CHAPTER 7

DISCUSSION

Animal usage in biomedical research has become critical and facing more challenges due to international and local regulatory agents rules and regulation on animal experiments, getting approval from ethical committee, use of quality animal in order to get reproducibility and repeatability of the experiment, preclinical data transfer to clinical trials and success of drug or chemical compound in wide usage among humans with safety and efficacy (Felton & Gaylor, 1989). However, the rise in animal activist organizations all over the world has become a serious threat to animal experimentation. Preclinical animal trial involves huge cost investment nowadays because of rise in cost of quality animals, vivarium operations and demand for scientist. Thomas Hartung (2009) elaborated the real problem of preclinical biomedical research by his famous article in Nature which reported that about ten billion Euros were spent on animal experimentation worldwide every year and how the money gets poorly used due to bad experimental design. He suggested adopting multistrain experiments in toxicological analysis.

These challenges made the government and nongovernmental organization (NGO) partnership to find out the alternative techniques of animal experimentation with huge investment in past few years to replace and reduce animals in experiments. Few of the leaders of such organization are John Hopkins university center for alternatives to animal testing, European Union reference laboratory for alternative testing to animal testing and Fund for the Replacement of Animals in Medical Research (FRAME). But the recent news on BBC (Melissa, 2013), reported that 8% increase in animal experiments was observed in UK as per the home office figures.

In this current scenario efficient animal usage has become inevitable in biomedical research in order to create reliable preclinical data which can be more efficiently used in clinical trials to roll out the drug or chemical compound into human society.

There are many publications about the effect of animal strains in the exhibition of toxic effects (Sam Kacew & Festing, 1996; Sam Kacew & Festing, 1999) and carcinogenicity (shellabarger et al., 1978 & Charles Irving, 1975) caused by various chemical and hormones. Sam Kacew et al. (1996) reported that strain differences in the exhibition of toxicity may be due to metabolic phenomenon which is limited to certain strain. Also the reactive oxygen differs between strains in the process of detoxification of acetaminophen. Moreover the increase in biliary Alpha-N-acetylg glucosaminidase activity, non-heme iron and protein carbonyl excretion in F344 rat strain differ drastically when compare to SD rat which leads to variations in the toxic effects of acetaminophen. The cytochrome P450 (CYP) mediated biotransformation may affect the pharmacokinetic and toxicity expression of acetaminophen between Wistar and SD rats. This may be due to various forms of CYP isomers availability in different strains of rats (Nelson et al., 1996). USFDA released an white paper (Critical Path, 2004) under critical path initiative stating that the current experiment toxicity study design may not give fruitful results as it involves outbred animals with single strain. Whereas it suggested to use multi-strain animals in a group with powerful statistical tool for the valuable and efficient preclinical data.

In spite of various publications on the role of strain difference in the exhibition of toxicity assay on various chemicals and drugs, the leading pharmaceutical industry and regulatory agency are yet to implement this valuable information. This may be because of paradigm shift resistance prevailing in the current pharmaceutical industry. The drug companies are using this as an opportunity in the evaluation of chemical compound especially the generic drug market by favoring the strain selection while conducting the animal experiment in order to get FDA approval (Birgitte et al. 2007). Vom Saal & Welshons (2006) reported that 92 % of government-funded reported observable adverse effects, while 100% of industry-funded studies reported no observable effects due to bias in study design.
The hidden toxicity due to strain variation might have a huge impact on human health on overall population but the drug industry gaining their marketing strategy by avoiding new scientific information with the help of various regulatory agencies. Particularly this is evident in acetaminophen usage in India very widely as OTC drug till date whereas it has been banned in Western countries. I remember a famous quote by Andre Gide “Everything has been said before, but since nobody listens we have to keep going back and begin all over again”. So the present study gives an opportunity to do such a unique study in India and it is an added advantage to pose a role of rat interstrain variations in toxicology to the scientific community which may impact the future decision of regulatory agencies, inspite of many researchers were already initiated in past years. In India the interstrain comparative animal study found to be rare which gave emphasis to the present study especially on acetaminophen toxicity assay (Shivbalan et al. 2010).

