# COMPARATIVE HISTOPATHOLOGY OF ACETAMINOPHEN INDUCED HEPATOTOXICITY IN ANIMAL MODELS OF MICE AND RATS

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## Abstract

Acetaminophen induced hepatotoxicity is one of the most popular and widely used model for evaluating synthetic and natural compounds that are assumed to have hepatoprotective effects. In this study, the hepatotoxicity of acetaminophen has been investigated in animal models of mice and rats. Liver injury was developed with toxic doses of acetaminophen in fasted mice (500 mg/kg) and rats (800 mg/kg). Hepatotoxicity was assessed after 12 hours of acetaminophen intoxication. In both mice and rats, acetaminophen produced considerable histopathological changes that were manifested as extensive hemorrhage, central vein congestion with endothelium disruption, hydropic degeneration, perivenular necrosis of hepatocytes, microvesicular steatosis and nuclear changes as karyolysis, pyknosis and karyorrhexis. These histopathological changes were more marked and exaggerated in mice compared to rats. The results showed that the extent of hepatic necrosis induced by acetaminophen was more severe in mice while rats appeared to be relatively resistant. These inter-species differences in susceptibility of acetaminophen induced hepatotoxicity supports the use of mice as an appropriate and clinically relevant hepatotoxic animal model for testing hepatoprotective agents and to investigate mechanisms of their therapeutic action.

Key words: Acetaminophen, hepatotoxicity, histopathology, animal model, rats, mice

## Introduction

Drug induced liver injury (DILI) is a most common reason for discontinuation of a new drug's development [1]. DILI is commonly classified into intrinsic *vs.* idiosyncratic hepatotoxicity and the latter further into allergic vs. non-allergic. Intrinsic hepatotoxicity is dose dependent and predictable, whereas idiosyncratic hepatotoxicity occurs without obvious dose dependency and in an unpredictable fashion [2]. Most common drugs involved in DILI are non-steroidal anti-inflammatory drugs [3], anti-tuberculosis drugs [4], antibiotics [5], anticonvulsants [6], anesthetics [7] and herbs [8]. Herbs hepatotoxicity has been associated with fulminant, acute and chronic hepatitis, cholestasis, veno-occlusive disease and cirrhosis [9].

Models of liver damage can provide useful tools for studying acute and chronic lesions of liver including necrosis (zonal, massive or diffuse), steatosis, hepatic venular lesions and cirrhosis of several morphological types [10]. The mouse *in-vivo* or primary mouse hepatocytes and the rat model are frequently used for investigation of compounds that are assumed to have hepatoprotective effects. Acetaminophen (APAP) overdose induced hepatotoxicity is one of the most popular and clinically relevant experimental *in-vivo* model as APAP is a dose dependant hepatotoxicant [11]. APAP hepatotoxicity involves increased apoptosis, cyclooxygenase-2 generation, reactive metabolite release and glutathione depletion [12-13].

APAP induced hepatotoxicity is a common consequence of overdose [14]. APAP hepatotoxicity is initiated by metabolic activation of APAP to a reactive metabolite that depletes cellular glutathione and causing the reactive metabolite to covalently bind to cellular proteins which results in increased cytosolic calcium levels and reduce activities of calcium ATPase. Increase uptake of calcium can lead to reduced mitochondrial respiration and ATP synthesis. In addition to reduced cellular ATP levels, mitochondria generate increased amount of superoxide which can react with nitric oxide to form peroxynitrite. In the absence of cellular glutathione, peroxynitrite causes extensive protein oxidation and nitration which may induce further mitochondrial dysfunction and eventually lead to irreversible damage and severe loss of cellular ATP. These events culminate in oncotic necrosis of hepatocytes [15].

In this study a comparison of histopathological changes induced by acetaminophen in the liver of animal models of mice and rats is presented.

