INFLUENCE OF ACUTE PANCREATITIS INDUCTION ON ZYMOGEN GRANULES OF PANCREATIC ACINAR CELLS USING IMAGE PROCESSING AND NUMERICAL ANALYSIS APPROACHES

Waslat W. Elshennawy

Department of Biology and Geology, Faculty of Education, Ain Shams University, Roxy, Cairo, Egypt
dr-waslat@hotmail.com

Abstract: Acute pancreatitis (AP) is a mild to severe inflammation of the pancreas with a clinical picture of a self – limited illness that sometimes progresses to a severe state leading to multiple organ failure eventually causing death. The initiating event of AP may be anything that injures the acinar cell and impairs the secretion of zymogen granules which enclose the digestive pro-enzymes. Therefore, the aim of this work was to assess the effect of acute pancreatitis on the ultrastructure of zymogen granules and to analyze this effect using image processing and numerical analysis approaches. **Material and Methods:** Ten male albino rats weighing 150-200g were divided into two groups; group [I] was treated with physiological saline injections *i.p.* as a control group. Acute pancreatitis was induced into group [II] by two injections of 250mg/100g b.w. of L-Arginine *i.p.* in an one hour interval as 20% solution in 0.15M Nacl. Electron micrographs obtained from zymogen granules were examined then were processed with CIS technique for image analysis and histographic analysis. **Results :** Ultrastructural results of zymogen granules of group [II] (rats received L-Arginine) revealed changes with different severity as; depletion, arrest towards the nucleus, dilatation or fusion together, some of them showed an atrophy in size, rupture of membranous boundary and eventually irregular in shape with loss of their rounded configuration and degraded. Obvious varieties appeared in coloured images and numerical values analysis that obtained from microphages of group [II] comparing with those of control group[I]. **Conclusions:** Influence of acute pancreatitis provoked deleterious effects in zymogen granules as consequence of intense inflammation. Despite of current knowledge, many hypothesis and questions remain unanswered concerning the effects of L-Arg. Image processing and numerical analysis which are considered to be valuable approaches in this study, may resolve some of mistiness of the impact of pancreatitis on the exocrine pancreas. Application of this technique gave more details of pathological changes which were unable to be seen by electron microscope only. So, it can be applied as good techniques for early diagnosis in the field of pathology to illustrate the fine details beyond that of electron micrographs.

1. Introduction:

Pancreatitis is a disease with a high mortality and no efficient treatment is available for it at present. Pancreatitis is inflammation of the pancreas, an organ that produces several enzymes to aid in the digestion of food. Pancreatitis may be either acute (sudden and severe) or chronic. Both types of pancreatitis can cause bleeding and tissue death in or around the pancreas. However, long-term damage to the pancreas is common, sometimes leading to malnutrition and diabetes. Necrotizing pancreatitis (in which pancreatic tissue dies) can lead to cyst-like pockets and abscesses (Schulz *et al*., 1999; Urunuela *et al*., 2002). Acute pancreatitis may occur when factors involved in maintaining cellular homeostasis are out of balance. Therefore, several possible causes of pancreatitis such as gallstones and certain drugs, including azathioprine, sulfonamides, corticosteroids, nonsteroidal anti-inflammatory drugs

(NSAIDs), antibiotics such as tetracycline, cisplatin anticancer drug and opiods (Kingsnorth and O'Reilly, 2006). Pancreatic acinar is specialized for the synthesis, storage, and release of digestive enzymes. Only in modeling studies, zymogen granule membrane constituents are likely to be essential for acinar cell function for two reasons. First, protein storing and packaging will, at least to some extent, depend on interactions between content and membrane components. Second, the granule membrane must contain trafficking proteins to ensure functioning of the zymogen granule as a trafficking organelle. The sequence of pancreatic acinar granules formation has been extensively studied (Seong *et al*., 2000; Malatesta *et al*., 2002; Andrzejewska *et al*., 2005). Secretory components are packed in the Golgi complex; the pro-granules bud-off the transcisternae and then fuse to form an immature granule, i.e. the condensing vacuole. The latter changes its

morphology and transforms into a mature electron-dense granule. The steps in granule formation involve massive membrane shuttling, a process which may be expressed in changes in the structure of internal organelles. Finally, mature granules are stored in the apical pole of the acinar cell.

L-arginine is a semi-essential amino acid found in our diet, and it is engaged in several metabolic pathways within the human body. The body converts L-arginine into nitric oxide (NO), which is a powerful vasodilator (relaxer of blood vessels) and increases blood flow. In pancreas, L-arginine is used to release insulin. Recently, there are numerous animal studies reported about L-arginine–induced acute pancreatitis (Czako *et al*., 2000; Tashiro *et al*., 2001; Takacs *et al*., 2002). Most of the authors, who studied the pathomechanisms of induced pancreatitis used 250mg/100g body weight of L-arginine twice at interval of one hour (Takacs *et al*., 1996 ; Varga *et al*., 1997; Czako *et al*., 1998; Hegyi *et al*., 2004). Therefore, this dose of L-arginine was applied in the present study to investigate the influence of acute pancreatitis induction on the zymogen granules of rat pancreatic acinar cells.

