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Laboratory models available to study alcohol-induced organ damage and immune variations; choosing the appropriate model

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Abstract

The morbidity and mortality resulting from alcohol-related diseases impose a substantive cost to society globally. To minimize the financial burden on society and improve the quality of life for individuals suffering from the ill effects of alcohol abuse, researchers in the alcohol field are focused on understanding the mechanisms by which alcohol-related diseases develop and progress. Since ethical concerns and inherent difficulties limit the amount of alcohol abuse research that can be performed in humans, most is performed in laboratory animals. This article summarizes the various laboratory models of alcohol abuse that are currently available and are used to study the mechanisms by which alcohol abuse induces organ damage and immune defects. The strengths and weaknesses of each of the models are discussed. Integrated into the review are the presentations that were made in the symposium "Methods of Ethanol Application in Alcohol Model – How Long is Long Enough" at the joint 2008 Research Society on Alcoholism (RSA) and International Society for Biomedical Research on Alcoholism (ISBRA) meeting, Washington, DC, emphasizing the importance not only of selecting the most appropriate laboratory alcohol model to address the specific goals of a project but also of ensuring that the findings can be extrapolated to alcohol-induced diseases in humans.

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Keywords

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Introduction

The adverse effects of short-term (binge and sporadic drinking, also termed acute intoxication) and long-term (chronic abuse) excessive drinking outweigh the reputedly beneficial effects. Both experimental and clinical findings indicate that acute ethanol (alcohol) intoxication has adverse effects on many organs, and even a one time episode of acute intoxication prior to another insult (e.g. traumatic injury resulting from driving under intoxication, burns and drug overdose) can exacerbate the suppression of the host defense and increase susceptibility to infections [Choudhry and Chaudry, 2008; Kawakami et al., 1989; Brown et al., 2006]. Chronic alcohol abuse in humans is associated with multiple diseases, including those of the liver (fatty liver, steatosis, steatohepatitis, fibrosis, and hepatocellular carcinoma) [O'Shea et al., 2009; Schütte et al., 2009; Seitz and Stickel, 2009], pancreas (pancreatitis and cancer) [Hanck and Whitcomb, 2004; Apte et al., 2009; Thrower et al., 2008], gastrointestinal tract (intestinal hyperpermeability, endotoxaemia, and cancer) [Keshavarzian et al., 2009; Benedetti et al., 2009], respiratory tract (airway diseases, acute respiratory distress syndrome, and lung infections) [Sisson 2007; Boé et al., 2009], muscle (alcoholic myopathy) [Fernandez-Solà et al., 2007], and the immune system [Jeong and Gao, 2008; Lau et al., 2009; Szabo and Mandrekar, 2009]. The pattern and duration of alcohol use, in association with other cofactors such as genetic predisposition, gender, dietary, and environmental, determine an individual's propensity for developing alcohol-related disease [Day 1997; Testino, 2008; Zakhari and Li, 2007; Pelucchi et al., 2008; Shankar et al., 2007].

The global disease burden and health care costs associated with alcohol abuse are high [Mathurin and Deltenre, 2009; Casswell and Thamarangsi, 2009; Room et al., 2005]. Therefore, research efforts continue to remain directed at understanding the mechanisms by which alcohol affects behavior, tolerance, physical dependence, immunity, and promotes organ damage. The many ethical concerns and inherent difficulties associated with performing alcohol research in humans have led to the development of several laboratory models [Stricker, 1991; Dolinsky and Babor, 1997]. These include the use of normal and genetically manipulated animals, animal tissues, isolated organs and cells, cell lines, lower vertebrates, and invertebrates. The intricacy of the human anatomy, along with the existence of many other variables in association with alcohol abuse in humans, makes it extremely difficult to replicate all facets of human drinking in laboratory models. Therefore, available models of alcohol abuse are either being constantly refined or new ones developed to mimic human drinking. From the numerous models available to date, none have reproduced exactly the clinical manifestations of any of the alcohol-related diseases seen in humans. However, despite their limitations, these models have served as very valuable tools in advancing our understanding of the many detrimental effects associated with alcohol abuse and for exploring the mechanisms by which alcohol induces organ damage and immune variations. After all, the ultimate purpose of these laboratory models is to identify potential targets for developing therapeutic interventions to combat the ill effects of alcohol abuse in humans. The objective of this mini review is to highlight the many laboratory models available to alcohol researchers to decipher the mechanisms by which alcohol induces organ and immune system damage, and promotes host susceptibility to a variety of infections. All other laboratory models, including those used for behavioral studies, will be alluded to briefly. The review is also designed to inform novice investigators regarding some of the more important factors involved in selection of an appropriate animal model, and is a starting