## Materials and Methods

### Chemicals

Acetaminophen (Bryon Pharmaceuticals Pvt Ltd), tween 80 (BDH Chemical Ltd*.,* Poole*,* UK*)*, commercial grade ethanol (Khazana sugar mills, Pakistan), xylene (Analytical reagent A3523, Lab-Scan, Ireland), paraffin wax (Bio-Optica, Milano, Italy), 10% neutrally buffered formalin and Harris hematoxylin and eosin stain were prepared according to the procedures of laboratory methods in histotechnology [16].

### Animals

Balb C mice (20-30 gm) and Sprague Dawley rats (150-200 gm) of either sex, maintained in a 12 hr light/dark cycle at 22 ± 2 °C were randomly selected and divided into four groups of six animals each. The animals were kept on fasting overnight before treatment. Experiments on animals were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and according to the rules and ethics set forth by the Ethical Committee of the Department of Pharmacy, University of Peshawar. Approval for the study was granted vides letter number Pharm/EC/446.

### Treatment

Mice and rats were assigned to groups I, II and III, IV respectively. Hepatic injury was induced with either APAP (500 mg/kg body weight, i.p., in 20% tween 80) in mice [17] or APAP (800 mg/kg body weight, i.p., in 1% tween 80) in rats [18]. Groups I and III served as control and were administered with 20% and 1% tween 80 vehicle respectively.

### Histological evaluation

After 12 hours of acetaminophen administration, the animals were killed by cervical dislocation and liver from each animal was removed and fixed immediately in 10% neutrally buffered formalin. The tissues were dehydrated in graded ethanol solutions (50, 70, 80, 90, two changes each of 100%), cleared in two changes each of 100% xylene and were infiltrated and embedded in paraffin wax. Tissue blocks were sectioned at 4 μm on a rotary microtome (SLEE Mainz CUT 5062, Germany) and were stained with Harris hematoxylin and eosin (H & E) for microscopic observation (Labomed Lx400 with digital camera iVu 3100, USA). Histological changes were scored as none (–), mild (+), moderate (++), or

severe (+++) damage.

## Results

### Acetaminophen induced histopathological changes in the liver of mice

Figure 1 (A1 and A2) shows the normal histology of liver after treatment with 20% tween 80 vehicle. The central vein (CV) appeared in the middle of hepatic lobule and was bounded by an intact endothelium. From the central vein, cords of hepatocytes extended toward the periphery of the lobule. The hepatocytes were separated by small sinusoidal spaces (small arrows) in which red blood cells and small number of lymphocytes were visible. These sinusoidal spaces were bounded by a discontinuous endothelium (small arrow). The hepatocytes have intact cell membrane and because of slight glycogen depletion, variation in the eosinophilic appearance of their cytoplasm was evident.

Figure 1 (B1 and B2) shows the major histopathological changes after treatment with acetaminophen. The hepatic lobules showed extensive centrilobular coagulative necrosis with increased eosinophilia. Severe hemorrhage was observed mostly in zone 1 of the hepatic lobule. The sinusoids were dilated and endothelium of the central veins was destroyed. The centrilobular hepatocytes showed severe ballooning degeneration. The sinusoids were heavily congested with red blood cells and lymphocytes. The cell boundaries were ill defined and most nuclei were darkly stained. The amount of heterochromatin increased at the periphery of the nuclei. The nuclei showed extensive karyolysis, pyknosis and karyorrhexis. Microvesicular steatosis was visible throughout the hepatic lobule.

### Acetaminophen induced histopathological changes in the liver of rats

Figure 2 (A1 and A2) shows the normal histological appearance of hepatocytes after treatment with 1% tween 80 vehicle. The central vein appeared in the middle of the hepatic lobule. Normal cords of hepatocytes radiate from the central vein toward the periphery of the lobule. The central vein was bounded by an intact endothelium. The central vein as well as the sinusoidal spaces between the hepatocytes contained numerous red blood cells. These sinusoidal spaces were lined by discontinuous endothelial cells. The hepatocytes have intact cell boundaries and their cytoplasm appeared granular

due to deposition of glycogen. Nuclei having interspersed chromatin material were visible in the middle of hepatocytes.

