**Aim of the Article**

This mini-review highlights our and others’ experience about liver injury models that are being used to follow up events of liver injuries under various conditions. Examples of applications of various *in vitro* and *in vivo* models in using synthetic chemicals or purified or semi-purified chemicals of herbal origins (i.e. chemically defined molecules) with reported experimental and/or clinical hepatoprotective activity are presented. The findings are likely to help further investigate hepatotoxic and hepatoprotective agents at both the experimental and the clinical levels. Based on these advances, a number of inflammatory targets have been identified with the potential for therapeutic intervention in various liver injuries by using certain compounds, presenting new opportunities and challenges for translational research.

Moreover, in this article, we consider briefly literature related to some studied active plant constituents of chemically defined molecules with potential hepatoprotective activity and the models most commonly used for their validation. We believe that these findings will certainly increase the likelihood of using hepatoprotective agents of well-defined molecules with less adverse effects, and will help design new molecules by using computational and synthetic chemistry. Naturally, this requires examination of the molecular aspects of studied compound mode of actions. This may seem more intricate since both the impairment of the liver is multifactorial (viral or protozoal infections, chronic use of excessive alcohol, drugs, xenobiotics etc.) and the chemoprotective agents are different in their chemical structures. Therefore, there is an urgent need to find out a range of efficient drugs that may be classified as hepatoprotective agents through the application of various *in vitro* and *in vivo* injury models.
In Vivo Experimental Hepatotoxic Models in Liver Research

There are several in vitro models like hepatocyte cultures and perfused hepatocytes that examine pathophysiological injuries due to various treatments (e.g., hepatotoxins, hypoxia or anoxia, anoxia/reoxygenation) [7-11]. Freshly isolated cells in suspension, primary cell cultures, and clonal cell lines are examples of well-established cellular models from various laboratories that have contributed significantly to the understanding of many aspects of mammalian cell physiology and molecular biology. Nevertheless, such cells may be metabolically less active than the corresponding cells in vivo, due to inefficient oxygenation and build-up of waste products [12,13]. Therefore, herein we focus on a developed perfusion method for isolated cells that improves the process of oxygenation and helps in end-product removal and is likely to be of considerable value in improving cell maintenance [12-15].

Short-term research-scale laboratory bioreactor

Hepatocytes, isolated by the collagenase method, are now being very extensively used in biological and biomedical studies [16]. The availability of an effective hepatocyte perfusion system would be of considerable value. More than 30 years ago, there were a few preliminary trials of hepatocyte perfusion followed by several reports on various methods of cell immobilization for perfusion studies, using either animal or plant cells. A description of hepatocyte immobilization for perfusion purposes in a small or research-scale laboratory bioreactor was described in detail previously [11,13,15,17,18] and is briefly outlined here.

Advantages and Shortcomings of Cellular Immobilization

Advantages
- Perfusion of immobilized cells facilitates the oxygenation process, and assists end-product removal, thus contributing to cell survival.
- Cells can be used for continuous monitoring of various metabolic and pathophysiological processes.
- Uncontaminated cellular products can be recovered readily for further processing.
- The flexibility of bioreactor design allows new approaches to the solution of special problems in biomedical research.
- The availability of a bioreactor reduces the need for whole organ studies.

The limited use of immobilized hepatocytes to date may reflect the following shortcomings:
- The lack of agreed optimal procedures for cell immobilization.
- Mechanical problems in achieving efficient and uniform perfusion.
- The relative instability of the immobilized hepatocyte systems so far developed.

Methods of cell immobilization

Currently cell immobilization may be summarized under five processes, namely, adsorption, covalent binding, encapsulation, entrapment and cross-linking [15,19]. Since there are large number of possible permutations between methods of immobilization and support material, it is easier to simplify the methods of cell immobilization into four major categories [12,14,20]:
- The thread technique: cells are mixed with a liquid gel (agarose gel, calcium alginate or matrigel) that is allowed to solidify, entrapping the cells in threads.
- Microcarrier beads: cells are cultured on the surface of solid or porous charged plastic beads.
- Hollow fiber systems: cells are grown using commercially available hollow fiber systems which are suitable for cellular proliferation.
- Perfusion of cells in culture dishes.

Isolation of rat hepatocytes

Small animals like rats or mice may be used for the isolation of hepatocytes by the standard two-phase perfusion method where collagenase is included in the second phase [16]. The hepatocytes are counted and examined for integrity. Hepatocyte suspensions in which Trypan blue is excluded by more than 90% of the cells should be used.