On the other hand, image processing is a technique for processing any image, it depends on the fact that the causes of colour in many structures are in response to the structural irregularities (Fortner and Meyer, 1997; Fraser *et al*., 2003; Gendler, 2003; Rector *et al*., 2004). Such use of this property can be considered as the key factor for mapping the way in which the electron beam of TEM interact with the internal structure of organelle to produce the digital image. This digital image was further used for better characterization of the differences in fine structural field. However, the digital image consists of a square array of image elements or pixels; at each pixel, the image brightness was sensed and assigned with an integer value (from 0 to 255 in the case of gray scale image) that was named as the gray-level. For better visualization of the image, the gray-level image is transformed into colour image and converted into hue, saturation and intensity (HIS) using a discoloured technique. The simplest way of obtaining a pseudo colour image from a gray-level image is to use the RGB mode. An RGB colour consists of three individual images exposed through Red, Green and Blue filters, which are eventually combined into a single composite colour image. Note that, the individual RGB images are not in colour. They will still be gray scale images until combine them into the final colour image. This is recognized by many of the popular image processing programs like Photoshop or Paint Shop Pro (Parker, 1997; Sonka *et al*., 1998; Myler, 1999). These programs are excellent tools when you want to crop, resize and perform final

adjustments to your colour images and hence. Also, it can offer both more feasible and practical performance at simple tasks and good implementation, which would be impossible by TEM or other tools alone. However, the purpose of RGB colour model is to facilitate the specification of colours in some standard, generally accepted way, and is the most commonly used model in graphics devices (MacDonald, 1999; Lynch and Livingston, 2001). This model however, allows offering colour range for the pixels from an integer value 0 to 16777215 (number of colours: 256x256x256). This can be used as an additional parameter for identifying the fine details of the differences in the ultrastructure features.

On the light of this, the present study has been carried out to apply image processing and numerical analysis techniques on the TEM images to visualize the coloured images on the nanosize structure and to analyze numerically and histographically the zymogen granules ultrastructure post L-arginine – induced pancreatitis in pancreatic acinar cell.

2. Material and Methods
1. Experimental Animals and Ultrastructural Preparation of Pancreatic Acinar Cells

Ten male albino rats *(Rattus norvegicus)* ranging in weights from 150-200g were acquired from Schistosoma Biological Supply Program (SBSP) Theodor Bilharz, Research Institute, Cairo, Egypt. Housed in clear plastic cages (one rat/cage) with wood chips as bedding and were kept at constant room temperature (25ºC) in a 12h light/dark cycle with free access to pellet rodent diet and water. After one week of acclimatization, the rats were divided into two groups: Group[I] was treated as control and received physiological saline injections *i.p*. In group [II] pancreatitis was induced with 250mg/100g body weight of L-Arginine (Sigma-Tec. El Salam City, Cairo, Egypt, under license of: Merck K GaA, Darmstadt, Germany) by injection *i.p*. twice at an interval of one hour as a 20% solution in 0.15M NaCl. All rats were scarified by decapitation 24h after the second L-Arginine injection.

The pancreas were rapidly excised and were processed for ultrastructural evaluation by electron microscopy as described previously by Dykstra *et al*. (2002) as follows: Freshly excised pancreas were cut into small blocks ($1\times1mm^3$) fixed in cold 4F:1G (i.e. 4% formaldehyde and 1% glutaraldehyde adjusted at pH 2.2) for 24h, and post fixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.3), dehydrated in an ethanolic series culminating in 100% acetone, and infiltrated with epoxide resin. After polymerization overnight at 60ºC, semithin

sections (0.5μm) were stained with 1% toluidine blue in 1% sodium borate and examined with a light microscope. Areas of exocrine acinar cells were selected and the blocks trimmed accordingly. Ulrtathin sections (80-90nm) were cut, placed on 200 mesh copper grids, and stained with uranyl acetate and lead citrate. The grids were examined and photographed using JEOL.JEM-1400EXELECTRON MICROSCOPE at the Central Laboratory of Faculty of Science, Ain Shams University, Cairo, Egypt. The photographs were printed on KODABROMIDE F5s GLOSSY Black and White- Schwarzweib- Kodak.

The determination of pathology was made blind from the electron micrographs that showed the most characteristic changes of L-Arginine–induced pancreatitis in rats of group [II].

2. Computer-assisted Examinations:

The electron micrographs of pancreatic acinar cells of the two groups were visualized and examined by applying Cartographic Information System software (CIS) technique (Shulei and Yufen, 2004). Combination of image processing; numerical analysis; artificial intelligent and expert system with general vision software were used to colourize; analyze and reveal the morphological and ultrastructural alterations in zymogen granules by using Adobe® ImageReady® CS Middle Eastern Version "8".

3. Results

As illustrated in figure (1) of electron micrograph that obtained from group [I], pancreatic acinar cells of control rats are characterized by numerous zymogen granules varying in size, smoothly rounded formations with a homogeneous content of high density and are crowded mainly at the apical region of the cytoplasm towards the lumen. Image analysis of this electron micrograph after being processed by CIS technique reveals that each zymogen granule is blue in colour but in different degrees of blue density starting from deep to light, which reflect that colour image can elucidate the presence of different components in the granule (pro-enzymes) as seen in figure (2), while electron photograph displays only a homogeneous electron density.

Regarding histographic analysis, it is well known in CIS technique as reported by Rector *et al*., 2004 and Shulei and Yufen, 2004, as previously mentioned in material and methods, that the colour which has appeared, is not randomly colour, but it is related to the formation of the structure, i.e. it means that the constituents of the structure is formed of its individual elements in different colours which combine and give the specific colour of the structure.

The histographic analysis is formed of two dimensions (2D); the X and Y axis. In this study, the Y axis represented the amount or the concentration (quantitatively) of the elements or compounds, whereas X axis represented the different elements or compounds of the structure. Therefore, histographical analysis of a magnified zymogen granules that obtained from the coloured image and marked in white dots are coloured in blue having a value of 116.42 and pixelation area is 21210 as clearly shown in figure (3). The arrangement of the peaks of this histogram are green, yellow, red and blue, which may reflect that they are different types of pro-enzyme constituents in zymogen granules.