As shown in figure 2 (B1 and B2), treatment with acetaminophen was associated with extensive hemorrhage throughout the hepatic lobule. The sinusoidal spaces were dilated and were heavily lodged with red blood cells and lymphocytes. Cell boundaries of majority of hepatocytes remained intact however their cytoplasm appeared whitish due to depletion of glycogen. The hepatocytes showed extensive eosinophilia with occasional macrovesicular as well as generalized microvesicular steatosis. Perivenular lymphocytic aggregations appeared throughout the hepatic lobule. The central veins were congested with red blood cells and their endothelium was detached into the lumen. The nuclei showed hyperchromatosis with the presence of pyknosis and karyorrhexis.

### Severity of acetaminophen induced hepatotoxicity in mice and rats

Table 1 shows the severity of hepatotoxicity induced by acetaminophen in mice and rats. Acetaminophen treatment in mice was associated with more exaggerated histopathological changes and includes extensive hemorrhage, central vein congestion with endothelium disruption, hydropic degeneration, perivenular necrosis of hepatocytes, microvesicular steatosis and nuclear changes. In comparison to mice, acetaminophen treatment has only modest effects on the liver morphology in rats other than marked glycogen depletion, hemorrhage, congestion of central veins and lymphoid aggregates in the portal tract. Furthermore, acetaminophen hepatotoxicity in rats was associated with increase lymphocytic infiltration and macrovesicular steatosis. These results showed that the extent of coagulative necrosis of hepatocytes induced by toxic doses of acetaminophen was more severe in mice as compared to rats.

## Discussion

In this study, animal models of acetaminophen induced liver injury were compared in mice and rats. Experimentally induced *in-vitro* and *in-vivo* liver damage models have been widely tested that allows studies of liver diseases [19-21], accidental and industrial toxicity [22-24], studies of hepatic physiology [25-26], pathology [27-28] and regeneration [29-30], development of diagnostic tools [31] and screening of medicinal agents [32-34].

*In-vitro* models include the use of cultured hepatocytes derived from rodent [35-36] or human [37-38], liver slices [39], immortalized cell lines [40-41] and isolated liver [42-43], while the *in-vivo* models used whole animals such as mice [44], rats [45], guinea pigs [46], hamsters [47], minks [48], dogs [49], calves [50], cats [51], pigeons [52], rabbits [53], horses and sheep [54]. These models have their own advantages and disadvantages, for example the use of cultured hepatocytes play an important role especially in the earliest part of the pre-clinical studies but due to genetic instability, limited replicative capacity, short life and donor dependant variation have reduced their applications [55]. The use of small animals such as mice or rats are useful as they are easily managed and present minimal logistical, financial or ethical problems, however, the results obtained from these animals are of limited applicability to human beings due to differences in the anatomical features of the liver and their more rapid metabolic capacity. Similarly, large animals such as pigs, sheep or dogs have anatomical and physiological features similar to those of human beings however; their use is restricted by serious logistical and financial difficulties and is often ethically questionable [56]. Animal models of hepatic injury are the best tools to investigate liver disease processes and to test drugs with the ability to interfere with the hepatic injury process [10].

The most frequently employed hepatotoxins for induction of liver damage include carbon tetrachloride [57], pyrogallol [58], aflatoxins [59], d-galactosamine [60], ethanol [61], thioacetamide [62] anti-tubercular drugs [63] and acetaminophen [15]. Carbon tetrachloride ($CCl_4$) is a classical hepatotoxin, which induces liver cell injury by metabolites that arise from its cytochrome P450-dependent metabolism [64]. Considerable liver injury can be induced by using different doses of $CCl_4$ [65]. D-galactosamine exerts its hepatotoxicity by causing intracellular deficiency of uridine metabolites, presumably in conjunction with other factors such as endotoxinaemia [66]. D-galactosamine induces fulminant liver failure by intraperitoneal injection in doses of 200 mg/kg [67], 400 mg/kg [68], 500 mg/kg [69] and 800 mg/kg [70]. Aflatoxins are well known for their hepatotoxic [71-72] and hepatocarcinogenic effects [73-74]. The formation of highly reactive intermediate by hepatic cytochrome P450 enzyme system which binds to nucleophilic sites in DNA, is regarded as a

