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RESEARCH ARTICLE

Development of Histological Techniques for Metabolic Syndrome Studies

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Abstract

Histology is an important branch in biology studies by studies the microscopic structures of human, animal and plant tissues. By histology, biologist can understand the biological changes that occur in cells by any disses lead to organ dysfunction such as metabolic syndrome. This study evaluates the biological changes that occur in tissue by metabolic syndrome. Haematoxylin and eosin staining was used to determine inflammatory cells and fat deposition in heart and liver. Further, a picrosirius red stain was used to distinguish the collagen deposition in heart. The tissues were taken from rats which fed by either a corn starch–rich (C) or high-carbohydrate, high-fat (H) diet for 16 weeks, and the C or H were supplemented with 0.8 g/kg quercetin diet for 8 weeks as treatment to metabolic syndrome, so the rats groups will be CQ and HQ. Histology is useful in many sectors such as health sector; for example it use hospital to diagnosis the diseases such as tumors and cancers. Also, it use in researches that related to health sector to help a researchers in that see the biological changes in tissue to confirm the results that obtain from the experiment. For instant, in this study by histology we see what the biological effects for quercetin in metabolic syndrome rats model. Therefore, by this way we can confirm that quercetin reduced the symptoms of metabolic syndrome such as gollagen deposition, fat deposition, and infiltrated inflammatory cells in liver and heart or quercetin has not any effect on symptoms of metabolic syndrome

Keywords: Histological techniques, Rats, high-carbohydrate diet, high-fat diet, Quercetin diet, Metabolic syndrome

1. Introduction

By definition, histology is the branch of anatomy that studies microscopic anatomy of cells and tissues of plants and animals. The word 'histology' consists of two parts which are *histo* (meaning tissue) and *logy* (meaning study), so histology means study of tissues in both a structural and functional perspective. In addition, histology is an important method to examine cells and tissues by sectioning and staining, followed by examination under a light microscope or electron microscope (Rolls 2011). Tissue is the fundamental unit that associate to form organs and systems. There are four indispensable tissues in mammal, connective tissue, epithelial tissue, nervous tissue and muscular tissue. Each tissue consists of elementary multicellular components. Therefore, the cell is the fundamental unit in all tissue of animals and plants. Although Marie François Bichat from French (1771-1802) is the first histologist, Marcello Malpighi (1628-1694), an Italian anatomist is considered "Father of Histology". Because Bichat did not use a microscope whereas Malpighi used microscope, and Malpighi described a series of microscopic structures never seen until then;

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for example, he was the first scientist to observe the capillaries. In addition, Malpighi preceded Bichat in a number of years. In 1819, A. Mayer coined Histology term. The first Histology textbook was written by Rudolph von Kölliker (1817-1905), and it was published in 1852.

Histology is useful in many fields such as education, archaeology, researches, diagnosis, forensic investigation, and autopsy. Histology is used in education to teach students about the microstructures of humans, plants and animals biological tissue (Brachtel and Yagi 2012). Furthermore, it is used in archaeology to provide information about ancient history and to distinguishing between human and non-human bone (Crescimanno and Stout 2012). Moreover, it is significant method that used in research to show what the biological changes are that occur in tissues during pathophysiological changes. Also, histology is used in hospitals to diagnose the diseases by taking the biological tissue sample from patient and prepare the sample for histology; then the medical experts can understand the causes of disease, and they can make the recommendation for treatment of the disease (Mukhopadhyay 2011). In addition, histology is used in forensic investigation to clarify the reason of sudden unexpected death and another issue in forensic science, and it is used in autopsy to know about the circumstances and possibly reason of death. Thus, histology is a significant method that people can use in many fields.

Histological stains are used to distinguish certain types of biological structure for tissues and cells, and stains are normally selected according to the type of tissue that histologist want to observed. There are many different types of histology staining such as haematoxylin and, eosin, picrosirius red, Milligan's, oil red "O", toluidine blue, alizarin red, and Bielschowsky stain. Haematoxylin and, eosin and picrosirius red stains were used to show the biological changes which occur by metabolic syndrome in cells.

Picrosirius red stain is used to determine the extent of collagen deposition in selected tissue sections. For instance, high amount of bright red colour in picrosirius red stain indicates high amount of collagen deposition (Panchal et al 2011). Moreover, Haematoxylin and, eosi were used to determine infiltration of inflammatory cells and lipid vacuoles. For example, dark spots surrounding the myocytes refer to inflammatory cells (Panchal et al 2011).