Acetaminophen is a widely used over the counter analgesic and antipyretic drug (Shivbalan et al. 2010). Oral administration of acetaminophen has been shown to be at least as effective as intravenous administration of an equivalent dose of acetaminophen, and the target concentration achieved more rapidly and with less variability in plasma concentrations compared with enteral formulations (Holmer et al. 2004 and Anderson et al. 2005). The target organ of acetaminophen toxicity is liver and uncommon in kidney in case of over dosage (Sheen et al. 2002; Deepak et al. 2007; shivakrishnan & Kottaimuthu 2013). So acetaminophen were chosen as test substance to understand the inter strain expression of toxicity in between the Wistar and SD rats. These two strains are most commonly used rat strain all over the world and are also accepted by all regulatory agents including USFDA.

The acute toxicity analysis of acetaminophen in Wistar and SD rats revealed that the LD50 of oral dosing of acetaminophen were found to be more than 2000mg/kg b.wt as per the OECD guideline 425 (2001) limit tests which accord the results of drug data bank (Drug Bank, 2014). The high dose group of 2000mg/kg b.wt in both Wistar and SD rat revealed clinical signs lethargy within few hours of dosing which may be due to gastric irritation caused by drug and subsequently all rats recovered normally and active without any other clinical signs throughout the study.
In the present investigation of sub acute toxicity assay of acetaminophen in Wistar rat, there was no mortality in male and female rats, at and up to the dose of 1000 mg/kg body weight. No incidence of any abnormal clinical signs in either sex, at and up to the dose of 1000 mg/kg body weight. No significant effect on the body weight gain of male and female rats, at and up to the dose of 1000 mg/kg body weight. In group five of female showed there was a drop of two gram means body weight in between day 28 and day 35 which was due to one female had a less body weight gain during the mentioned period which was found to be normal gain on subsequent week. No treatment effect on the daily food consumption by the male and female rats treated at and up to the dose of 1000 mg/kg body weight. On day 28 all the groups of male revealed a drop in feed consumption which was comparable with recovery group of five and six. It may be due to age factor of the animals or due to continuous oral gavage stress might have reduced the appetite of the animals.

Based on the laboratory investigation the following observation was noticed. No toxicologically significant effect on the haematological parameters of male and female rats treated at and up to the dose of 1000 mg/kg body weight. No toxicologically significant effect on the biochemical parameters of male and female rats treated at and up to the dose of 1000 mg/kg body weight. No toxicologically significant effect on the urinalysis parameters of male and female rats treated at and up to the dose of 1000 mg/kg body weight. No effect on the absolute and relative organ weights of male and female rats, at and up to the dose of 1000 mg/kg body weight. No treatment related gross pathological alterations in the tissues of male and female rats treated at and up to the dose level of 1000 mg/kg body weight. One male rat showed unilateral testis in group I which might be genetic abnormality. No treatment related histopathological findings were observed except for a mild increase in the numbers of tingible body macrophages in the thymus in the treated groups which was considered to be of less pathological significance. These changes may therefore constitute an adaptive response.

Based on the findings of this study in Wistar rat, the No Observed Adverse Effect Level (NOAEL) of acetaminophen in Wistar rats, following oral administration at the doses of 250, 500 and 1000 mg/kg on daily basis found to be 1000 mg/kg body weight. The results of this study accords the results of Garey et al. (2009) where he found the NOAEL of Wistar rat is 1000mg/kg body weight.
Similar methods and procedure were used to find out the sub acute toxicity assay of acetaminophen in SD rat and the results were concluded. The aim of the present study was to evaluate the sub-acute oral toxicity of acetaminophen in Sprague Dawley (SD) rats at 250 to 1000 mg/kg body weight. The following observations were noticed during the study. No mortality in male and female rats, at and up to the dose of 1000 mg/kg body weight (b.wt.). There were abnormal clinical signs observed on female animals at 1000mg/kg b.wt. dose level except one female of group IV showed severe lethargy, hunched back within few hours after the dosing which was similarly observed in acute dosing of Wistar and SD rats of female also. There were no difference in body weight gain and no effect on the daily feed consumption.

Based on the laboratory investigation the following observations were made. No toxicologically significant difference on the haematological parameters but liver and kidney related biochemical parameter showed significant difference at 1000mg/kg b.wt. in females. In biochemical parameters male rat significant difference in total cholesterol and creatinine at group III and other parameters were not significant and comparable with control group. In female group II showed significant level in BUN; group III showed significant difference in glucose, BUN and ALT(P<0.05); group IV showed significant in glucose, total protein, globulin, BUN, creatinine, AST, ALP, ALT and total bilirubin (P<0.01).

