CHAPTER – 4

4. Histopathological examination of liver, apoptotic and antiapoptotic mRNA transcripts expression in control and experimental rats.

4.1. Introduction.

Electron microscopy is a proven technique in the field of cell biology and a very useful tool in biomedical research. Innovation and improvements in equipment together with the introduction of new technology have allowed us to improve our knowledge of biological tissues, to visualize structures better and both to identify and to locate molecules. Of all the types of microscopy exploited to date, electron microscopy is the one with the most advantageous resolution limit and therefore it is a very efficient technique for deciphering the cell architecture and relating its function. This chapter aims to provide an overview of the most important techniques that we can apply to a biological sample, tissue or cells, to observe it with an electron microscope, from the most conventional to the latest generation. Processes and concepts are defined, and the advantages and disadvantages of each technique are assessed along with the image and information that we can obtain by using each one of them. Histopathological studies used to show architecture and the lobules of neoplastic hepatocytes exhibited focal area of fatty change. Neoplastic cells have vesicular to hyper chromatic nuclei. It also can sinusoidal dialation with cords of neoplastic hepatocytes (Janani et al., 2010).

Apoptosis is a selective and genetically programmed cell death process which occurs and leads to normal cellular differentiation and development. Defects in apoptosis contribute too many diseases, including cancer, autoimmune and neurodegenerative disorders. The process of apoptosis is mediated by a complex mechanism involving intracellular proteases, the caspases, activators and inhibitors of these cell death proteases. The characteristics of apoptosis are blebbing of cytoplasmic membrane, morphological cell shrinkage, nuclear fragmentation and nuclear condensation, DNA cleavage internucleosomes. In liver, apoptosis plays a prominent part in the pathogenesis of toxic liver injury various factors such as chemical agents, viral hepatitis. Apoptosis is initiated by binding of Fas ligand (FasL, CD95L) to Fas, tumor necrosis factor (TNF) to TNF receptors 1 and 2, transforming growth factor b (TGFb) to TGFb receptors, or TNF-related apoptosis inducing ligand (TRAIL) to its receptors.1, 2 Fas and TNF receptor 1
attach to cytosolic adaptor proteins (FADD, MORT, RIP, TRADD), which in turn recruit caspase-8 to activate caspase-3. Caspase-3 (also - known as CPP32, YAMA, and apopain), a cysteine protease, is related to interleukin-1b-converting enzyme and is the human homologue of nematode Caenorhabditis elegans Ced-3.3,4 Caspase-3 gene has been localized to chromosome 4q33–35.1,5,6. The active form of caspase-3 is generated after proteolytic cleavage of the procaspase-3 at specific aspartic acid residues to generate two subunits with molecular mass of 17 and 12 kDa. The two subunits associate with each other to form active caspase-3 complex. Caspase-3 is ubiquitously expressed in human tissue including liver and in human cell lines. Over expression and loss of expression of caspase-3 has been reported in a variety of human cell lines and malignancies. Therefore, the expression of caspase 3 in HCCs and associations with the clinicopathological features were carried out. In the present study, we examined over expression of caspase-3 by immunohistochemistry, immunoblot and functional activity in HCC and adjoining liver parenchyma, and compared over expression status with patient and tumor characteristics. (Rajendra Persad et al., 2004).

Research has indicated that bcl-2 is an inhibitor of apoptosis abnormal c-myc and bcl-2 expression is an important factor in biological behavior of gastric carcinoma and can regulate apoptosis (Li. et al 1997). The expression of bcl-2 increased in cells of gastric cancer. Bcl-2 appears to not only inhibit apoptosis, but the protein is an antagonist of apoptosis mediated by oncogenesis suppressor genes. When the expression of bcl-2 increased, cancer cells would resist the apoptosis induced by chemical drugs or γ-radiation during therapy. when non-classic proliferation occurs in gastric mucosa , the expression of bcl-2 increases significantly. Expression of bcl-2 reached the top at the early stage of gastric cancer and decreased in the progressive gastric cancer. Bcl-2 might do some work both in the triggering of gastric cancer and developing of early gastric cancer. Although bcl-2 was a strong inhibitor to apoptosis, it could not induce the cancer alone. However, cancer has been associated with bcl-2 (Guo Xu et al., 2001).