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point for further inquiry, since there is no one model that is perfect or universally accepted for examining the effects of alcohol alone or with other interacting factors on various organs and systems. Also, integrated in the review are the presentations that were made in the symposium "Methods of Ethanol Application in Alcohol Model – How Long is Long Enough" at the joint 2008 RSA/ISBRA meeting, emphasizing the importance of not only selecting the most appropriate laboratory model to address the specific goals of a project but also of ensuring that the laboratory findings can be extrapolated to human alcoholic diseases.

Alcohol research performed using laboratory animals, animal tissues, animal cells, as well as cell lines and, whenever possible, human tissues (e.g., biopsies and blood) has provided substantial insights into the possible mechanisms by which alcohol intake, acute and chronic, can promote the development of diseases, compromise immunity, and increase susceptibility to a variety of infections (e.g., bacterial, fungal and viral) [Apte et al., 2005, 2009; Fernandez-Solà et al., 2007; Kharbanda, 2009; Schaffert et al., 2009; Szabo and Mandrekar, 2009]. Alcohol, by directly affecting membrane integrity, allows the translocation of gut-derived bacteria into the portal and systemic circulation. This, in turn, promotes organ damage via activation of immune cells to release inflammatory mediators and free radicals [Bode and Bode, 2003]. Furthermore, alcohol metabolism, which takes place primarily in the liver hepatocytes, produces derangement in carbohydrate, protein, and lipid metabolism, and affects cellular signaling [Baroana and Lieber, 1979; Sabesin, 1981; Tuma et al., 1991; Den Boer et al., 1997]. Aldehyde, a major metabolite of alcohol, is known to form protein and DNA adducts that promote alcohol-induced organ damage [Niemelä, 1993; Brooks and Theruvathu, 2005; Thiele et al., 2008; Seitz and Stickel, 2009].

There are a variety of laboratory models used by investigators to study the effects of alcohol on health, and the choice of the model is usually dependent on the nature of the question asked. Examples of variations include manipulation of diet, if diet is the primary interest, or maximization of alcohol intake, if alcohol is considered to be the primary factor promoting diseases. Since most alcohol-related organ injury or diseases are seen only after heavy and sustained drinking, and often in association with poor nutrition, environmental factors, and genetic predisposition, consideration has to be given to ensure that the laboratory model selected allows for sufficient alcohol administration, adequate control over nutrients and alcohol intake, and the flexibility to test genetic and environmental factors.

Choosing the right Laboratory Model of Alcohol Abuse

To understand the pathogenesis of alcohol-related diseases in humans, researchers would prefer to use animals closest to humans, such as non-human primates. However, the cost, the duration for which these animals have to be kept exposed to alcohol, and the fact that not all will display the ill-effects of alcohol to the same extent, often preclude non-human primates from being either feasible or ideal as models [Tsukamoto, 1998]. Therefore, other *in vivo* and *in vitro* experimental models of alcohol administration have been developed. The methods most commonly used to study the effects of alcohol on various organs and systems (e.g. immune, endocrine and CNS) require the administration of alcohol to intact animals (*in vivo*) or exposure of isolated organs, tissues, and primary and transformed cell lines to alcohol in culture (*in vitro*).