Ultrastructural results of zymogen granules of group [II] (rats received L-Arginine) demonstrate alterations with different severity as; depletion (i.e. decreased in number, as only ten zymogen granules are seen in the acinar cell), arrest towards the nucleus, dilatation or fusion together, some of them reveal an atrophy in size, rupture and explosion of membranous boundary and eventually irregular in shape with loss of their rounded configuration and degraded as clearly observed in figure (4).It is also noticed, that there are many electron dense particles participated all over the acinar cell, which may be due to a chemical reaction of L-Arginine with the chemical components of the cell.

The ten zymogen granules appear in the coloured image of this electron micrograph with light green colour intermixed with yellowish colouration as shown in figure (5), which is totally different from the colour of the control one. The histographical analysis of each individual granule after marked by dots gave the same arrangement of the peaks as red, green and blue similar to the other granules with a mean value of the same which is 150 and the pixelation area is 120000. The slight difference in number can be neglected as clearly noticed in figures (6-15). To prove that, a histographical analysis of three zymogen granules together (ZG 4,6,8) was applied and the result was in the same manner as obviously seen in figure (16).

These histograms of figures (6-16) insured that the ten fine structures which are seen in figure (4) of the electron micrograph are zymogen granules that were impaired due to the impact of pancreatitis induction.

An interesting observation is seen in these histograms of the ten granules, that the peaks of the three colours appear at the peripheral of the X axis as single lines in concentration intermixed with each others in a massy disturbance manner. These results may explain the hazardous effect of pancreatitis induction on the role of the zymogen granules for assemblage and storage the pro-enzymes in normal

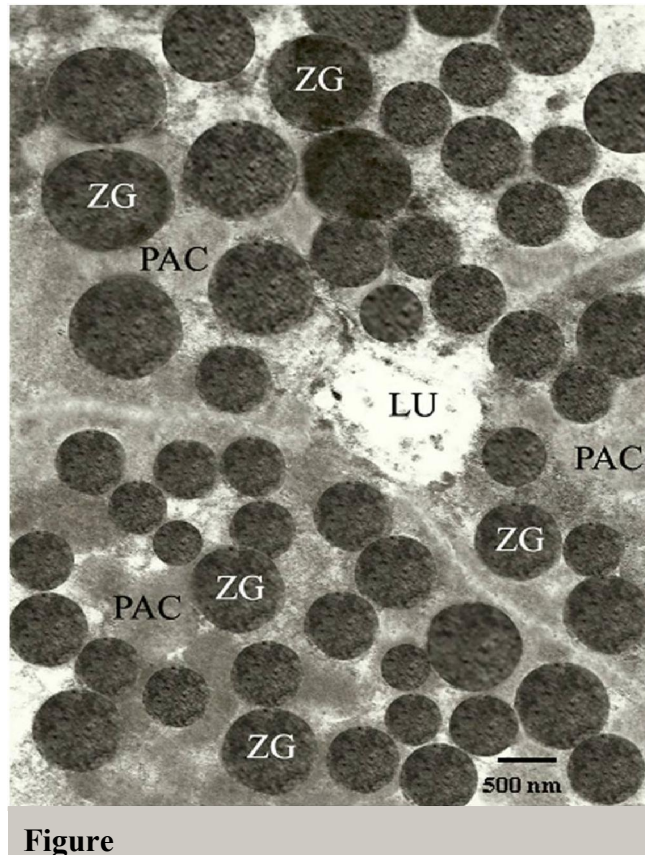way, which reflects the external symptoms of   inflammation.

Figure

Figure (1): An electron micrograph of the apical region of control pancreatic acinar cells **(PAC)**, revealing normal architecture of numerous zymogen granules **(ZG)** varying in size, smoothly rounded formations with a homogeneous content of high density. At the center of the acinus, clear and rounded lumen **(LU)** is located.   **(scale bar 1cm = 500 nm)**
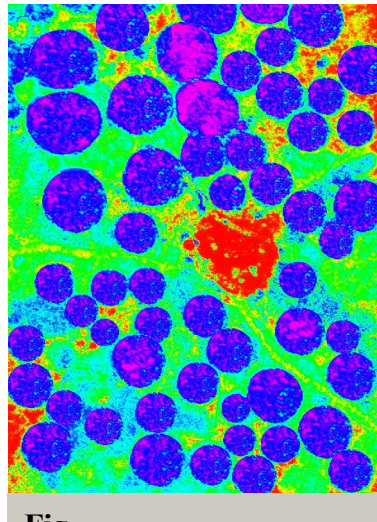