Hepatocyte immobilization in agarose gel threads and assessing hepatocyte functionality and integrity in the agarose gel matrix

Although the hollow fiber method is perhaps more physiological than other methods, and is used in bioartificial liver support systems, the thread method is outlined here because of its simplicity and the ease with which it can be performed after short practice. The details of the methods of immobilization in agarose gel threads and perfusion with the appropriate medium were outlined in reference [11,17,18].

An outline of some applications of immobilized cells

Among the applications of bioreactor is the study of nuclear magnetic resonance spectroscopy (NMR) of cells which allows for noninvasive and on-line analyses of many biochemical cell events which is known as real-time measurements. 31P-NMR can be employed to gain information about the intracellular pH and the energetic status of cells and some phosphorus-containing xenobiotics can be used for drug metabolism studies [8,21]. 13C- and 31H-NMR spectroscopy are also powerful tools for cellular metabolic studies [22]. Generally, further applications of immobilized cells include:
- In vitro biochemical, physiological, pharmacokinetic, pharmacodynamic and toxicity studies.
- Immobilized and perfused cells as bioartificial organs.
- Monoclonal antibody production and study of recombinant proteins. The feasibility of producing high amounts specific monoclonal antibodies was assessed and it was found that cells in a hollow fiber bioreactor can provide significant amounts of monoclonal antibody [23].
- Viral vectors. The use of mammalian cell bioreactor technology has enabled scientists to successfully implement bioreactor technology for the engineering of viral vectors [24].

More recently, we have used this model in the study of the resveratrol effects as compared to silymarin pretreatments on tert-butylhydroperoxide (tBH) induced apoptotic/necrotic markers in hepatocytes [7]. Hepatocyte in cultures (48 hours) and in perfused immobilized agarose threads (5 hours) were used as cellular systems. Resveratrol and silymarin reduced tBH-induced hepatocyte toxic effects in short term experiments as measured by a significant reduction in alanin-aminotransferase...
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This cellular model is also sensitive for detection of minimal enzyme metabolizing activity. It was reported that rat liver microsomes display, practically, no O-deethylating activity toward 7-ethoxycoumarin and on the other hand it was demonstrated an accumulation of 7-ethoxycoumarin de-ethylated product umbelliferone in the perfusate of induced and noninduced hepatocytes. The use of the present model demonstrated that even a minimum deethylase activity in rat liver could be detected in a hepatocyte bioreactor [11,17,25].

In Vivo Experimental Hepatotoxic Models in Liver Research

A number of in vivo models are depicted in Figure 1 that demonstrates common experimental setups in small animals. In the present article we discuss the model of D-galactosamine and lipopolysaccharide (D-GalN/LPS) combination with some detail since it is the most frequently used model for our in vivo studies. Other models shown in Figure 1 are used widely as well.

D-galactosamine/lipopolysaccharide-induced fulminant liver failure

The combination of both D-GalN and LPS produces significant hepatic injury due to combined prooxidant mechanisms and consequently oxidative stress. It was known earlier that D-GalN in vivo result in the depletion of uridine phosphate pool due to the formation of UDP-D-GalN derivatives which leads to the inhibition of mRNA and protein synthesis [26]. Later on, it was recognized that reactive oxygen species, perhaps, contribute mainly to D-GalN induced hepatotoxicity due to roles played by macrophages and Kupffer cells in vivo [27]. Moreover, LPS-induced cytokine(s) as TNF-alpha results in oxidative stress [28]. At present, it is clear that the combination of D-GalN and LPS causes definite oxidative stress in vivo and leads to fulminant liver failure.

Nowadays, D-GalN/LPS-induced hepatitis is a well established model of LPS-induced liver injury or septic shock [30-36]. Various dose combinations of LPS and D-GalN were used to produce sublethal liver failure which is relevant to clinical situations in viral, drug or alcohol-induced, immune-induced or under ischemia reperfusion hepatitis. In our studies, we used increasing doses of LPS from 0.5 µg/kg to 5 0 µg/kg combined with decreasing doses of D-GalN from 800 mg/kg to 200 mg/kg. The combination of 10 µg/kg LPS with 400 mg/kg D-GalN produced a nonlethal hepatitis as proved histologically and biochemically evidenced by increases in ALT, AST, alpha-GST, NO, and the mRNA levels of the studied genes [29]. Therefore, D-GalN/LPS combinations with different amounts of D-GalN/LPS appear very well reproducible model for in vivo experimental hepatitis studies.

Resveratrol and related compounds as antioxidants in D-GalN/LPS-induced hepatotoxicity: role of sirtuin 1 modulation in hepatoprotection

As described in the earlier section of this review (in vitro models), investigated naturally occurring cytoprotective agents such as resveratrol (trans-3,4',5-trihydroxystilbene) and other related compounds, probably with similar molecular mechanisms of action, have the potentials of applications in medical fields. Several physiological aspects have been ascribed to resveratrol and similar compounds including silymarin, curcumin, quercetin, and glycyrrhizin [6,37].