When selecting or developing a laboratory model of alcohol abuse, the research question is of paramount importance. The research question will dictate the choice of the species, the strain, the age and nutritional status of the animals, as well as the mode of alcohol administration and the duration of alcohol exposure. The most commonly used *in vivo* alcohol models have been developed in laboratory mice, rats, ferrets, guinea pigs, hamsters,

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rabbits, mini pigs, and monkeys. The importance of selecting the most appropriate species, strain, route of alcohol administration, and the laboratory animal to study the development and progression of alcohol-related diseases and immune defects will be discussed. In addition the advantages and disadvantages of the available laboratory models will be considered along with some cautionary notes for the benefit of novice investigators desiring to use any of these models.

The animal genus and species and strain

By far the most commonly used animals for studying alcohol-induced organ damage and immune variations are mice and rats. In addition to their well-defined genetic background and availability in diverse genetic traits, these are smaller, less sentient mammalian species whose physiological response to alcohol closely resembles that of humans. In the mouse species C57BL/6, BALB/c and ICR are the preferred strains and in the rat species Sprague-Dawley, Wistar and Long Evans. It is well known that the immune systems differ between mice and rats. Therefore, it is possible that different results could be obtained given a similar research protocol. For studies in mice where the amount of alcohol consumed is of paramount importance, the C57BL/6 strain is commonly used. This strain of mice presents features of higher voluntary alcohol consumption [Crawley et al., 1997]. BALB/c mice will also consume sufficient amounts of alcohol only if alcohol is phased in [Zhu et al., 2004]; however, there are immunological differences among strains of mice that need to be considered [Chen et al., 2008; Rymarchyk et al., 2008; Weinberg et al., 2004; Zhu and Gilmour, 2009]. In the rat species, the Wistar rats have less alcohol aversion and consume more alcohol than Sprague-Dawley rats. Through bidirectional selective breeding, lines of mice and rats that display high or low ethanol-drinking phenotypes have been developed. The alcohol-preferring (P) rat line developed from the Wistar rat can be used affectively in assessing alcohol-preference, a genetic predisposition for alcohol abuse and/or alcoholism and excessive drinking, using protocols of binge-like or relapse like drinking [Dyr and Kostowski, 2008; McKinzie et al., 1999; Bell et al., 2006]. The alcohol preferring rodent lines and genetically engineered mice with targeted gene mutations, such as transgenic and knock-out mice, are useful to identify candidate genes, pathways and mechanisms for alcoholism and other abuse-related effects of alcohol [Rodd et al., 2007; Crabbe et al., 2006; Hoffman et al., 2001].

Mini pigs are used in models that require voluntary and high alcohol intake which may be otherwise difficult to achieve in other small animal models. Pigs are similar to humans in body mass, feeding patterns, endocrine systems, heart and kidney structure and function, and the rate at which ethanol is cleared from the blood. In addition, many of the alcohol-induced biochemical changes reported in other models are reproducible in the mini pig model. Hanford and Yucatan miniature pigs are preferred due to their voracious appetite. In this model, the mini pigs are fed laboratory chow mixed with ethanol to account for 40% of the calories in the diet. Peak blood alcohol levels in the range of 130–229 mg/dl can be achieved, and with an increase in dietary polyunsaturated fat (33% of total calories) liver fibrosis can be induced [Halsted et al., 1993]. However, like the non-human primate models, mini pigs are high in cost and effort.

Non-human primate (baboon and rhesus macaques) models are used to mimic conditions like liver injury and HIV seen in alcohol abusers [Rubin and Lieber, 1974; Dufor et al., 2007]. In these models, alcohol is administered for extended period either orally, via surgically implanted gastric catheter [Rubin and Lieber, 1974; Bagby et al., 2003], or as a single bolus application using a gastric intubation tube [Srivastava et al., 2007]. These models are also employed for behavioral studies, examining alcohol dependence by self-administration [Mello et al., 1988].

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Age and gender

Age should be factored into the selection of the animal model; however, to a large degree this is determined by the research question. Gender/Sex is another consideration that the investigator must make since differences in hormonal status and alcohol metabolizing enzymes can impact the susceptibility to alcohol-induced organ damage [Thurman et al., 1998, Thurman, 2000]. A recently published study, using the drinking in dark (DID) procedure to mimic the kind of binge-type ethanol intake in human adolescents, demonstrates that adolescent C57Bl/6 mice consume more alcohol than their adult counterparts and, that, such adolescent ethanol exposure increases ethanol intake in adulthood. The study further demonstrates that both age and genotype are important influencers on ethanol consumption [Moore et al., 2010; Boehm SL 2nd et al., 2008].