2. Materials and Methods

Note: Due to improper fixing of the tissues at this stage from recent studies, the tissues collected in a previous study were used for this purpose. The method section describes the protocol used for the rats from which the tissues were obtained.

2.1 Animals

All experimental protocols were approved by the University of Southern Queensland Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia. Male Wistar rats (8–9 week old, 330-340 g, n = 12) were obtained from The University of Queensland Biological Resources facility. Rats were randomly divided into 4 groups: corn starch–rich diet-fed rats (C; n = 3), corn starch–rich diet-fed rats treated with quercetin (CQ; 0.8 g/kg food; n = 3; MP Biomedicals), high-carbohydrate, high-fat diet-fed rats (H; n = 3), and high-carbohydrate, high-fat diet-fed rats treated with quercetin (HQ; 0.8 g/kg food; n = 3). The compositions of the diets were previously described in detail (Panchal *et al*., 2011; Poudyal *et al*., 2012). C and H rats were fed with corn starch–rich and high-carbohydrate, high-fat diets, respectively, for 16 weeks. CQ and HQ rats were fed with corn starch–rich and high-carbohydrate, high-fat diets, respectively, for the first 8 weeks and the respective diets were supplemented with quercetin (0.8 g/kg food) for a further 8 weeks. All the rats were individually housed under temperature-controlled, 12-h-light/-dark conditions and consumed food and water *ad libitum* (Panchal *et al*., 2012).

2.2 Terminal experiments and histology

For terminal experiments, rats were euthanized with Lethabarb (pentobarbitone sodium, 100 mg/kg, IP; Virbac). After euthanasia, heparin (200 IU; Sigma-Aldrich Australia, Sydney, New South Wales, Australia) was injected through the right femoral vein. Heart, liver, kidneys, skeletal muscle, pancreas and retroperitoneal fat were stored in 10% neutral buffered formalin for fixing these tissues. The samples were then dehydrated and embedded in paraffin wax. Different staining procedures were used for different tissues to characterise the pathological changes.

2.3 Tissue processing and embedding in wax

Tissues were processed before they were embedded in wax (Table 1) using Thermo Scientific Microm STP 120 Spin Tissue Processor.

Solutions	Time in the solution (minutes)
70% ethanol	60
70% ethanol	60
90% ethanol	45
90% ethanol	60
100% ethanol	60
Xylene	60
Xylene	60
Paraffin	75
Paraffin	75

2.4 Cutting

Tissues were cut into 5mm sections and were placed on slides for staining.

2.5 Staining procedures

There are basic steps in staining and mounting paraffin sections which are dewaxing, hydration, removal of mercury pigments wherever needed, staining, dehydration and clearing, and mounting.

Picrosirius red staining: The slides were put in xylene solution three times for three minutes in each. This step is done to remove the wax completely. Then slides were put alcoholic solutions from higher concentration to lower concentration (100%- 100%- 90%- 70%) for two minutes in each. Then the slides were put in running water for two minutes, and it was put in distilled water for 30 seconds. This step is done to hydrate the sample. After that, the slides were put in phosphomolybdic acid for five minutes then it put in running water again. After that, the slides were put in pisrosirius red (0.1%) for 90 minutes then the slides were transferred to HCl 0.1N for two minutes. Then the slides were put in alcoholic solutions again, but in this time from lower concentration to higher concentration (95%- 100%-100%-100%) for two minutes in each this step is called dehydration and clearing. Then the last step is cover slipping and mounting which coverslips were put on the slides this step is done after 2nd or 3rd xylene. In addition, the following diagram is showing how the staining is done.