critical step in the initiation of aflatoxin B1 induced hepatocarcinogenesis [75]. Thioacetamide is a hepatotoxin which is conventionally used for induction of liver cirrhosis [76-77] and acute hepatic encephalopathy [78-79]. Prolonged oral administration of thioacetamide induces macro liver nodules, liver cell adenomas, cholangiomas and hepatocarcinomas, histologically similar to that caused by viral hepatitis infection [80]. Pyrogallol is a strong generator of free radicals [81] and exhibits hepatotoxicity by causing membrane disintegration and vacuolation with cytoplasmic rarification, necrosis and inflammation [82]. Pyrogallol induced hepatotoxicity can be used as a model to evaluate hepatoprotective agents that have an antioxidant property [58]. Antitubercular drugs particularly isoniazid, pyrazinamide and rifampicin are potential hepatotoxic [83]. The exact mechanism of anti-tubercular drugs induced hepatotoxicity is unknown but metabolism to toxic metabolites are suggested to play a crucial role at least in the case of isoniazid [84]. Evidence of liver injury appears on treatment with isoniazid at a dose of 100 mg/kg for 21 days in rats [85] and combination of isoniazid (50 mg/kg) and rifampicin (100 mg/kg) in mice [86]. Acetaminophen hepatotoxicity is manifested after an overdose that leads to the formation of toxic metabolite N-acetyl-benzoquinoneimine by the cytochrome P450 oxidase system, which causes apoptosis and necrosis by formation of radicals and activation of Kupffer cells [15, 87]. A standardized experimental model of acetaminophen induced liver damage is difficult to devise because of considerable species and age dependant variation in the presence and efficacy of the cytochrome P450 oxidase system of the hepatocytes [56].

The liver is the largest gland in the human body and is a unique organ anatomically located to serve its dual role in metabolic and biochemical transformation reactions. The vulnerability of the liver to injury is a function of its anatomical proximity to the blood supply and digestive tract and to its ability to concentrate and biotransform xenobiotics [10]. Drugs, or their active metabolites, may have a direct toxic effect or induce an immune reaction to cellular proteins. Direct effects lead to predictable, dose dependent toxicity while immune reactions are dose independent, occur rapidly and are associated with hypersensitivity phenomena [1]. Intoxication by acetaminophen is among the most frequent causes of acute liver failure [88] and is widely used as a model of liver damage [89-91].

Clinically fulminant acetaminophen hepatotoxicity is manifested as confluent centrilobular coagulative necrosis, hydropic vacuolization and macrophage infiltration followed by regeneration activity [92-95]. In this study, treatment with an overdose of acetaminophen in mice and rats was associated with extensive centrilobular coagulative necrosis of hepatocytes, severe hemorrhage, destruction of endothelium, dilatation of sinusoids with infiltration of lymphocytes and red blood cells, microvesicular as well as macrovesicular steatosis and glycogen depletion.

The nuclei exhibited chromatin condensation, karyolysis, pyknosis and karyorrhexis. These results are similar to those observed previously in mice [96-97] and rats [98]. Acetaminophen induced histopathological changes started in the centrilobular zone and increased in severity and distribution over time [99]. The elicited ultrastructural changes include proliferation, dilatation and fragmentation of endoplasmic reticulum and Golgi apparatus, giant mitochondria with pleomorphism having paracrystalline inclusions and dense matrical granules, cytosol exhibits vacuolization, glycogen depletion and steatosis while the nucleus showed karyolysis, chromatin condensation/ margination (apoptosis) [92]. Acetaminophen induced liver damage can be a useful model for the study of various histopathological changes including necrosis (zonal, massive or diffuse) [94-95, 100], steatosis [96], cirrhosis and lipofuscin deposition [101], lymphoid aggregation and neoplastic nodules [102].

