Appendix. H&E stained from the section of appendix shows suppurative inflammatory processes (sever and purulent). All sections of the appendix are infiltrated with acute inflammatory cells and hyperemia can be seen in some area of tissue. Image was captured using the light microscope at 10X magnified areas

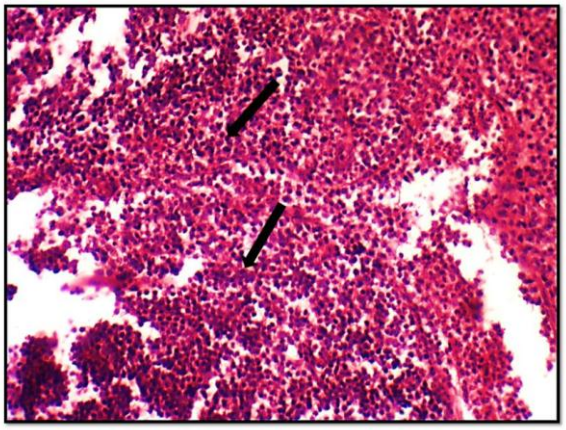
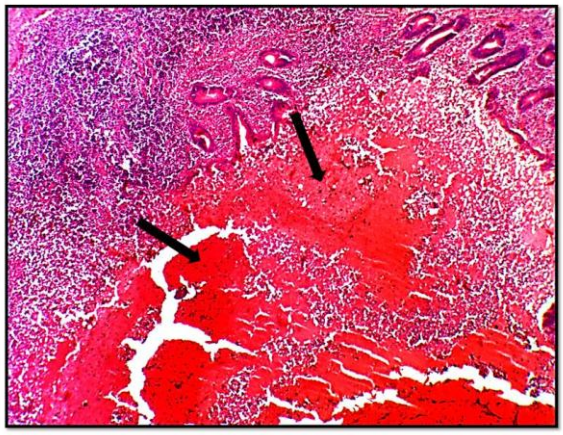
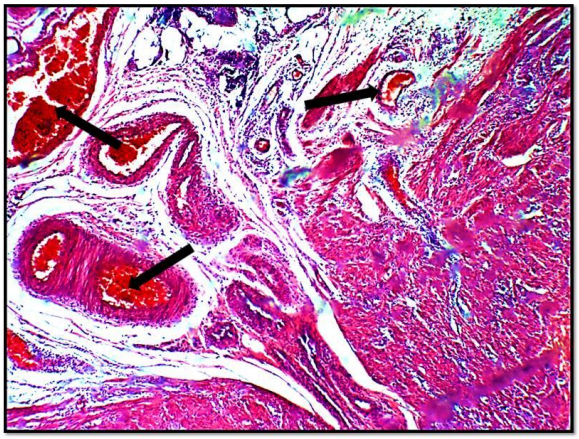


Figure (6). Representative Histopathological Section of Appendix.H&E stained from the section of appendix shows fibrinous exudate due to inflammatory cells infiltration, mainly neutrophils (appendicitis). Image was captured using the light microscope at 10X magnified areas.



Figure(7). Representative Histopathological Section of AppendixH&E stained from the section of appendix , the pointer oriented to the blood vessels in the submucosal area are greatly dilated associated with sharply increasing in the thickness of blood vessel walls and its engorged with RBCs. Image was captured using the light microscope at 10X magnified areas.



Figure(8). Representative Histopathological Section of Appendix. H&E stained from the section of appendix shows sever haemorrhagic changes in the mucosal area of appendix (haemorrhagic appendicitis) with infiltration of the mucosa by large number of polymorph leukocytes. Image was captured using the light microscope at 10X magnified areas.