To investigate the mechanisms of alcohol-induced organ damage many investigators prefer to use laboratory animals of female gender. This is primarily due to greater susceptibility of females to alcohol than males [Thurman, 2000; Gallucci et al., 2004]. It is important to note that variations exist in alcohol metabolism not only between the species (e.g. rats and mice) but also between different strains in a given species [Livy et al., 2003]. In addition, differences in immune responses after alcohol exposure are also described [Giberson et al., 1997; Kovacs et al., 2004; Weinberg and Jerrells, 1991].

In summary, many studies have been published using various animal models to advance our understanding of alcohol-induced organ damage and behaviors that characterize alcoholism in humans. The results of these studies and interpretation, however, have not always been consistent. The intricacies of the experimental design and the model used (e.g. the species, strain, gender, and the route and amount of alcohol administered), may all contribute to the apparent discrepancies.

Alcohol Administration

The research question will govern to some extent the method by which alcohol is given, as this can influence the blood alcohol concentration [Livy et al., 2003; Moore et al., 2010]. One should also consider whether blood alcohol concentration is an important variable to the research question asked. Alcohol's suppression of natural killer (NK) cell cytolytic activity in mice given 20% w/v alcohol in the drinking water, for example, is not correlated with blood alcohol concentration [Abdallah et al., 1988; Blank et al., 1991; Meadows et al., 1989]. These data suggest that the effect of alcohol consumption on NK cell activity is indirect, and this is supported by the fact that alcohol added to NK cells *in vitro* does not inhibit the cytolytic activity of these cells (Blank et al., 1994a).

Choosing the appropriate control groups

The route of alcohol administration dictates the treatment of control groups. The same procedure has to be performed for control groups. This applies as much to *in vivo* or *ex vivo* animal experiments as for *in vitro* cell cultures. Depending on the procedure used, the *in vivo* controls may need to be matched to the alcohol groups for variables such as the caloric intake, volume loading, osmolarity, and fluid intake. In addition, some experimental designs may warrant the use of additional control groups (e.g. animals allowed ad libitum access to standard rodent chow and/or the liquid diet).

Nutritional Status

Many of the parameters investigated to study alcohol-induced organ damage and immune system dysfunction are affected by nutritional status. The modulatory effects of macro- and micronutrient imbalances on parenchymal and non-parenchymal cell responses are numerous and well described in the literature; therefore, it is imperative that nutritional

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status in the alcohol consuming animal is maintained. Several published studies suggest that nutritional status and/or composition of the diet are two important variables that can either accentuate or dampen down many of the adverse effects of alcohol [Baumgardner et al., 2007; de Meijer et al., 2010; Reitz, 1993; Chen, Xi, Cohen, 1995; Weinberg and Bezio, 1987; Fisher et al., 2002]. To emphasize this point, mice given 20% w/v alcohol in the drinking water along with free access to standard laboratory chow exhibit suppressed natural killer (NK) cell cytolytic activity; however, when chow intake is reduced by 30–40% of control, there is no differential effect of alcohol consumption on NK cell cytolytic activity [Blank et al., 1991]. Furthermore, even if nutritional adequacy is assured through sufficient diet consumption, chronic alcohol abuse can affect the absorption of macro- and micronutrients by altering the permeability of the gastrointestinal tract [Bode and Bode, 2003; Puorohit et al., 2008]. The alcohol-nutrient interactions and their effects on the immune response is an under-explored area of investigation.