No toxicologically significant effect on the urinalysis parameters, absolute and relative organ weights and gross pathological alterations; whereas histopathological alterations were observed in female liver at dose level of 1000mg/kg b.wt. were observed. In histopathological examination, focal lymphocytic infiltration and/or centrilobular necrosis in liver were observed which was considered as treatment related changes when compare to the control group histopathological sections which were similar to the hepatotoxic lesions of acetaminophen reported by Theodore et al. (1996). Based on the findings of biochemical parameters and histopathological lesions in this study, the No Observed Adverse Effect Level (NOAEL) of acetaminophen in SD rats, following oral administration at the doses of 250, 500 and 1000 mg/kg on daily basis was found to be 500 mg/kg body weight. The above results in SD rats matched the previously reported findings of Joan et al. (1996); Laura et al. (2003) and Monira et al. (2012).
Joan et al. (1996) observed that acetaminophen (APAP) at 1250 mg/kg induced nephrotoxicity (as indicated by elevations in BUN concentration) in 3-month-old females but not males, whereas APAP induced hepatotoxicity (as indicated by elevations in serum ALT activity) in 3-month-old males but not females. Sex differences in APAP toxicity were no longer apparent in 18-month-old rats. APAP at 750 mg/kg ip produced liver and kidney damage in 18-month-old but not 3-month-old male and female rats.

Monira et al. (2012) and Robert (2008) observed the acetaminophen toxicity after its administration to rats induced marked disturbance of hepatic and renal functions, characterized by a significant increase in the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, total protein, blood urea and serum creatinine (p < 0.01) and injured the hepatic and renal cells evident from increased level of malondialdehyde (MDA) (p < 0.01) along with depletion of super oxide dismutase (SOD), catalase (CAT), activities and reduced glutathione (GSH) levels (p < 0.01). Histopathological changes showed that paracetamol caused significant structural damages to liver and kidneys. But the present study did not reveal any signs of nephrotoxicity which contradicts the result of Norman & Barry (1992) where they observed renal impairment due to paracetamol toxicity in the absence of hepatotoxicity.

The liver is the vital organ of paramount importance involved in the maintenance of metabolic function and detoxification of drugs (Satish et al, 2012 and Theodore et al, 1996). After the intake of toxic dose of acetaminophen causes P450-dependent hepatotoxicity in man and various laboratory animals as observed by the release of serum alaninie aminotransferase (ALT) into the serum (Jos et al. 2001; Sam Kacew & Festing, 1999; wallace, 2000). Liver damage is always associated with cellular necrosis, increase in tissue liquid peroxidation and depletion in the tissue GSH levels. In addition, serum levels of many biochemical markers like SGOT, SGPT, ALP and billirubin are elevated (Mcjunkin et al. 1976 & Mossa et al. 1991). The laboratory features of hepato-toxicity induced by paracetamol resemble other kinds of acute inflammatory liver disease with prominent increase in levels of SGOT, SGPT, and ALP. Hepato-toxicity is the most remarkable feature of paracetamol overdose (Rumack & Mathew, 1975; Mahesh et al. 2009; Walker et al. 1981). The basal values of triglycerides, AST and glucose levels of SD and Wistar rats were compared with historical control data of the animal source and found to be within the range among the sexes.
However, in the present study, there was no significant change in the levels of hepatic enzymes AST, ALT, GGT and ALP in acetaminophen treated groups of either sex as compared to the respective control group. Studies in the human and animals report that overall incidence of acute renal failure with acetaminophen toxicity (Blakley & Donald, 1995). In the present study, biochemical parameters related to kidney function were evaluated and no significant differences were observed in blood urea, creatinine, glucose and proteins with respect to control. However, it has been reported that certain strains of rats that have high concentrations of microsomal cytochrome P450 in their kidneys developed acute tubular necrosis after a single, nonlethal dose of paracetamol (Anurag Pyasi, 2010). It has been observed that conditions that are associated with glutathione depletion or increased activity of P-450 microsomal oxidase enzymes enhance acetaminophen toxicity even at the therapeutic dosages. Examples include chronic alcohol use, starvation, fasting or ingestion of drugs that induce these enzymes, such as anticonvulsants. It has been reported that the proximal tubules are the target of APAP toxicity because of their active absorptive and secretory activities (Seham & Awatef, 2008). There were no signs of toxicity seen in any of the organs in histopathological analysis. Thus histopathological studies provide supports to the safety data of other physiological, biochemical and hematological parameters of acetaminophen treatment.