Discussions

Appendicitis occurs as a result of obstruction of its tip (4) which result in the accumulation of appendix secretions that lead to enlargement and pain **(3)** ,impaired of blood and lymphatic flow would cause local edema ,so that appendicular epithelium failed to act as a barrier against commensal bacteria **(19)** which result in bacterial invasion into the sub-mucosal layers **,**that lead to the initiation of a fast immune response, which involves the activation of Pathogen Recognaition Receptor)PRR) by microbial pathogens and their products (8)**.** All the previous factors lead to secretion of pro-inflammatory cytokines and antimicrobial peptides (AMP),the expression of AMP stimulated different types of TLRs and NOD-2 depending on the

costive agents(20). Then activation of immune defense mechanisms and locally interleukins and chemokine were released to recruit T cells, monocytes, and natural killer cells(19;21)**.**Naive lymphocytes (T cell) could be differentiated into one of three effector helper T cell Th1,Th2 &Th17 T lymphocyte depending on cytokine that caused naive T cell activation**.**Th1 cells induced by Interleukin-12,whereas interleukin-4 induce the differentiation of naive T cells into Th2 cells, While a combination of transforming growth factor β (TGF-β) and interleukin-6 will induced the differentiation of naive T cells into Th17 cells (22).

Interleukin-17 (IL-17)

IL-17 produce from Th17 cells after the activation of naive T cells that have particular important at epithelial and mucosal surfaces **(23)**, its receptors were found on neutrophils , keratinocytes, and other non-lymphoid cells. Most of IL-17 family share the property of operating at the interface of innate and adaptive immunity and serving to coordinate the release of pro-inflammatory and neutrophil-mobilizing cytokines (16).Also human T_H17 cells had distinct migratory capacity ,antigenic specificities & establish a link between microbial products, T helper cell differentiation and homing in response to fungal antigens**(24)**.

After investigation of infection agent the target organ ,recognition occur by PRR2 which lead to triggering of NF-κB and other downstream signals and release of IL-1β, IL-23, and IL-6 that cause activation of naive T cell in to Th17 (25). (26) demonstrated that T_H17 cells could produce different cytokine depending on the causative agent that causes the infection for example in *Candida albicans* infection T_H17 cells produce IL-17 and IFN-γ, but not IL-10, while in *Staphylococcus aureus* infection T_H17 cells produced IL-17 and IL-10. IL-6, IL-23 and IL-1β contributed to T_H17 differentiation induced by both pathogens but IL-1β was essential in *C. albicans*-induced T_H17 differentiation to counteract the inhibitory activity of IL-12 and to prime IL-17/IFN-γ double-producing cells. In addition, IL-1β inhibited IL-10 production in differentiating and in memory T_H17 cells, whereas blockade of IL-1β *in vivo* led to increased IL-10 production by memory T_H17 cells.

Our study suggested that increasing level of IL-17 might be due to appendicitis occur as a result of bacterial or fungible infection because it had an important role in providing protection against infection and in inducing and maintaining chronic inflammatory diseases**(23).**

Interferon Gamma(IFN-γ)

Interferon Gamma(IFN-γ) produced by activated NK cells and T cells after triggering by IL-2&IL-12,the mean effect of IFN-γ were antiviral, immune-regulatory, anti-proliferative (enhance MHC class I and II, macrophage activation)(17)**.** our suggestion that elevated level of IFN-γ might be due to some memory helper T cells produce both IFN-γ and IL-17 , this elevation in appendicitis occurred for many reasons: first as a result of bacterial or fungal infection (23),second : it seems due to the mucosa-associated lactobacilli in human gastrointestinal mucosa could be potent stimulators of IL-12, which in turn cause elevated in IFN-γ (27)**,**third: the *elevation* of both IFN-γ and IL-17 might be because that T cell precursors(CD4+&CD161+) in the thymus in response to IL-1β&IL-23 might give rise to Th1,Th17 and Th17/Th1 which caused the elevation of IL-17& IFN-γ(28) **.**

Our result detected the elevated level of IFN-γ which induced by IL-18 (a member of the IL-1 family)and synergizes with IL-12 to activate T cells and NK cells (29).

IL-18 induced gene expression and synthesis of IL-8 and IL-1 β by Macrophages in Intestinal Lamina Propria cells(CD14) **(30).**

Interleukin-8(IL -8)

IL -8 was a chemokine produced mainly by macrophages and keratinocytes because it was one of the first cells that responded to an antigen, and a potent chemo-attractant for neutrophils and induced degranulation and morphological changes.