Circadian rhythm

Most of the biological processes in the living organisms of both animals and man are known to be of rhythmical nature. Variability of enzymatic activity in the circadian cycle depends on many factors including age, sexual maturity, and diet. Recent studies reveal that the circadian rhythms of physiology and behavior are controlled by clock genes; the effectiveness and toxicity of many drugs including alcohol vary depending on dosing time associated with 24 hour rhythms of biochemical, physiological, and behavioral processes under the control of the circadian clock [Esquifino et al., 2007; Moore et al., 2010; Arjona et al., 2004; Arjona and Sarkar, 2006]. Circadian rhythm is also linked to the autonomic nervous system and the neuroendocrine system. Together these systems can modulate a myriad of immune parameters related to leukocyte physiology [Arjona and Sarkar, 2008]. Therefore, the time of the day, for example, when the alcohol is administered and one determines a parameter (s) of interest has to be kept consistent when designing experiments.

Level of alcohol intake - how long is enough?

Another important consideration in designing an appropriate experiment to examine any biological, hormonal or behavioral changes induced by alcohol is to decide on the amount of alcohol to feed and for how long to give it. Again, this will somewhat be dictated by the research question that is being asked. Many investigators utilize an amount of alcohol that mimics consumption by human alcoholics. It is generally accepted that this equates to 30% or more of the total caloric intake derived from alcohol. For C57Bl/6 mice allowed 24 hour access to rodent chow and 20% w/v alcohol in the drinking water (Single bottle-no choice model), the range of alcohol intake in 24 hours is between 22–45 g/kg [Blank et al., 1994a]. This is similar to the intake in mice given 24 hour access to 5% w/v alcohol in the Lieber-DeCarli liquid diet [Meadows et al., 1992] and unpublished observations (Meadows et al). On the other hand, in C57Bl/6 mice allowed limited access (2 to 4 hours) to alcohol in drinking water (DID model); the alcohol intake within that period is around 4–6 g/kg [Ehringer et al., 2009]. Findings using various animal models of alcohol abuse reveal that ethanol consumption, dose and duration-dependently, produces damage to a variety of organs including the liver, gastrointestinal tract, and brain [Siegmund et al., 2003, 2005; Yin et al., 2009; Ward et al., 2009]. With regards to alcohol-induced immune variations, alcohol intake ranging between 20–25 g/kg/day is reported to be associated with reduced NK cytolytic activity for female C57BL/6 mice (free access to 20% w/v alcohol in drinking water and laboratory chow) independent of the duration of alcohol intake [Blank et al., 1991, 1993, 1994b; Meadows et al., 1989]. On the other hand, mice given 10% w/v alcohol in the drinking water consume about 16 g/kg/day and show no signs of reduced NK cell activity [Abdallah et al., 1983; Meadows et al., 1993b]. It is possible, however, that these observations may be cell specific. The effects of low levels of alcohol on the immune

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response have been inadequately explored; however, there is some evidence that enhanced NK cell cytolytic activity is associated with the duration of low alcohol consumption [Meadows et al., 1993a].

Duration of alcohol intake – how long is enough?

There are many studies in the literature that report the effects of "acute" or "chronic" alcohol administration on various organs. However, there is no universal definition as to what constitutes "acute" and "chronic" treatment or what delineates the two. Typically alcohol given within a 24 hour period by the various methods of administration is easily defined as acute administration. Conversely, many studies view one or two weeks of alcohol administration, or even longer periods of time also as acute studies. The duration at which an acute study becomes chronic is still subject to investigator interpretation. Most studies reported in literature, using laboratory animals, to mimic many of the adverse effects of long-term drinking in humans have used a minimum of four to six weeks of alcohol feeding regimen. Therefore, it is probably safe to say that most investigators would view alcohol feeding for one month or more as chronic administration. If the goal of the experiment, for example, is to design a model in mice that is equivalent to a middle-aged human alcoholic this will entail administering alcohol for up to 8 months [Coleman et al., 2008]. Chronic disease induced by alcohol, such as alcoholic liver disease, is a multi-step disease process which typically progresses through stages of alcoholic steatosis, alcoholic hepatitis, and alcoholic cirrhosis to end-stage liver disease. What phase of the multi-step process the investigator desires to capture will determine the duration for which the alcohol is to be administered. Depending on the species, strain, gender and model of alcohol administration used, the earliest step of the disease may manifest as early as three to four weeks after alcohol exposure. On the other hand, livers from C57BL6 female mice gavaged orally with a single large dose of alcohol (30% ethanol in saline at 6 g/kg body weight) are reported to show increased hepatic lipid deposition, steatosis, and an up-regulation of genes involved in fibrinolysis eight hours after the gavage [Seth et la., 2008].