Comparative analysis of inter strain difference of acetaminophen toxicity in Wistar and SD rats were carried out by combining the toxicology data of both SD and Wistar rat. The inter strain comparison statistical analysis were performed with IBM SPSS- 20.0 version for Windows software. Group mean and standard deviation, standard error of mean were calculated for all the data of intra group variances. The order of analysis was descriptive, ANOVA on females, males and combination serially. The treatment groups were analysed by Duncan multiple range test.

The following differences were observed in between strains during the treatment of acetaminophen. Female and Male body weight significantly differ between strains which may be due to physiological and genetic nature of each strain. Henrik & Per Thomsen (1988) reported that body weight of Wistar rat was not affected during acetaminophen toxicity upto 12 weeks chronic study but it was affected after 18 weeks of continuous dosage. Serum glucose value showed that male was significantly difference in between strains which may be due to high excretion of glucose in females than male. Joan et al.,
(1996) reported age and sex related toxicity exhibition with acetaminophen dosage in SD rats, where he observed that glucose was excreted in urine at high levels in female at high dose of acetaminophen.

Because of hepatotoxicity observed in SD rats and not in Wistar rat at 1000mg/ kg b.wt. of oral acetaminophen dosage continuously for 28 days. This study revealed obvious interstrain difference in NOAEL. The inter strain toxicity exhibition was clearly revealed in the outcome data of statistical analysis of combined data analysis as well. So the acetaminophen administration under same environmental condition, same group of scientist, same methodology adopted, and same investigation methods followed with only exception of differences in the two animal strains revealed that significant difference in the toxicity outcomes. The hepatotoxic parameters showed the following findings. Blood urea nitrogen in female had significantly different in between strains. Creatinine levels in male showed significantly different in between strains. Protein in male and female showed significant in between strains. Liver enzyme of female showed significantly different in between strains. Lipid profile triglycerides of female and male were found to be highly significant different between strains whereas total cholesterol was not significant.

Sam Kacew & Festing, (1999) reported that susceptibility of any chemical dependent on target organ sensitivities which may dependent on different strain of animals. The authors reviewed in detail about the inter strain differences in toxicity studies. The SD rat and Wistar rat showed differences in toxicity assay when treated with acetaminophen on same dosage which correlates with present study. McMurtry et al. (1978) reported acetaminophen induced necrosis in SD rat which was more susceptible but highly resistant in F344 rat strain. The strain difference in the exhibition of toxicity may be due to cytochrome P450 involvement during metabolism of acetaminophen. There were many isomers of P450 enzymes reported in different rat strains (Tomoyuki et al. 2008; Sam Kacew & Festing, 1999; Sam Kacew et al. 2000; Kacew & Festing, 1996).

Walberer et al. (2005) studied the inter strain role in the susceptibility of cerebral ischaemic stoke between SD and Wistar rat. The authors found that ischaemic lesion was more accentuated in Wistar rat than SD rat. Sam Kacew et al. (2000) reported that genetic variation among strain in the formation of biotransformation enzymes which play a vital role in the toxicity of any compound. Hepatotoxicity due to N-hydroxy-2-
acetylamino fluorene observed in Wistar and F344 rats than in an SD rat which was due to metabolite which played major role in the toxicity of the compound. Koster et al. (2003) pointed out the importance of isozyme responsiveness within a strain in the exhibition of variability of toxicity. It was evident that inter strain differences exist with respect to drug induced liver peroxisomal proliferation and the factors involved are complex, which makes it difficult to extrapolate to humans for liver toxicity and carcinoma prediction. Patel et al., (2012) reported that acetaminophen treatment in female rats has raised the glucose levels significantly. Also the differences in glucose levels between sexes may be due to hormonal influence.

So, the role of rat strain in the exhibition of toxicity of any compound is very important which need to be considered while designing toxicology experiments. Wrong experimental results could lead to poor dosage or formulation information to clinical trial which will cause the molecule to fail and in turn cause huge loss of investment for the drug industry. Moreover the bias in the toxicity exhibition in between strain can be misused by pharmaceutical industry until it would be revealed in pharmacovigilance or by sacrificing more number of humans which will be intolerable human ethics violation. I hope the present study will add value to rise the voice of scientific community which already existing and insisting the government and regulatory agents to change the experimental study design by using multi strain usage instead of single strain usage in toxicity studies by avoiding the bias in toxicology, thereby improve the quality of preclinical data which in turn helps in clinical study design and ultimately helps in drug discovery.