In another side our result detected significant elevation of IL-17 which was produce from Th17 , because of the activation of these cells could directly chemoattract neutrophils through the production and release of IL-8, **(31)**. that may be explanation the elevated level of both IL-17 & IL-8.

Cancer Antigen-125 (CA - 125)

CA - 125 was a glycoprotein with molecular weight (225 kDa) which found in the normal epithelium of the female genital tract ,gastric and colonic mucosal cells, and at the luminal surface of mesothelium lining the peritoneum **(32).**(33) explained the increased secretion of CA-125 by Mesothelium cells occurred as a result of one of three activator(rIL-1Jl ,LPS and TNF-α) and stimulated were introduced to the apical or basolateral side it would lead to stimulation of mesothelial cell monolayer to secret CA-125 towards the apical side of the monolayer. Also indicated that CA-125 was preferentially secreted towards the abdominal cavity, and it was likely that CA 125 reached the circulation through lymphatic absorption via the large fenestrae in the diaphragmatic peritoneum.

our suggestion that there were correlation between increased level of CA-125and increased level of IFN-γ , IL-17 concentration might belong to bacterial infection(LPS),because CA-125occurred as a result of LPS activator **(34)**.;therefor,our recommended that In the future another study may carry out on the causative agent that cause the appendicitis and its relationship with the elevation of each type of the immune marker, also multiple researches should be done on measuring of these immune marker in pregnant ,smoker, and patient with UTI, and Sample size must be larger to avoid bias, finally study the possibility of the present of genetic finger (HLA) that may determine susceptibility of persons for appendicitis.

macroscopic examination

Grossly features of serosal appeared hyperaemic, severely inflamed, distended, severely congested , enlarged (edematous) and loses its glistening appearance, in addition the inflammatory process could extend into the cecum or involved the meso-appendix (35).We cannot depend on gross examination alone ,because of misdiagnosis with the other disease ;therefore ,he recommended that microscopic with gross examination were good indicator for appendicitis(36).

microscopic examination

The pathological evaluation was the gold standard method for diagnoses of appendicitis by doing the routine histopathological evaluation which performed to confirm the diagnosis of appendicitis and it might reveal other important pathological details **(37)**.

Appendicitis occurs as a result of Appendiceal luminal obstruction by fecalith, tumours or Enlarged lymphoid follicles as a result of viral or worm infection **(36)** which lead to accumulation of appendix Secretions that result in appendix

enlargement and pain **(3)**,Also impaired of blood and lymphatic flow would cause local edema , Soon the appendicular epithelium fails to act as a barrier against commensal bacteria which result in bacterial invasion into the sub-mucosal layers**(19).**

All samples in our study showed an inflammation of the appendix which were noted by hypertrophy (increasing in the size of lymphoid follicle) which also known as reactive hyperplasia that seems to be agree with **(38,39)**,that might be occurred as a result of viral gastroenteritis or mesenteric adenitis **(40)** while **(41)**demonstrate that lymphoid hyperplasia might occur as a result of bacterial infection, also that would explain increasing in numbers of different types of leukocyte such as lymphocyte and neutrophil.

Also we had severe haemorrhagic changes in the mucosal area of appendix (haemorrhagic appendicitis) with infiltration of the mucosa by large number of polymorph leukocytes , the appearance to be agreed with (42) who explained the presence of focal sloughing of the appendix mucosa with hemorrhage as a result of Erosion of Appendiceal mucosa and demonstrated that Appendiceal hemorrhagic was a very rare cause of lower gastrointestinal bleeding which occur as a result of appendicitis in addition of several pathological conditions such as Crohn's disease.
Our conclusion the result of histopathological examination would prove our immunological result by increasing in the level of different cytokine (IL-17,IFN-γ and IL-8) , which corresponded with **(8)** who concluded were that neutrophil, monocyte and lymphocyte infiltration, that not presented in the normal appendix ,but the time course for the development of these infiltrates was unknown.

Conclusion:

Our conclusion that the serum concentration of IL-17,IFN-γ, IL-8,and Cancer Antigen (CA-125)can be used as a predictor for the diagnosis of appendicitis.

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