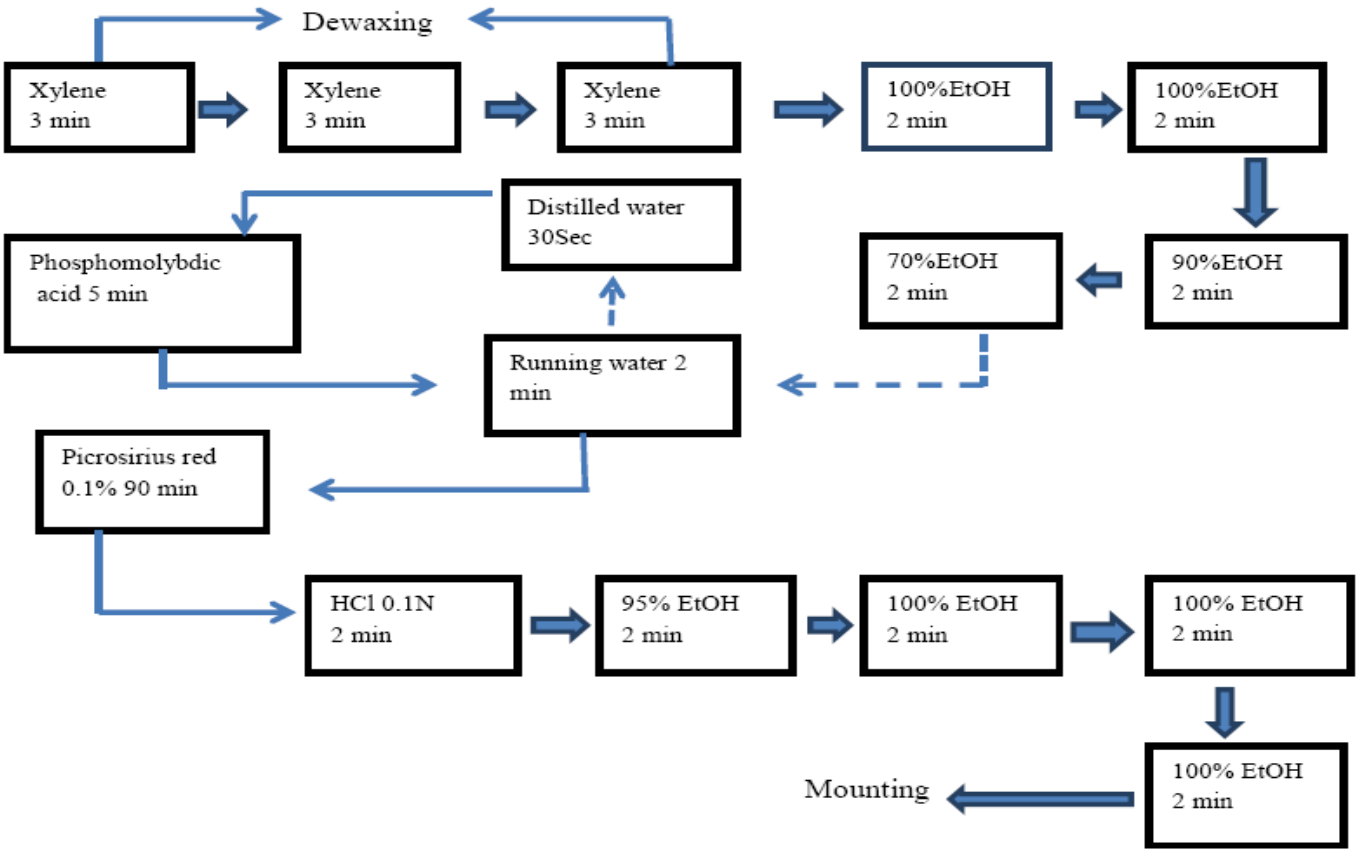

Figure1. showing the protocol of Picrosirius red staining for Heart

Haematoxylin and eosin staining: dewaxing and hydration is done as in pisrosirius red staining. Then the slides were put in haematoxylin staining for six minutes, and it was transferred to running water for two minutes. After that the slides were moved to 70% alcoholic for two minutes. Then the slides were transferred to eosin staining for six to seven minutes. After that the last step is done as above. Also, the following diagram is showing how the staining is done.