Figure (2) : Coloured image of the previous electron micrograph of control rat designating the blue colouration of zymogen granules with an extend of various density of the colour in each granule.
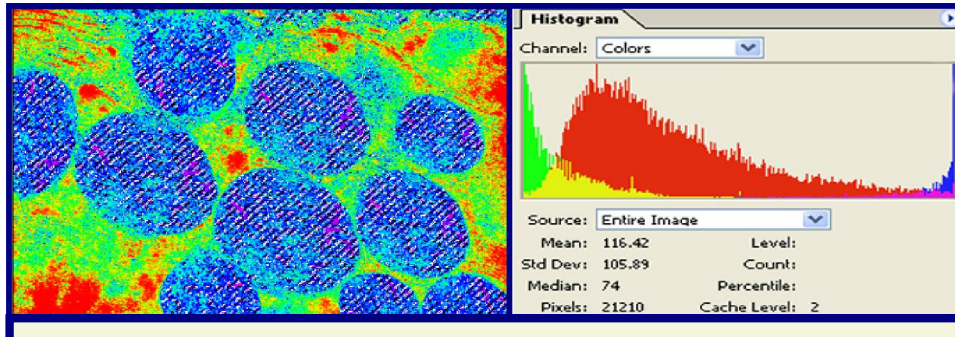
Figure (3): A magnified part of the previous coloured view of control zymogen granules after marked with white dots for histographic analysis, showing the arrangement of the peaks as green, yellow, red and blue with mean value of 116.4 and 21210 pixels.
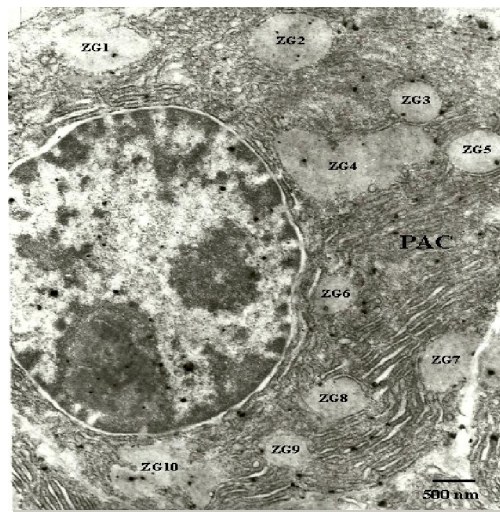

Figure (4) : Electron micrograph of a pancreatic acinar cell (PAC) obtained from group (II) of rats received L-Arginine revealing deleterious changes in zymogen granules with different severity as; arrest at the nuclear region, decrease in number, since only 10 granules are seen (ZG1 – ZG10) which reflect a sort of depletion. Some of them display an atrophy in size (ZG3, ZG6 & ZG9), while some others dilate or fuse together (ZG1, ZG4, ZG7 & ZG8) with loss of their rounded configuration. Also (ZG10) showing rupture and explosion of its membranous boundary. Many dense particles are obviously noticed all over the cell. (scale bar 1cm = 500 nm)
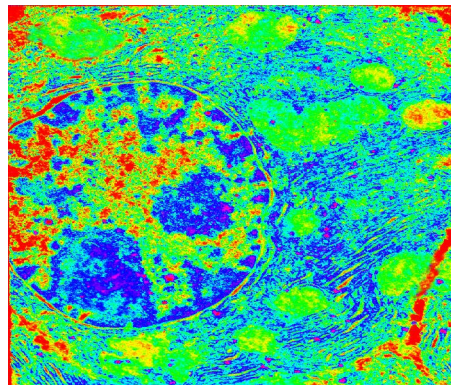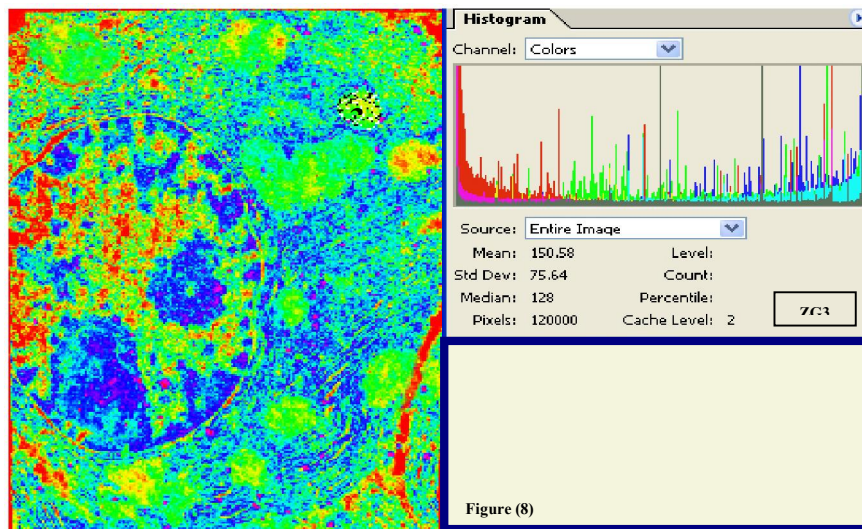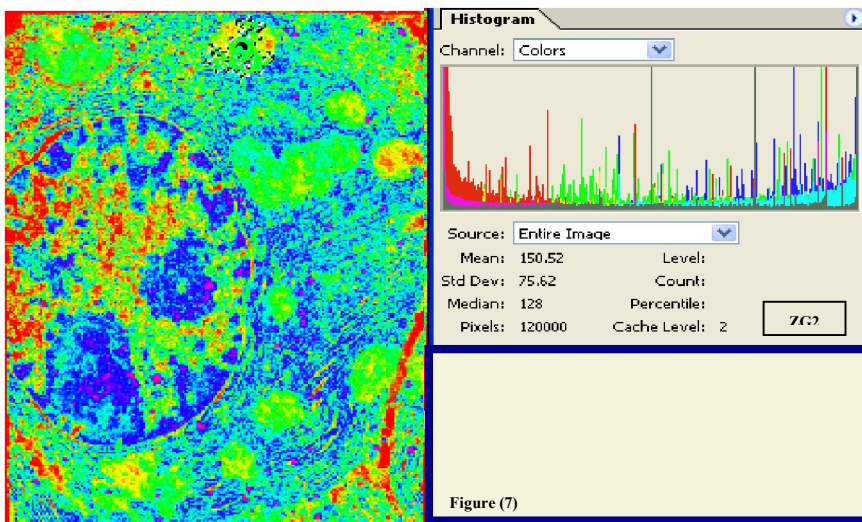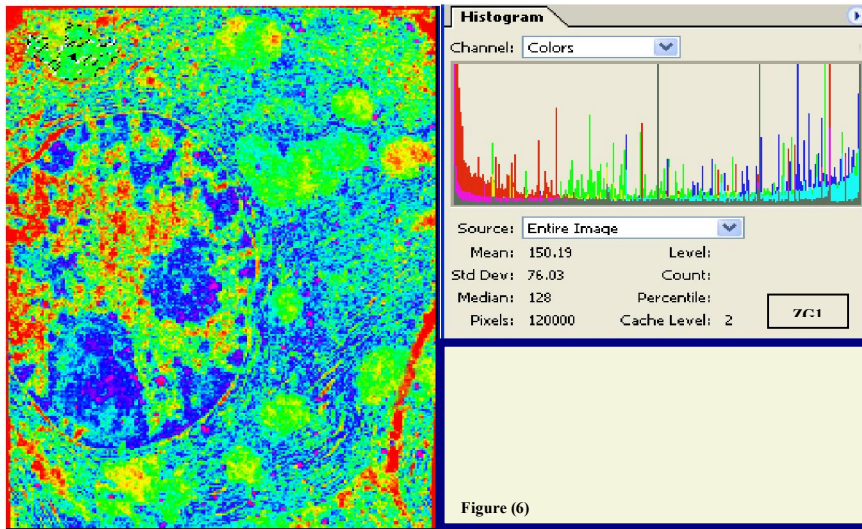