RNAi-mediated gene silencing using small interfering RNAs (siRNAs) is an effective technique used for varied applications from primary academic research to therapeutic discovery. While phenotypic observations may elucidate the effect of target-specific knockdown on biological systems, silencing efficacy should be confirmed to
ensure confidence in phenotypic results. Efficacy is commonly reported as relative percent knockdown of mRNA levels compared to controls and can be determined in high throughput with easy-to-use, commercially available qPCR gene expression assays (Ross et al., 1997).

4.2. Materials and methods.

4.2.1. Preparation of liver tissue homogenate.

The liver tissues were excised and rinsed in ice-cold saline. Known amount of the tissue were homogenized in 0.1 M TrisHCl buffer, pH 7.4 at 4°C in a Potter–Elvehjem homogenizer with a Teflon pestle at 600 rpm for 3 minutes. The homogenate was centrifuged at 3000 rpm for 10 minutes. The supernatant was collected as tissue homogenate, which was used to assay various parameters.

4.2.2. Histological studies (Kleiner et al., 2005).

The classic paraffin sectioning and haematoxylin eosin staining techniques were used for the histological studies. The various steps involved in the preparation of tissues for histological studies are as follows:

4.2.2.1 Fixation.

In order to avoid tissue by the lysosomal enzymes and to preserve its physical and chemical structure, a bit of tissue from each organ was cut and fixed in bouin’s fluid immediately after removal from the animal body. Bouins fluid, which is the commonly used fixative, was prepared by mixing the following chemicals. 2.5 gm copper acetate, 4gm of picric acid, 10ml of formalin and 1.5ml of glacial acetic acid were added to 100ml with water. The tissues were fixed in bouin’s fluid for about 24 hours. The tissues were then taken and washed in tap water for a day to remove excess of picric acid.

4.2.2.2. Dehydration.

The term dehydration means the removal of water from the tissues by alcohol of varying grades. For dehydration ethanol was used. The tissues were kept in the following solutions for an hour each
Inadequately dehydrated tissues cannot be satisfactorily infiltrated with paraffin. At the same time over dehydration results in making the tissues brittle, which would be difficult for sectioning so the tissues were carefully dehydrated.

4.2.2.3. Clearing.

Dealcoholization or replacement of alcohol from the tissues with a clearing agent is called as clearing. Xylene was used as the clearing agent for one or two hours for two or three times. Since, the clearing agent is miscible with both dehydration and embedding agents, it permits paraffin to infiltrate the tissues. So, the clearing was carried out as the next step after dehydration to permit tissue spaces to be filled with paraffin. The tissues were kept in the clearing agent till they become transparent and impregnated with xylene.

4.2.2.4. Impregnation.

In this process the clearing agent xylene was placed by paraffin wax. The tissues were taken out of xylene and were kept in molten paraffin embedding bath, which consists of metal pots filled with molten wax maintained at about 50 °C. The tissues were given three changes in the molten wax at half an hour intervals.

4.2.2.5. Embedding

The paraffin wax used for embedding should be fresh and heated upto the optimum melting point at about 56°C-58°C. A clear glass plate was smeared with glycerine. L-shaped mould was placed on it to form a rectangular cavity. The molten paraffin wax was poured and air bubbles were removed by using a hot needle. The tissue was placed in the paraffin and oriented with the surface to be sectioned. Then the tissue was pressed gently towards the glass plate to make settle uniformly with a metal pressing rod and allowed the wax to settle and solidity room temperature. The paraffin block was kept in cold water for cooling.
4.2.2.6. Section cutting

Section cutting was done with a rotatory microtome. The excess of paraffin around the tissue was removed by trimming, leaving ½ cm around the tissue. Then the block was attached to the gently heated holder. Additional support was given by some extra wax, which was applied along the sides of the block. Before sectioning, all set screws holding the object holder and knife were hand tightened to avoid vibration. To produce uniform sections, the microtome knife was adjusted to the proper angle in the knife holder with only the cutting edge coming in contact with the paraffin block. The tissue was cut in 7 μm thickness.

4.2.2.7. Flattening and mounting of sections

This was carried out in tissue flotation warm water bath. The sections were spread on a warm water bath after they were detached from the knife with the help of hair brush. Dust free clean slides were coated with egg albumin (not for histochemistry) over the whole surface. Required sections were spread on clean slide and kept at room temperature.