Necrosis may predominantly involve a particular liver zone because the enzymes involved in drug metabolism are often zonally distributed or because toxicity depends on the oxygen gradient across liver zones. The clinical manifestations of necrosis depend on its extent and duration. Microvesicular steatosis occurs as a consequence of direct toxicity on the mitochondria and their oxidative processes. Macrovesicular steatosis corresponds to triglyceride accumulation, due to defects in lipoprotein metabolism, damage to plasma membrane or increased lipid delivery to hepatocytes consequent on increased synthesis or mobilization. In hepatic venular lesions, there is direct acute or chronic injury to the venular endothelium and zone 3 hepatocytes [1].

Acetaminophen induced hepatotoxicity varies considerably among species. It is generally believed that the major determinant of species differences is the rate of conversion of acetaminophen to a toxic metabolite, *N*-acetyl-*p*-benzoquinoneimine [103]. Therefore, sensitive species form higher amounts of covalently bound adducts and glutathione conjugates of acetaminophen and lose hepatic glutathione more rapidly than resistant species [104]. The interspecies differences in xenobiotic metabolism are due to the absence or presence of a particular reaction unique to a single species, but more often they are a reflection of variations in the relative extents of competing reactions for the compound [105]. Hamsters and mice are most sensitive, while rats, rabbits and guinea-pigs are resistant to acetaminophen induced liver injury [106]. Moreover, the severity of toxicity is increased or decreased by cytochrome P450 inducers or inhibitors respectively [107-108].

The pharmacological activity, metabolism and toxicity of many drugs or xenobiotics often depend on the gender of all strains of rats and some strains of mice [10]. In this study, the severity of acetaminophen induced histopathological changes in liver was compared in mice and rats. The extent of coagulative necrosis was more severe in mice and was exemplified by extensive endothelium disruption lining the central veins, hydropic degeneration and cytolysis of hepatocytes, microvesicular steatosis and karyolysis, pyknosis and karyorrhexis of nuclei. Although the basic integrity of hepatocytes in rats remained intact, however the liver was more vulnerable to glycogen depletion, dilatation of sinusoidal spaces with lymphocytic infiltration, macrovesicular steatosis and perivenular aggregation of lymphocytes after intoxication with acetaminophen.

Miller and others [109] studied liver slices obtained from rat and hamster after intoxication with acetaminophen. They observed that acetaminophen induced discrete damage to the centrilobular region of the liver in hamster but not in rat species. The lack of susceptibility to the hepatotoxic effects of acetaminophen in rats can be explained by metabolic differences. Peter Moldeus [110] studied acetaminophen metabolism and toxicity in isolated hepatocytes from rat and mouse. He observed that only hepatocytes isolated from mouse lost integrity, measured as increased permeability of the cell membranes, upon incubation in the presence of acetaminophen.

Moreover, the rate of glutathione conjugate formation increased about three times in rat hepatocytes however, only half of that is induced in

hepatocytes from control mouse. The increase susceptibility of mouse to acetaminophen induced hepatic injury is due to the rate of *N*-hydroxylation of acetaminophen by the hepatic microsome. An increase in *N*-hydroxylation enhances the need for reduced glutathione, and glutathione depletion in the liver precedes marked increases in covalently bound acetaminophen. Formation of metabolites covalently bound to microsomal proteins and depletion of hepatic glutathione were highest in the mouse but only minor extents of covalent binding and depletion of glutathione were observed in the rat [111]. The rat is protected by a relatively low capacity to metabolically activate acetaminophen to its toxic metabolite [109] as well as a high capacity to clear acetaminophen via the nontoxic pathways of sulfation requiring the availability of 3'-phosphoadenosine 5'-phosphosulfate [112] and glucuronidation due to the involvement of multiple UDP-glucuronosyltransferase isoforms especially 1A7 [113]. Acetaminophen induced liver injury, especially in mice, is an attractive, experimentally convenient and clinically relevant model for evaluating the therapeutic potential of synthetic and natural products purported to be hepatoprotective [11].