Figure (5): Coloured image of the previous micrograph of L-Arginine group displaying light green colour of the ten zymogen granules intermixed with some yellowish colouration.

Figure (6)

Figure (7)

Figure (8)

Figure (10)

Figure (9)

Figure (11)

Figure (12)
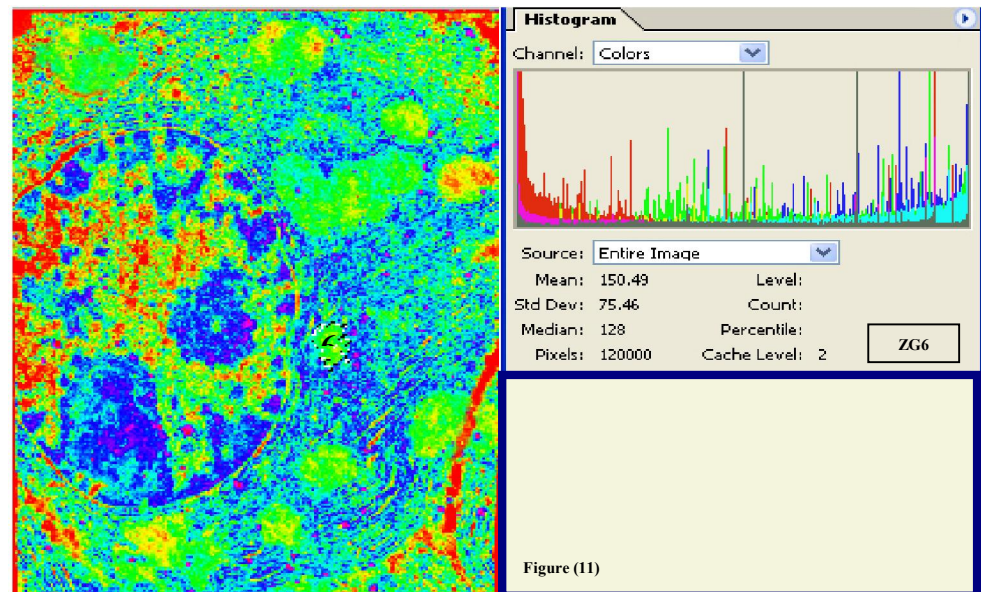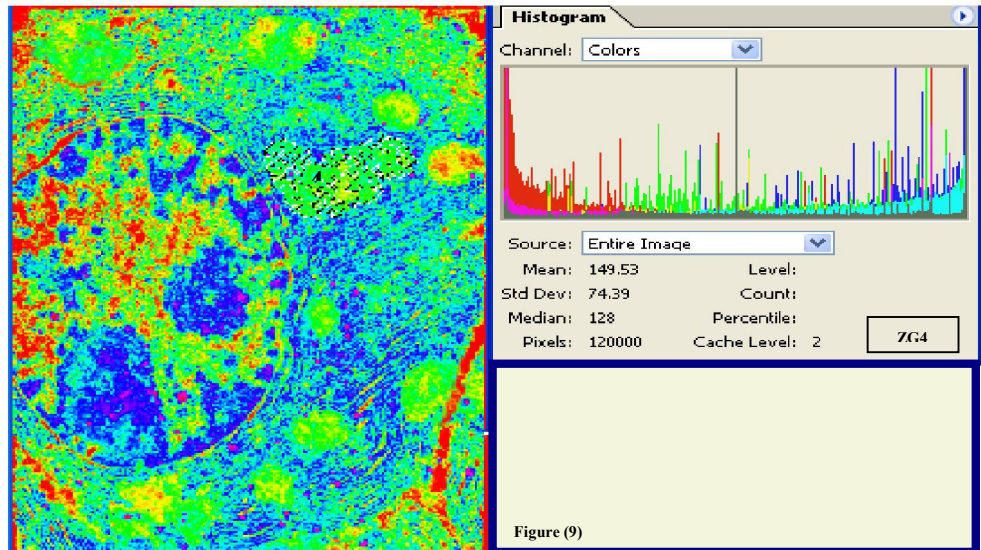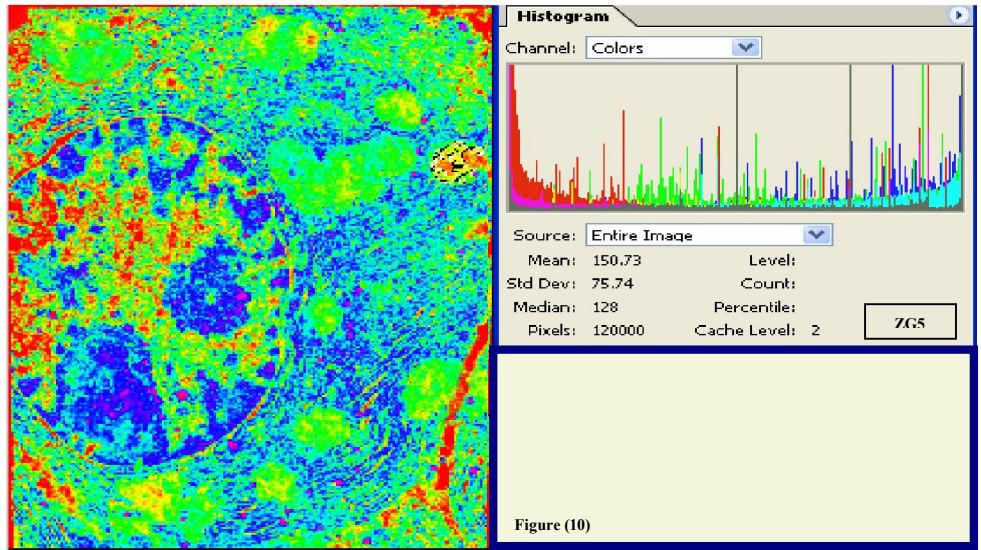
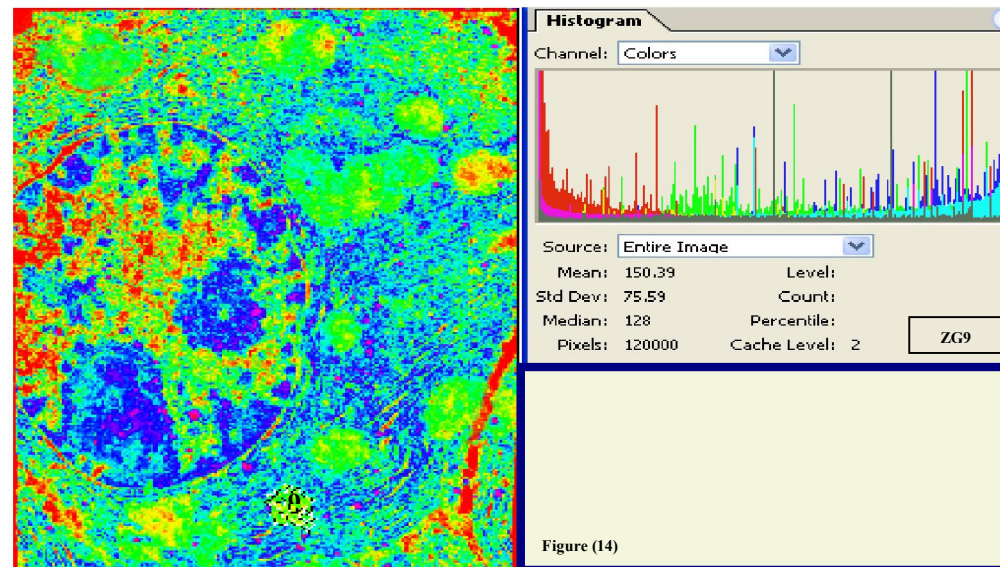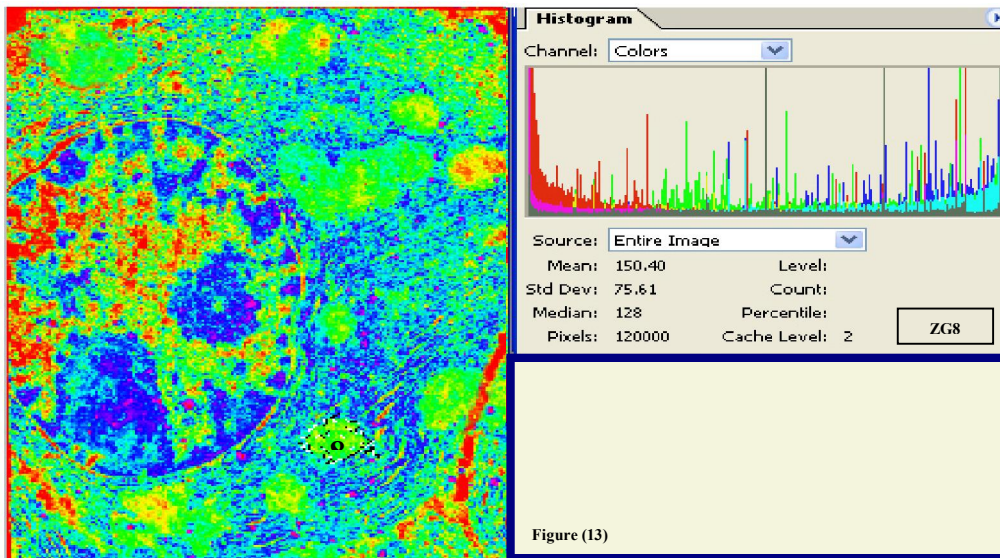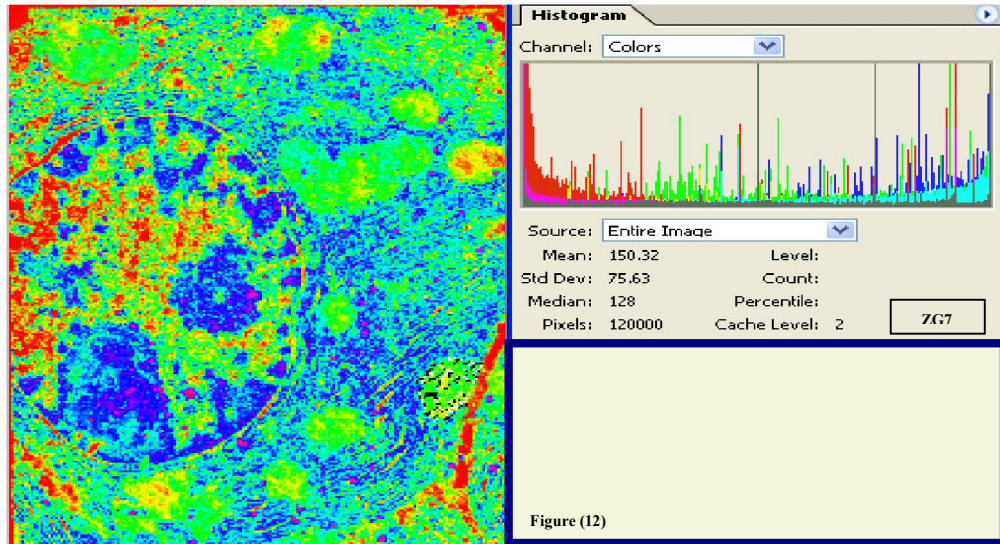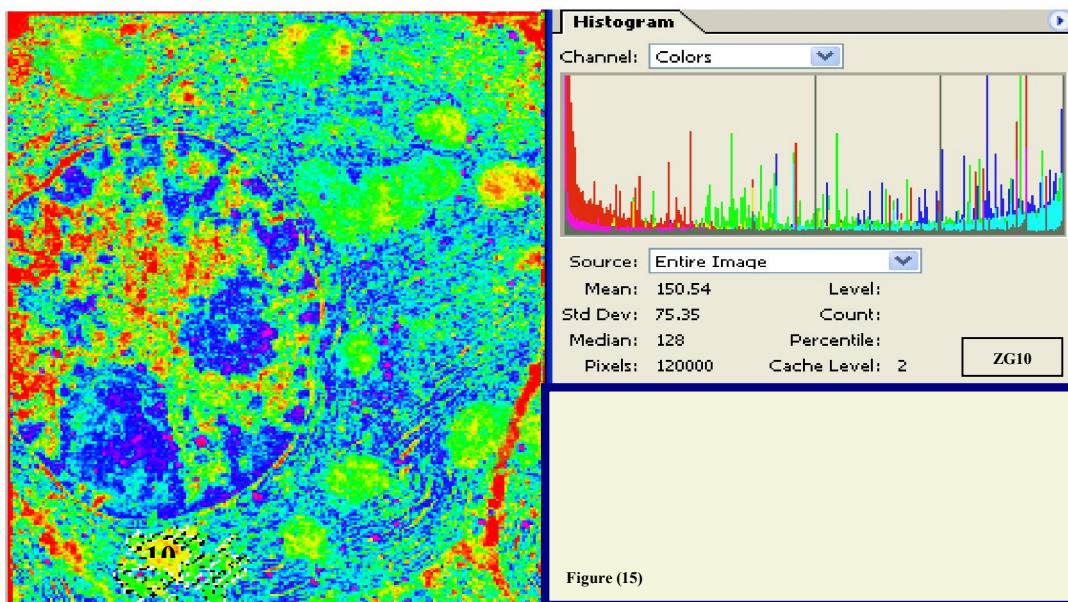Figure (13)