4.2.2.8. Staining

The sections were stained as follows; deparaffinization with xylene two times each for five minutes. Dehydration through descending grades of ethyl alcohol

<table>
<thead>
<tr>
<th>Alcohol Grade</th>
<th>Time</th>
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<tbody>
<tr>
<td>100% alcohol (absolute)</td>
<td>2 minute</td>
</tr>
<tr>
<td>90% alcohol</td>
<td>1 minute</td>
</tr>
<tr>
<td>50% alcohol</td>
<td>1 minute</td>
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Staining with Ehrlich’s haematoxylin for 15-20 minutes. Thoroughly washed in tap water for 10 minutes. Rinsed with distilled water, stained with eosin. Dehydration was done again with ascending grades of alcohol.

<table>
<thead>
<tr>
<th>Alcohol Grade</th>
<th>Time</th>
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<tbody>
<tr>
<td>70% alcohol</td>
<td>2 minute</td>
</tr>
<tr>
<td>90% alcohol</td>
<td>2 minute</td>
</tr>
<tr>
<td>100% alcohol</td>
<td>1 minute</td>
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Cleared with xylene two times, each for about 3 minutes interval.
4.2.2.9. Mounting

On the stained slide, DPX mountant was applied uniformly and microglass cover slides were spread. The slides were observed in Nikon microscope and microphotographs were taken.

4.2.2.10. Ultrastructural Studies

The tissues were dissected out and washed in physiological saline, cut into pieces of desired size and fixed in Bouin-Hallande fixative for 24 hours. After that excess of picric acid was removed and dehydrated in graded series of alcohol. The tissues were infiltrated with molten paraffin at 58-60°C through three changers and finally embedded in paraffin, 3-5 micron thick sections of all the tissues were obtained using a rotary microtome (Weswox, India) and stained in Haematoxylin and Eosin as counter stain. The slides were mounted using DPX mountant

4.2.2.10.1. Ultrastructural studies by Light, transmission electron and scanning electron microscopy.

The colon tissue from control and experimental groups of rats were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 18 hrs. Post fixation was done using 2% osmium tetroxide in 10mM sodium phosphate buffer (pH 7.4) and left over night. Then sections were dehydrated using series of ethanol solutions. Liver sections of 1 μm semi thin sections were cut, picked up on glass slides and stained with toluidine blue for light microscopic examination prior to the final examination. The tissue was embedded in a mixture of (1:1) 1, 2-epoxy propane and Epon (Epikote resin). The tissue was then hardened using dodecyl Succinic Anhydride (DDSA) and Methyl Nodic anhydride (MNA). A diamine catalyst N-benzyl-N diethylamine was used for hardening. The specimen was kept in a block holder and placed in hot air oven at 60°C for 48 h. Ultrathin sections were cut, stained with uranyl acetate and lead nitrate and collected on mesh grids coated with a thin film and viewed in a Philips EM201C transmission electron microscope. Intact liver sample was examined using Philips Scanning Electron Microscopy. The images were taken (15000 magnification) in each animal tissues according to methods described previously (Couteur et al., 2001 and McLean et al., 2003).
4.2.3- Analysis of Gene expression by qPCR and general PCR (Caspse-3, caspase-9, Bel-2 and Bel- XL).

The total RNA was isolated according to the manufacture protocol (RNA lipid tissue mini kit, Qiagen USA). The isolated RNA was estimated using UVS-99 Micro-volume UV/Vis Spectrophotometer-ACT Gene. 1 µg RNA was reverse transcribed using oligo (dT) and III reverse transcriptase is a version of M-MLV RT (superscript III first stand synthesis system for RT-PCR - Invitrogen Life technology). Real-time PCR was performed with an ABI 7500 Real-Time PCR System. Target cDNA levels were determined by SYBR green-based real-time PCR in 20 µL reactions containing 10 µL Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 1 µL cDNA, 1 µL 10 pmole forward (FP) and reverse primers (RP). Caspase3- FP: ATG TCG ATG CAG CTA ACC TC RP: TCC TTT TGC TGT GAT CTT CC, Caspase9 - FP: TCC TGC TTA GAG GAC ACA GG, RP: TGC TCC TTT GAT TTG AGT CC, Bel-2- FP: GAC TCA CTA TAG GCG GGA GAT CGT G, RP: CAC TAT AGA GAA GGG CGT CAG GTG C, Bel-XL- FP: GAG CCA GAT CAT GTT TGA AGC CTT, RP: GGT GAC CGT AAC ACT ACC TGA G. The general PCR (Gene Amp PCR system 9700) was conducted according to the Maxime PCR Mix Kit (i-Taq) manufacture protocol. All the genes expression was normalized against β-actin transcript signal.