## Conclusion

There are a large number of chemical agents and animal models that have been used for the induction of hepatotoxicity of several pathological types. Each of these chemicals and models has significant advantages with respect to specific scientific questions. The animal model of acetaminophen induced hepatic injury is the best and widely used experimental model. In this study acetaminophen intoxication in mice was associated with severe histopathological changes in liver while the rats appeared to be relatively resistant. These findings conclude that the toxicity induced by acetaminophen overdose highly depends on the animal model applied and support the use of acetaminophen induced liver injury in mice as an appropriate model for testing hepatoprotective agents and to investigate mechanisms of their therapeutic action. Further studies should be conducted to improve the validity of this animal model as the mechanisms of acetaminophen hepatotoxicity are extremely complex and the interpretation of *in-vivo* data is difficult.

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**20% Tween 80**          **Acetaminophen (500 mg/kg)**
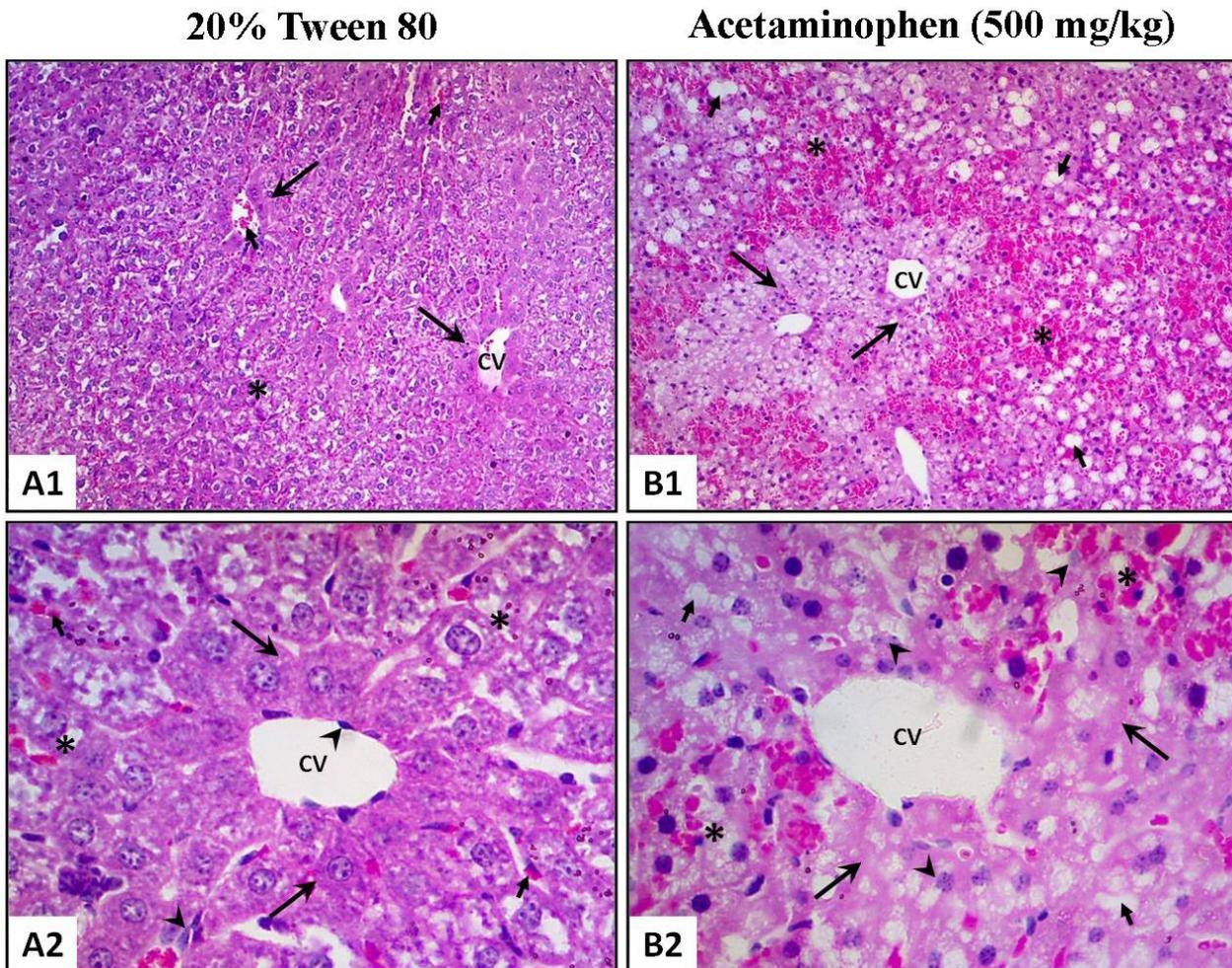