Figure (14)

Figures (6-15): Illustrating histographical analysis of the ten zymogen granules (ZG1 – ZG10) respectively. Each granule in its figure with marked dots give the same results as the others in arrangement of the peaks as red, green and blue with mean value of 150 and 120000 pixels. The slight difference in number can be neglected. Notice, that the three colours in all figures are intermixed in a massy disturbance manner at the peripheral part of X axis.
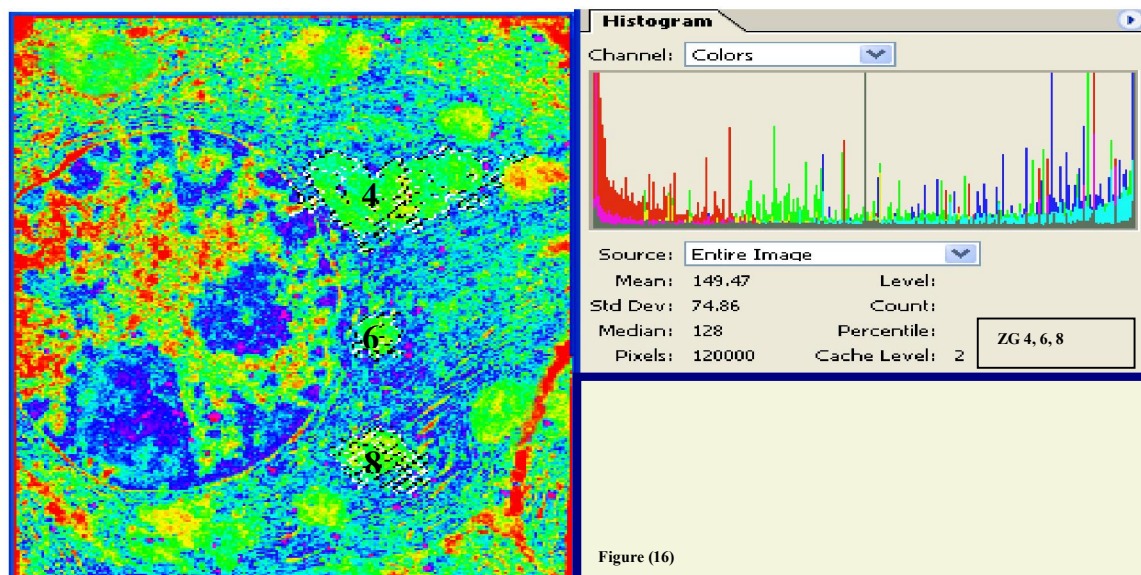