Indeed, we have shown that rat pretreatment with resveratrol before D-GalN/LPS application not only modified most of the oxidative parameters as thiobarbituric acid reacting substances and conjugated dienes or the antioxidants as catalase, but more importantly, NO-2 and HO-1 gene-expression were significantly modulated [29]. Thus, it was revealed that this naturally occurring cytoprotective compound exhibited a degree of hepatoprotection in a liver disease model of reversible fulminant hepatic failure.

Resveratrol, among others, has been recently described as a silent information regulator T1 (SIRT1 or sirtuin 1) activator that increases AMP-activated protein kinase (AMPK) phosphorylation and reduces the oxidative damage biomarkers during aging in laboratory settings. The reports on resveratrol and other SIRT1 activators from various sources are encouraging. The pharmacological strategies for modulation of sirtuins by small molecules through allosteric mechanisms should gain a greater momentum including human research. Resveratrol and resveratrol-like molecules seem to fulfill the requirement of anewhorizon in drug

Figure 1: Common in vivo models of liver damage.
research since these molecules cover a growing research means as antioxidants with allosteric mechanism in epigenetic drug targets. However, one should keep in mind the challenges of extrapolation of basic research into clinical results. Overall, the issue of sirtuins in biology and disease provides an insight on therapeutic potentials of sirtuin-based therapeutics and demonstrates the high complexity of drug-targeting these modalities for human applications.

The study of Kemelo et al. [38] reported that D-GalN/LPS-induced hepatotoxicity downregulates SIRT1 in rat liver and discussed the role of sirtuin 1 modulation in hepatoprotection. The role of sirtuin 1 in this model has not been documented before. However, there have been vast numbers of studies about the cytoprotective effects of resveratrol, a SIRT1 activator, in the liver and other tissues. This study was directed to elucidate the roles of SIRT1 protein expression or catalytic activity in D-GalN/LPS model of hepatotoxicity. Some groups of animals were pretreated with resveratrol and/or EX-527 (specific SIRT1 inhibitor). The effects of these treatments were evaluated by biochemical and Western blot methods. D-GalN/LPS treatment was able to induce a dramatic decrease of SIRT1 protein levels in liver tissue. Resveratrol pretreatment attenuated D-GalN/LPS-induced hepatotoxicity. EX-527 blocked the cytoprotective effects of resveratrol. Collectively, these results suggest that downregulation of SIRT1 expression is involved in the cytotoxic effects of D-GalN/LPS model and SIRT1 activity contributes to the cytoprotective effects of resveratrol in the liver.

Recent Developments of In Vitro and In Vivo Hepatotoxic Models in Liver Research

An in vitro platform for evaluating liver toxicity was described by Bale et al. [39]. The authors addressed a predictive human in vitro model and introduced a paradigm of microfluidic culture systems with the goal to mimic the liver with physiologically relevant dimensions, cellular structure, perfusion, and mass transport by taking advantage of micro and nanofabrication technologies. High-content analysis/screening for predictive hepatotoxicity and the evaluation of database-derived pathway development for enabling biomarker discovery for hepatotoxicity were also recently described [40,41].

Although, higher vertebrate organisms (for example rodents and pigs) are physiologically similar to humans, and have been traditionally used for studying in vivo drug-induced liver injury, smaller, lower order vertebrates, such as the zebrafish (Danio rerio), can be also used for this approach [42]. The zebrafish can accurately model human physiology and offer; beside its limitations, some significant advantages (rapid development of liver; large numbers of transparent embryo, low overall cost etc.), compared to rodents and other larger animals. However and as reported, before the model can be applied on wider scale, more validation is needed to confirm the translatability of the model to humans [42].

Discussion and concluding remarks

The application of in vitro and in vivo experimental hepatotoxic models in liver research is essential to understand and assess of mechanisms of liver toxicity and to study potential hepatoprotective drugs. Isolated hepatocytes in suspension, primary cell cultures, and clonal cell lines are examples of well established cellular models from various laboratories that have contributed significantly to the understanding of many aspects of liver physiology and molecular biology. Nevertheless, such cells may be metabolically less active than the corresponding cells in vivo, due to inefficient oxygenation and build-up of waste products. Therefore, we focus on a developed perfusion method for isolated cells that improves the process of oxygenation and helps in end-product removal that is likely to be of considerable value in improving cell maintenance in the so-called short-term research scale small laboratory bioreactor.

**Figure 2:** Steps in development of potential hepatoprotective therapy for liver diseases.
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