Long-term alcohol administration in mice causes lymphopenia and other immune changes [Cook et al., 2007; Zhang and Meadows, 2005]. For example, changes in specific subpopulations of NK cells are seen as early as 8 weeks during prolonged alcohol administration [Blank et al., 1993]. Additionally, it is important to realize that short-term and long-term alcohol administration can differentially modulate immune parameters. For example, mice consuming 20% w/v alcohol in the drinking water for two weeks exhibit decreased splenocyte proliferation; whereas, the splenocyte proliferation is increased when determined at 3 months [Zhang and Meadows, 2009].

From these and other studies, it is apparent that not all findings reported in the literature using various animal models of alcohol are consistent. The discrepancies could be the result of many variables including the use of different animal models, route of alcohol administration and feeding periods. Therefore, it is critical that investigators conduct their experiments choosing the most appropriate species, strain, gender, route of alcohol administration, and use carefully defined time periods after initiating alcohol feeding. They must, of course, also report all these details accurately.

Models of Acute Alcohol Intoxication

Acute alcohol intoxication encompasses taking a single intoxicating drink either in a single sitting or in a binge situation where several drinks are consumed within a few hours and/or consecutively for several days. Like chronic alcohol abuse, acute alcohol intoxication also has detrimental effects on many organs. Understanding the effects of acute alcohol intoxication, especially on the immune system, can be relevant to treating patients who have

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trauma injuries immediately after/during acute alcohol intoxication. Using *in vitro* and *in vivo* models, acute alcohol intoxication is shown to affect carbohydrate, protein and lipid metabolism, and impair immune (innate and adaptive) responses (organ specific and systemic) to a variety of immune stimuli (second hit) in a dose and time-dependent manner [Karavitis et al., 2008; Ochshorn-Adelson et al., 1994; Taieb et al., 2002; D'Souza et al., 1989].

In vitro models of acute alcohol intoxication

Exposure of primary cells or cell lines to alcohol in culture—The model involves incubating cells (primary or transformed) in a culture medium containing alcohol of desired concentration. Briefly, the cell suspension is plated in tissue culture plates, and the plates are incubated at 37°C in a sealed chamber of a tissue culture incubator filled with a gas mixture (95% O_2 + 5% CO_2). An open Petri dish containing alcohol (twice the concentration used to incubate the cells) is placed at the bottom of the modular chamber [Szabo and Mandrekar, 2008]. The sealing of the chamber and the placing of an open alcohol containing Petri dish in the chamber helps to minimize alcohol evaporation and maintain a constant concentration of alcohol in the culture medium. Exposure of cells to alcohol for less than 24 hours *ex vivo* is usually referred to as an acute exposure. The amount of alcohol used in published studies ranges from 1 to 500mM. Typically, exposure of cells *in vitro* to a concentration of 25mM alcohol extrapolates to a blood alcohol concentration of approximately 115 mg/dl (115%) in humans.

The data published under acute alcohol intoxication range from exposure of cells to alcohol *in vitro* for less than an hour to several hours. Thus, by exposing different types of cells and/or cell lines to varying concentrations of alcohol for short durations, it has been shown that alcohol at concentrations below 100 mM affects redox state of cells, alters intercellular junctions, increases cell membrane fluidity, and affects the composition of lipid rafts, all of which, in turn, alter cell functions. Furthermore, in vitro models of acute alcohol intoxication have expanded our understanding of the possible cellular and molecular mechanisms by which alcohol induces oxidative stress, modulates inflammation, increases susceptibility to infections, and promotes organ damage [Dolganiuc and Szabo, 2009; Ramachandran et al., 2003; Gutierrez-Ruiz et al., 2001; Fernandez-Lizarbe et al., 2009; Spurzem et al., 2005; Bailey et al., 2010; Dai and Pruett, 2006; Mandrekar et al., 2008; Stolz et al., 2000; Chen et al., 2008].