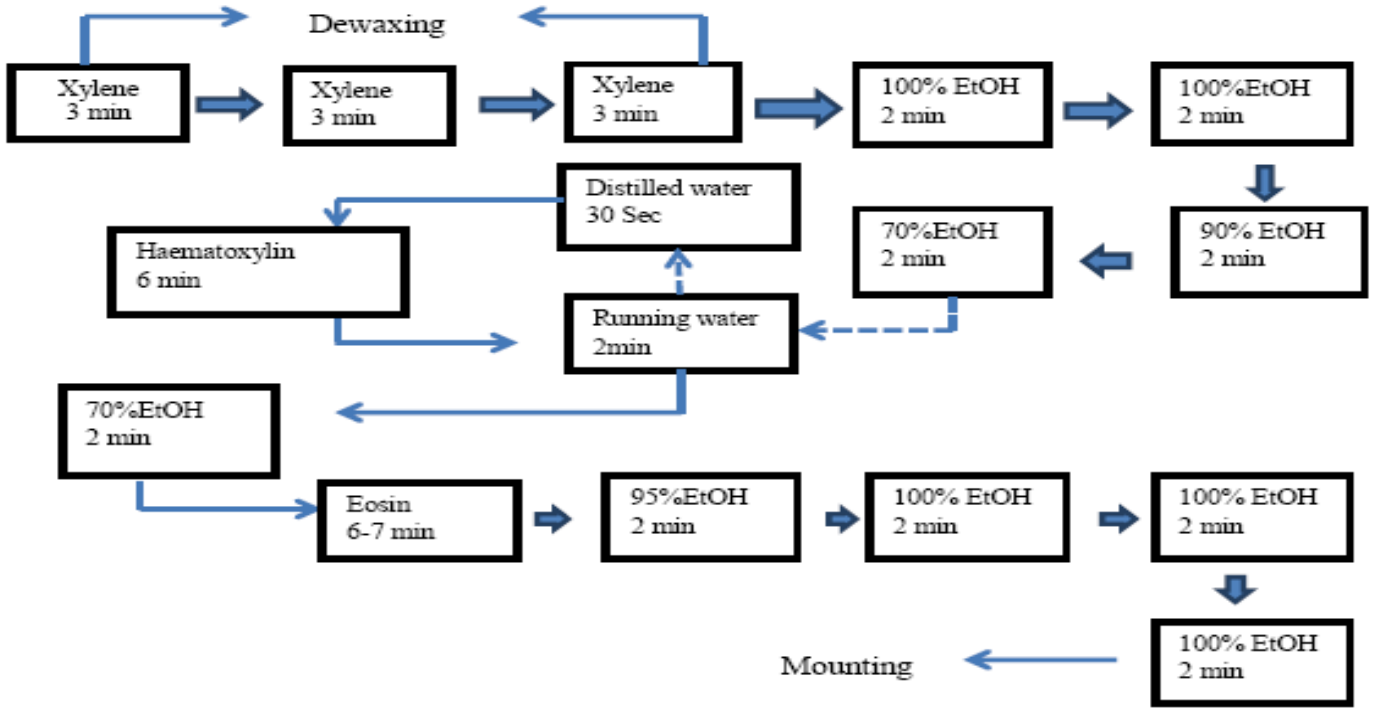

Figure2. showing the protocol of Haematoxylin and Eosin staining for liver and heart

2.6 Imaging

After staining, the slides were allowed to dry and then were used to capture pictures for different section.

3. Result

In Figure 3A-3D, haematoxylin and eosin staining was used to show the inflammatory cells in the heart. The presence of inflammatory cells is marked as “in”. In addition, in figure 3E-3H Picrosirius red staining was used to show the collagen deposition in left ventricular. The presence of fibrosis and hypertrophy are marked as “fi” and “hy”.