**Figure 1:** Histopathological evaluation of acetaminophen induced hepatotoxicity in mice (H & E; A1, B1; 100x and A2, B2; 400x original magnification). (A1 and A2): Photomicrograph of a section of liver from a mouse treated with 20% tween 80 vehicle showing normal cords of hepatocytes (large arrows) radiating from the central veins (CV) which are bounded by an intact endothelium (arrow head). Mild glycogen depletion (asterisk) and sinusoidal congestion (small arrows) was evident throughout the hepatic lobule. (B1 and B2): Photomicrograph of a section of liver from a mouse treated with acetaminophen showing extensive centrilobular necrosis (large arrows), hydropic degeneration (small arrows), severe hemorrhage (asterisks) with congestion of sinusoidal spaces, destruction of central vein (CV) endothelium, karyolysis, pyknosis and karyorrhexis of nuclei (arrow heads).
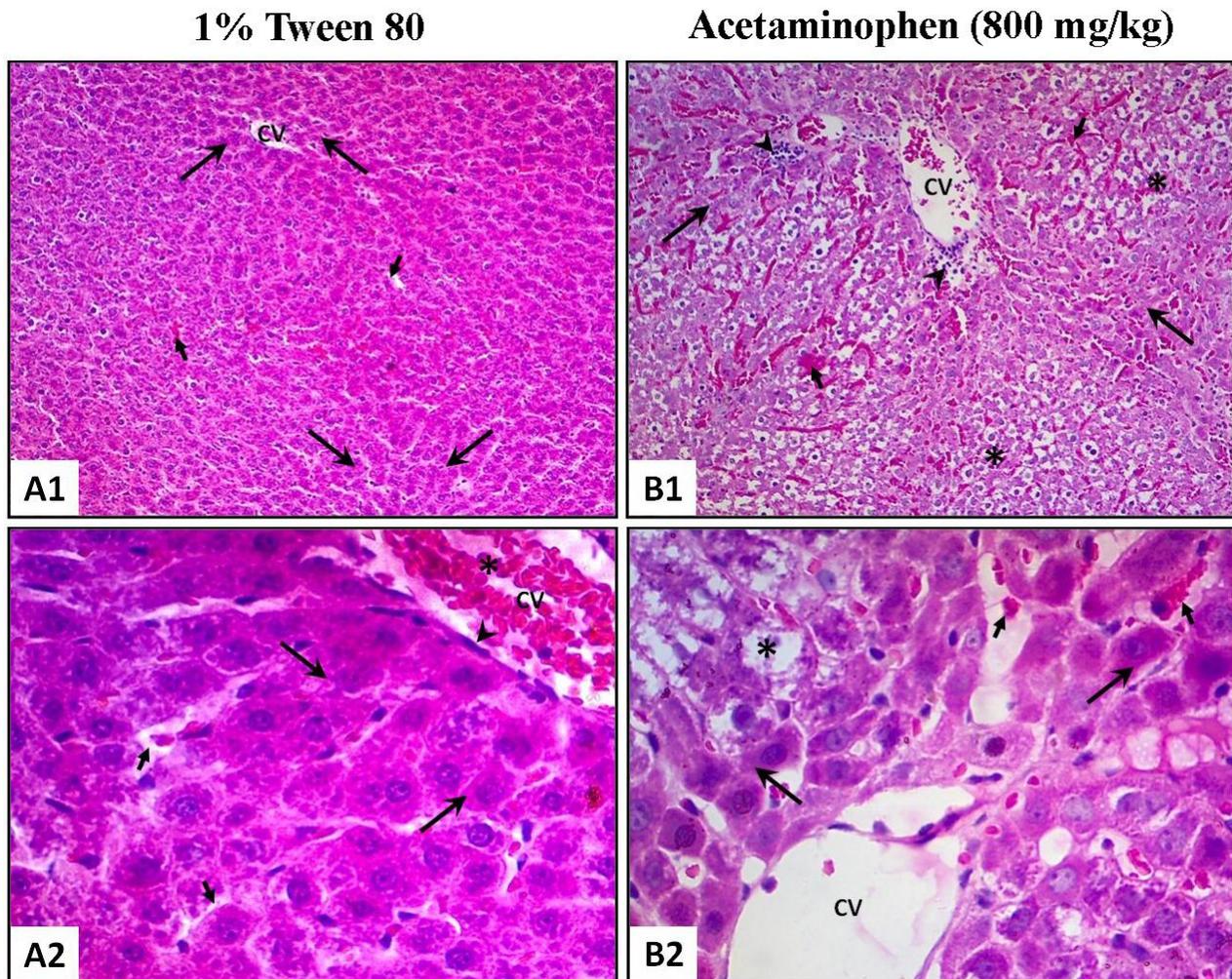
**Figure 2:** Histopathological evaluation of acetaminophen induced hepatotoxicity in rats (H & E; A1, B1; 100x and A2, B2; 400x original magnification). (A1 and A2): Photomicrograph of a section of liver from a rat treated with 1% tween 80 vehicle showing normal appearing central vein (CV) bounded by an intact endothelium (arrow head) having red blood cells in its lumen (asterisk). Parallel cords of hepatocytes radiate from the central vein towards the periphery of the hepatic lobule and are separated by sinusoidal spaces (small arrows) which exhibit mild congestion. (B1 and B2): Photomicrograph of a section of liver from a rat treated with acetaminophen showing extensive glycogen depletion (asterisks), perivenular aggregation of lymphocytes (arrow heads), chromatin condensation in nuclei, severe hemorrhage with mild dilatation and congestion of sinusoidal spaces (small arrows) and central vein (CV).