In situ organ perfusion with alcohol—The technique involves in situ perfusion of an organ/tissue (e.g. the liver or placental villous tissue) with either oxygenated Krebs Ringer solution or Dulbecco's Modified Eagle Medium (DMEM) at pH 7.4. After stabilization, the organ is infused with alcohol or alcohol metabolites added to the perfusate [Kay et al., 2006; D'Souza et al., 1993]. The method has been used to study the effects of alcohol on physiological processes such as carbohydrate and lipid metabolism, the metabolic response of the liver to phagocytic stimuli, and to decipher the role of alcohol-induced oxidative stress in the development of fetal alcohol syndrome (FAS) [Topping et al., 1979; Lieber et al., 1975; D'Souza et al., 1993; Kay et al., 2006].

Advantages: The *in vitro* models are suited to study the direct effect of alcohol or its metabolites on the functions of a specific cell type or an organ. The experiments can be controlled for the amount of and exposure time to alcohol or its metabolites. The models allow the flexibility to expose naïve or previously *in vivo* exposed cells or organs *ex vivo* to alcohol alone and/or in combination with other diverse pharmacological agents or other cell types (co-cultures). Finally, the reproducibility of the data generated is better than with the *in vivo* models.

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Disadvantages: The removal of *in vivo* effects of communication between an organ, organ systems, and various cell types within an organ make extrapolation of the findings to *in vivo* events somewhat difficult. The exposure of cells to alcohol at concentrations above 100 mM may be toxic. The exposure of cells to high alcohol concentrations can affect the expression of adhesion molecules and, thereby, cell adherence; this may be a cause for concern if adherent cells are required. If not adequately controlled, the evaporation of alcohol may produce fluctuations in alcohol concentration to which the cells are exposed; this in itself may be good if one wants to study events when alcohol levels are waning but not when constant alcohol levels are desired. Finally, there is a possibility for loss of *in vivo* primary cell function(s) due to the limited *ex vivo* environment.

Cautionary note: Not all findings from *in vitro* experiments using cultured primary cells and/or cell lines or organ perfusion may translate to *in vivo* situations. In particular, concentrations of ethanol used to produce damage in vitro are often far greater than those experienced in vivo. Hence, careful consideration should be given when designing *in vitro* studies to ensure that the findings can be extrapolated to adverse effects of alcohol abuse observed in humans.

In vivo models of acute alcohol intoxication

In general, these methods are usually used in studies where effects of a single intoxicating drink are evaluated or in studies designed to mimic binge drinking wherein the animals are administered alcohol consecutively for a few days. The data generated using these models are likely to be more informative and extrapolative to human acute alcohol intoxication compared to the *in vitro* models. There are several human and non-human (vertebrate and invertebrate) models of acute alcohol intoxication that have been developed. However, most of the published data on the effects of acute alcohol intoxication on organ damage and immunity have been generated using small laboratory animals (Table 1). This is due to the feasibility of generating reliable data quickly and at a much lower cost compared to non-human primates. In addition to the most commonly used animal species, the use of invertebrates *Drosophila melanogaster*, *Caenorhabditis elegans*, and the lower vertebrate, Zebrafish (*Danio rerio*) have served as valuable tools to evaluate basic cell signaling mechanisms [Wolf and Heberlein, 2003;Guarnieri and Heberlein, 2003].

Oral gavage (I/G) or intraperitoneal (IP) injection—Alcohol (20% w/v) is administered either as an oral gavage directly into the stomach or as a single bolus injection given IP [Plackett and Kovacs, 2008; D'Souza El-Guindy et al. 2007; Nelson et al. 1989]. The control animals receive an equivalent volume of the vehicle (water or saline). The peak blood alcohol concentration is seen at around 30 minutes after alcohol administration, and the peak level attained depends on the amount of alcohol administered as well as the species and the age of the animals used [Walker and Ehlers, 2009].