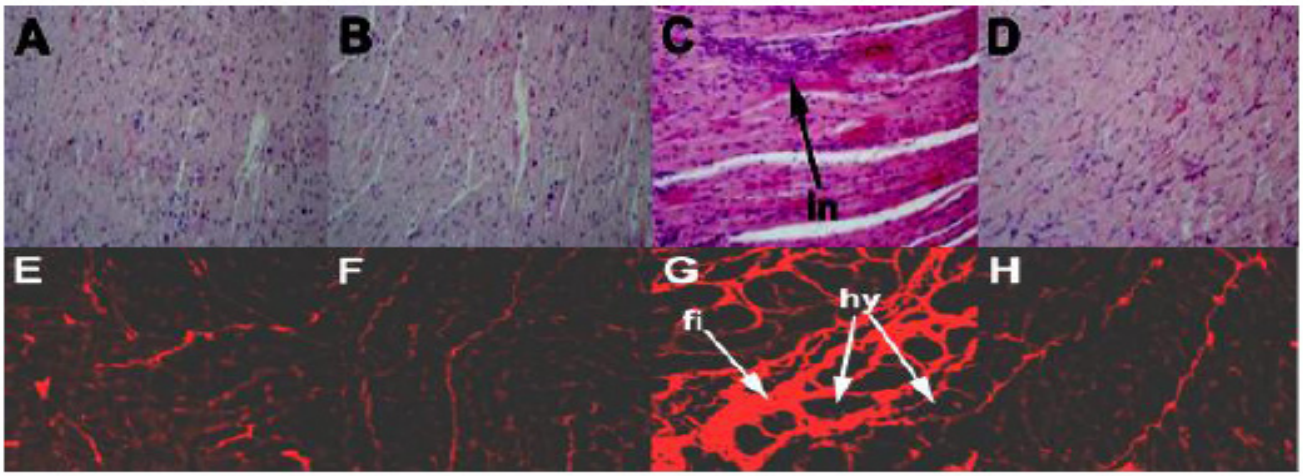

Figure 3. Collagen deposition and inflammation in the heart from rats tissue that mentioned in abstract. Picrosirius red staining is shown that collagen deposition and hypertrophy in left ventricular (E-H, fibrosis marked as “fi” and hypertrophy as “hy”; □40) from C (E), CQ (F), H (G), HQ (H) rats. Hematoxylin and eosin staining is shown that the infiltration of inflammatory cells in left ventricular (A-D, inflammatory cells marked as “in”; □20) from C (A), CQ (B), H (C), and HQ (D) rats..C, corn starch-rich diet-fed rats; CQ, corn starch-rich diet-fed rats treated with quercetin; H, high-carbohydrate, high-fat diet-fed rats; HQ, high-carbohydrate, high-fat diet-fed rats treated with quercetin. (Figure taken form Panchal et al. 2012 The Journal of Nutrition, 142:1026-32)

In Figure 4A-4H, haematoxylin and eosin staining was used to show the inflammatory cells and enlarged fat vacuoles in liver. The presence of inflammatory cells is marked as "in", and the enlarged fat vacuoles are marked as "fv". Furthermore, in figure 4I-4L Milligan's trichrome staining was used to show the collagen deposition in the hepatic portal region. The presence of fibrosis is marked as "fi".

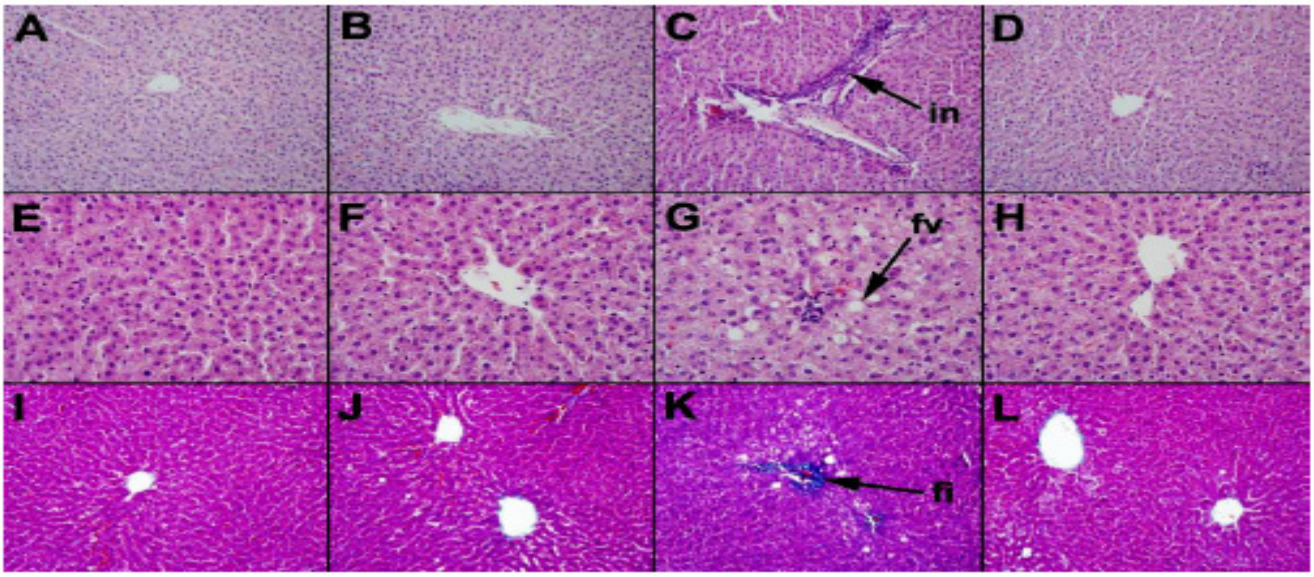

Figure4. Collagen deposition, inflammation, and fat deposition in the liver from rats tissue that mentioned in abstract. Hematoxylin and eosin staining is shown that inflammatory cells (A-D, marked as "in"; ′20) and enlarged fat vacuoles (E-H, marked as "fv"; ′40) in liver from C (*A*,*E*), CQ (*B*,*F*), H (*C*,*G*), and HQ (*D*,*H*) rats. Milligan's trichrome staining is shown that collagen deposition in the hepatic cells (I-L, marked as "fi"; ′20) from C (*I*), CQ (*J*), H (*K*), and HQ (*L*) rats. C, corn starch-rich diet-fed rats; CQ, corn starch-rich diet-fed rats treated with quercetin; H, high-carbohydrate, high-fat diet-fed rats; HQ, high-carbohydrate, high-fat diet-fed rats treated with quercetin. (Figure taken form Panchal et al. 2012 The Journal of Nutrition, 142:1026-32)