**Table 1:** Severity of acetaminophen induced hepatotoxicity in mice and rats

| Histopathological findings | Mice | | Rats | |
|---|---|---|---|---|
| | Group I | Group II | Group III | Group IV |
| Glycogen depletion | + | ++ | - | +++ |
| Hemorrhage | - | +++ | - | +++ |
| Congestion | + | +++ | + | +++ |
| Endothelium disruption | - | +++ | - | ++ |
| Sinusoidal dilatation | - | + | - | ++ |
| Hydropic degeneration | - | +++ | - | + |
| Cytolysis | - | +++ | - | + |
| Lymphocytic infiltration | - | + | - | ++ |
| Perivenular necrosis | - | +++ | - | ++ |
| Microvesicular steatosis | - | +++ | - | ++ |
| Macrovesicular steatosis | - | + | - | ++ |
| Karyolysis | - | +++ | - | + |
| Pyknosis | - | +++ | - | + |
| Karyorrhexis | - | +++ | - | + |
| Lymphoid aggregates in the portal tract | - | - | - | ++ |

(–) none; (+) mild; (++) moderate; (+++) severe
Group I (20% tween 80), Group II (APAP 500 mg/kg in 20% tween 80), Group III (1% tween 80), Group IV (APAP 800 mg/kg in 1% tween 80)