Figure (16) : Displaying the histographical analysis of three zymogen together (ZG4, ZG6 & ZG8). They give the same results of the arrangement peaks and of numerical value as the individual histogram of each one of them.

4. Discussion

Primarily, it should be recalled that the most of the work carried out on pancreatitis have been emphasizing mainly on its medical application together with some physiological and biochemical aspects. A little attention has been paid to investigate the possible impact of pancreatitis on the fine structures of the body organ - in general- and exocrine pancreas - in particular. Despite medical treatment, the lethality of severe acute pancreatitis is still high (20-30%). Therefore, it is very important to characterize the events of this severe disease on the level of the fine structure of the acinar cell, specially zymogen granules which are the main sites of assembling and storage of the digestive enzymes.

L-arginine is considered a good choice for inducing pancreatitis in animal models like rats. Arginine has gained recent attention in critical care nutrition and is considered a conditionally essential amino acid. Arginine is the specific precursor for nitric oxide production and a potent secretagogue for anabolic hormones such as insulin, prolactin, and growth hormone. Under normal conditions, arginine is considered a nonessential amino acid because it is adequately synthesized endogenously via the urea cycle (Saka *et al*., 2004).

L-Arginine – induced pancreatitis is a slowly developing experimental model in which characteristic laboratory changes are observed 24h after induction of the disease. By this time, administration of high doses of L-Arginine can cause severe necrotizing pancreatitis confirmed by the significant elevations in the serum amylase level, as observed by Szabolcs *et al*. (2006). Acinar cell ultrastructure after taurine treatment in rat acute necrotizing pancreatitis was studied by Ates *et al*. (2006) and they found degree of injury in rough and smooth endoplasmic reticulum, Golgi apparatus, mitochondria and nucleus of acinar cells.

On contrary, recently, some authors illustrated that exogenous L-Arginine intake has multiple beneficial pharmacological effects when taken in doses larger than normal dietary consumption, as reported by Gad (2010) who illustrated that L-arginine has a positive role as an anti-aging. Also, El-Demerdash *et al*. (2010) assessed the positive effect of modulation of nitric oxide (NO) on peptic ulcer healing using L-arginine as NO precursor.

On the other hand, many cases had been reported of acute pancreatitis due to different drugs as explained by (Singh *et al*., 2004; Memis *et al*., 2005; Trivedi and Pitchumoni, 2005; Magill *et al*., 2006).

Some authors were concerned with the impact of acute pancreatitis on other organs rather than pancreas, such as Camargo *et al*. (2008) who elucidated that acute pancreatitis provoked deleterious effects in endothelium-dependent relaxing response for Ach in mesenteric rings that were strongly associated with high plasma NO level as consequence of intense inflammatory responses, and they found that the subsensitivity of contractile response to PHE in mesenteric and pulmonary rings might be due to the complications of pathological condition in the early stage of pancreatitis. Moreover, Abd-Hady *et al*. (2010) found that high level of Asymmetric Dimethyl L-arginine (ADMA) as an endogenous inhibitor of nitric oxide (NO) synthase in congestive heart failure, can explain endothelial dysfunction in heart failure in spite of increased total NO production. In addition, the effects of splenectomy on spontaneously chronic pancreatitis in mice with alymphoplasia / alymphoplasia (aly/aly) mutation were studied by Wang *et al*. (2010) and they reported that inflammation and development of the pancreatitis in aly/aly mice were suppressed effectively after splenectomy.

So, it is obvious, on the light of collecting data from these reports that the impact of pancreatitis on the fine structure of zymogen granule did not receive good attention despite of its important role in storage, docking and transient of the digestive enzymes present in pancreatic juice to a specialized plasma membrane structures called porosomes or fusion pores, to discharge its vesicular contents.

In conclusion, the present investigation indicated that pancreatitis provoked deleterious effects in zymogen granules as consequence of intense inflammation. It is therefore, worth mentioning that the present study tried to illuminate new aspects, concentrating on the fine structure of zymogen granules and determinate the pathological changes of acute pancreatitis that affected it by using image processing and numerical analysis techniques as a new method to colourize the control and treated TEM images of zymogen granules and to analyze histographically their numerical values which give good results for detection of pathological changes that occurred in their morphology and their inner fine structure which were unable to be seen in electron micrographs. Therefore, this method may resolve some of mistiness of the impact of pancreatitis on the acinar cells of exocrine pancreas, and it may be applied for early diagnosis in the field of pathology to illustrate the fine details beyond that of electron micrographs.

Corresponding author
Waslat W. Elshennawy
Department of Biology and Geology, Faculty of Education, Ain Shams University, Roxy, Cairo, gypt
dr-waslat@hotmail.com

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6/2/2011