4. Discussion

We planned the project about the effects of a small molecule 2-(3,4-dihydro-2H-pyrrolium-1-yl)-3oxoindan-1-olate (DHPO) in the sings & symptoms of metabolic syndrome, so in this study we wanted to do histology for tissues that were collected from C8, H8, and HD12rats (C, corn starch-rich diet-fed rats; H, high-carbohydrate, high-fat diet-fed rats; HD, high-carbohydrate, high-fat diet-fed rats treated with DHPO). However, we could not finish histology for these rats due to improper fixing of the tissues at this stage from this study. Therefore, the tissues collected in a previous study were used for this purpose. Unfortunately, after we did the cutting and staining, we faced another problem which was related to the microscope. Therefore, we have taken the figures from previous study (Panchal et al., 2012).

By using the histology researchers can assist the biological changes that occur in the tissue. In metabolic syndrome, the heart and vascular, liver, kidney, and pancreas will have biological changes (Viscogliosi et al. 2012). Metabolic syndrome induce hypertension, increase infiltration of inflammatory cells, increase collagen deposition, increase diastolic stiffness, left ventricular hypertrophy, decrease fractional shortening and ejection fraction, and decrease contractility in heart and epithelial dysfunction in blood vessels (Panchal et al.2011). Also, metabolic syndrome elevates the infiltration of inflammatory cells and lipid deposition in liver. In kidney, the glomerular and tubular damaged by metabolic syndrome. In addition, the metabolic syndrome elevates the inflammatory cells and the size of islets of Langerhans in pancreas (Panchal et al.2011). Collagen deposition has significance effete on organ function. When, collagen deposition occurs in organ such as heart and liver lead to organ dysfunction. For example, collagen deposition in left ventricle generates fibrosis and stiffness which lead to attenuate contractility in heart. An inflammatory response is a normal defence mechanism, but when it takes prolonged period change to pathological. Therefore, infiltration of inflammatory cells enhances the development of cardiovascular remodelling and hepatitis (Brown et al. 1999) . Fat deposition leads to obesity and increase cholesterol which induce atherosclerosis and impaired glucose tolerance. Liver fibrosis occurs by excessive accumulation of extracellular matrix proteins such as collagen (Bataller et al. 2005). This procedure leads to liver dysfunction.

From the results, the haematoxylin and eosin staining is shown that infiltration inflammatory cells in heart and liver were attenuated in CQ and HQ rats, and it is shown that enlarged fat vacuoles in liver were decreased in CQ and HQ rats. In addition, by using picrosirius red staining is shown that collagen deposition in heart was reduced in CQ and HQ rats. Moreover, Milligan's trichrome staining is shown that collagen deposition in the hepatic portal region was there but it was decreased in CQ and HQ rats. Therefore, quercetin was effective against the symptoms of metabolic syndrome in liver and heart. Due to it attenuate the collagen deposition, infiltrated inflammatory cells, and fat deposition (Panchal et al., 2012). Quercetin was inhibited the Pb-induced kidney inflammation, and it modulate the MAPK and NF-kappa B signaling pathway (Liu et al. 2012) .These results suggest that quercetin is significant component that can used to treat the sings and symptoms of metabolic syndrome.

5. Conclusion

Histology is useful in many sectors such as health sector; for example it use hospital to diagnosis the diseases such as tumors and cancers. Also, it use in researches that related to health sector to help a researchers in that see the biological changes in tissue to confirm the results that obtain from the experiment. For instant, in this study by histology we see what the biological effects for quercetin in metabolic syndrome rats model. Therefore, by this way we can confirm that quercetin reduced the symptoms of metabolic syndrome such as gollagen deposition, fat deposition, and infiltrated inflammatory cells in liver and heart or quercetin has not any effect on symptoms of metabolic syndrome. In this study, we faced some problem that was related to the tissue fixing and microscope, so if the researchers have all histology requirements such as the solution for fixing, stains, and microscope the results will be more accurate, and the histology will be easier.

6. Acknowledgement

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