4.3. Results.

The appearance of the liver in control animal shows normal morphology (Fig.4.1 A). DEN alone administered animals showing enlargement and several grayish white nodules and foci on the peripheral surface of the liver (Fig.4.1C). Liver shows normal morphology in 1, 3 BPMU alone treated animals (Fig.4.1B). Most of the foci and nodules disappeared in the liver from DEN induced then 1, 3 BPMU treated group of rats compared showing the effect of anti cancer activity like sylimarin treated group of rats (Fig.4.1D and 4.1 E).
Light microscopic examination of the liver cells showed normal architecture in both normal control group (Fig 4.2A) and 1, 3 BPMU supplemented rats (Fig 4.2B). However, cellular damage with malignancy was obvious in the DEN treated liver. The liver showed loss of architecture, neoplastic hepatocytes with large cells, vesicular nuclei and prominent nucleoli. It showed nodular arrangement (Pseudo lobule formation) surrounded by lymphocytic infiltrate (Fig 4.2C). In contrast, DEN with 1,3 BPMU treatment showed nearly normal hepatocytes with lymphocytic infiltration formed around the central vein without disruption of the liver architecture (Fig 4.2D) same like sylimarin treated (Fig 4.2 E).

Fig. 4.1- Morphological observations in control and experimental rats liver.

A- Control, B- 1, 3 BPMU alone treated, C- DEN induced, D-DEN+1,3 BPMU treated, E-Sylimarin treated.
Normal hepatic cells were polyhedral in shape; arranged in chords; they consist of interconnecting plates of hepatocytes that radiate towards a central vein (Fig. 4.3A and 4.3B). In cancer bearing rats fed series hepatic cells appeared unhealthy and damaged. Phagocytes were also found in large numbers as compared to normal group. Blood liver barrier did not appear to be intact. Scattered RBCs were found (Fig 4.3C). In compound fed series apparent decrease of necrosis was evident. Blood liver barrier appeared restitute. Blood vessel was present in its normal shape. Hepatic cells appeared healthy and arranged properly. Fibrosis appeared to decrease in comparison to carcinogen fed series (Fig. 4.3D).

Fig. 4.2-Light microscopic observation of control and experimental group of rats liver.
A-Control, B-1, 3 BPMU alone treated, C- DEN induced, D-DEN+1, 3 BPMU treated, E-Sylimarbin treated.
The transmission electron microscopic examination of hematoxylin and eosin staining of hepatic tissues of control and experimental rats are depicted, the hepatic tissue of control (Fig.4.4A) animals revealed normal architecture and cells with granulated cytoplasm and uniform nuclei. Likewise, the hepatic tissues of rats treated with 1, 3 BPMU alone also revealed an equivalent architecture with sylimarin treated (Fig.4.4B, Fig.4.4.E).

![Fig.4.3. Scanning electron microscopic observation of control and experimental group of rat liver.](image)

A-Control, B-1, 3 BPMU alone treated, C- DEN induced, D-DEN+1,3 BPMU treated, E-Sylimarin treated.

Fig.4.4C depicts the hepatic tissues of HCC induced rats which exhibited distortion in the arrangement of hepatocytes around the central vein, periportal fatty infiltration with focal necrosis of hepatocytes, congestion of sinusoids around central vein regions, granular degeneration, microvesicular vacuolization, focal necrosis and portal tract inflammation. The hepatic section tissues of rats treated with 1, 3 BPMU maintained near normal architecture with fewer neoplastically transformed cells (Fig.4.4D).
Fig-4.4. Transmission electron microscope of control and experimental group of rats liver.  
A-Control, B-1, 3 BPMU treated, C- DEN induced, D-DEN+1,3 BPMU treated, E-Sylimarin treated.

Figure 4.5 shows apoptotic and anti apoptotic gene expression in control and experimental animals. The apoptotic genes such as caspase -3 and caspase-9 expression was down regulated by DEN alone administrations compared with control group of rats. However, DEN induced rats treated with 1, 3BPMU significantly upregulated the caspase -3 and caspase-9 expression compared to the DEN induced group of rats, up regulation of these caspase genes that leads to stimulate the apoptosis. For antiapoptotic genes such as Bcl-2 and Bcl-XL increased the expression in DEN alone treated animals as compared with control group of rats whereas it down regulated by the rats administered with 1, 3BPMU as compared to DEN treated group of rats.
4.4. Discussion

Liver plays a pivotal role in regulation of physiological processes such as metabolism, secretion and storage. Unfortunately, it is a common target for a number of toxicants. The multitude of pathological changes caused by the progression of tumor as well as its inhibition through chemotherapy is expected to be reflected in the biochemical and histological parameters of the host system, particularly pertaining to the liver which is known to be the major organ affected in carcinogenesis. Electron microscopic studies play a prominent role in histological observation that shows the degeneration of hepatocytes ultrastructurally. The depressed granules of glycogen, disturbed mitochondria and disintegration of hepatocytes rough endoplasmic reticulum observed in DEN induced group of rats when compare with control.

Histopathological and transmission electron microscopic investigations were performed for evidence of intracellular morphological changes in experimental rats. The rats treated with DEN exhibited irregular shaped nucleus and cytoplasm. Metastatic stages were seen due to the excessive free radical generation after DEN administration. Control and 1, 3BPMU alone treated rats exhibited a similar kind of
DEN-induced rats showed irregular nuclei, membrane changes with irregular cytoplasm. DEN treatment caused disorganization of hepatocytes, deformation of surface membranes and more population of hepatic cells. Moreover, administration of the 1, 3BPMU after DEN reduced such alteration and maintained the organ quite similar to that of control group of rats. Also, rats treated with 1, 3BPMU show signs of stimulation of apoptosis such as shrunken nucleus, condensed chromatin membrane and formation of apoptotic bodies. This indicates that administration of 1, 3BPMU restores the hepatic cellular architecture and hepatic cellular function through free radical scavenging mechanism and apoptotic mechanism.

Caspases are groups of aspartate specific cysteine protease which regulates the apoptosis induced by a different kind of stimuli (Earnshaw et al., 1999). The caspase-3 is an effector gene that involves in the apoptotic process and caspase-9 is an initiator of caspase-3 in the mitochondria-dependent pathway (Jiang et al., 2005). Generally, the caspases regulate the apoptosis through DNA fragmentation, chromatin condensation and nuclear fragmentation. The caspase -3 activation could be done by upstream protease either caspase -3 in the intrinsic pathway or caspase-8 in the extrinsic pathway in the death receptors (Ryter et al., 2007). In the present study, DEN treated rats show down-regulation of caspase-3 and caspase -9 as compared with control rats. It indicates that the DEN slows down the apoptosis program through down-regulation of caspase-3 and caspase-9 genes, but rats administered with 1, 3BPMU show significantly up-regulated expressions of caspase-3 and caspase-9. This up-regulation of these genes enhances the apoptotic process. Bcl-2 genes are important regulators for cytochrome C release from mitochondria and caspases activation. These groups contain both apoptotic and antiapoptotic genes. The Bcl-XL expressions prevent the mitochondrial cytochrome c release, protecting the cells from apoptosis by inhibiting the availability of cytochrome c in the cytosol (Kharbanda et al., 1997). In our study, DEN up-regulates the Bcl-2 and Bcl-XL mRNA transcripts in experimental rats as compared with control group due to induced cell proliferation and inhibits cell apoptosis; it causes the tumor and cancer, where as in rats treated with 1, 3BPMU, the enzyme significantly down regulates the Bcl-2 and Bcl-XL mRNA transcripts as compared with DEN-induced rats. From these results, it is concluded that the 1, 3 BPMU significantly activates the apoptotic process through up-regulation of apoptotic genes and inhibition of anti-apoptotic genes.