Intravenous (IV) bolus injection followed by a continuous infusion—The model consists in giving an IV bolus injection followed by a continuous infusion of alcohol at a lower concentration. The continuous infusion allows maintenance of the desired blood alcohol levels at a constant level throughout the study period [D'Souza et al., 1989; Bautista et al., 1991]. The control animals receive equivalent amount of saline similarly.

Advantages: With training, the methods described above are easy to perform and allow precise control on the amount of alcohol given. The oral gavage and the IP procedures, although most suitable to mimic acute alcohol intoxication (single episode or binge drinking), have been used successfully also to mimic long-term alcohol use. Finally, the IV

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method allows the maintenance of the desired blood alcohol levels for hours or throughout the study period.

Disadvantages: The methods are somewhat stressful, and the IP and IV modes of alcohol delivery are not physiological. The learning curve may be more difficult for the oral gavage, and the IV procedure involves implanting a catheter in the inferior vena cava under general anesthesia and aseptic conditions.

Cautionary note: When deciding on the amount of alcohol to be administered to the animals, using the above techniques, consideration should be given to the species, strain, gender, body weight, and the nutritional status of the animals used. There is a threshold for alcohol tolerance, beyond which alcohol may become lethal. As previously mentioned, the tolerance to alcohol is not the same in all species or even amongst different strains within a given species, even when controlled for nutritional status and body weight. Females are more sensitive to alcohol than males. In addition, the blood alcohol concentration attained with a given dose of alcohol will depend upon the route by which the alcohol is administered. For example, peak blood alcohol levels will be achieved faster when alcohol is administered IV vs. IP or via an oral gavage and on an empty stomach. It is also important to note that forced alcohol administration can initiate a stress response, manifested by an increased release of glucocorticoids, which can be immunosuppressive [Collier et al., 2000; Meadows et al., 1993a; D'Souza El-Guindy et al., 2007]. Unlike the IP or IV methods, the oral gavage method of administering alcohol to laboratory animals is closer to human drinking; however, if not performed correctly, it has the probability of introducing a greater stress response than the IP or IV methods.

The above described methods of acute alcohol intoxication superimposed with a second hit (e.g. live bacteria, the cell-wall component of microorganisms, and viruses) administered locally (e.g. intratracheal or intraperitoneal) or systemically (e.g. intravenous) are used to elucidate the mechanisms by which acute alcohol intoxication affects immunity and/or induces organ damage. It is important to note that alcohol-induced changes in an inflammatory response, for example, are dependent not only upon the amount of alcohol in the systemic circulation at the time of the second hit but also on the type and the amount of stimuli used. Since the above models of acute intoxication induce stress, the stress response must be considered a variable when interpreting the results of experiments that utilize these delivery forms of alcohol. However, in some studies the stress response is an appropriate component of the experimental design; for example, those experiments involved in determining the immune effects associated with binge drinking [Albright et al., 2009; Collier et al., 2000].

The acute *in vivo* models of alcohol intoxication are most clinically relevant to health conditions such as those seen in humans suffering from traumatic injuries while intoxicated [Hadfield et al., 2001; Maier 2001]. Published animal data indicate that if alcohol is in the systemic circulation before a traumatic injury, the immune responses are suppressed. However, if alcohol is present in the systemic circulation 48 hours after a traumatic injury, the immune responses may be heightened [Plackett and Kovacs, 2008]. Acute or chronic alcohol consumption in humans produces changes in both the endocrine and the immune systems with interactions between each system affecting the other. The experimental models described above have been used to explore these interactions (Table 1). Despite comparable metabolism in the different models, the potential exists for differences in end-organ response. For example, in mice administered alcohol as a single dose (IP or as an oral gavage) for 5 consecutive days, to attain peak blood alcohol levels in the range of 80–100mg/dl each day, the serum cortisol levels rise almost four-fold in both models, 1 hour after alcohol administration. However, while with the IP method the serum cortisol levels

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return to baseline at the end of 24 hour, the levels remain persistently elevated at 24 hour in the animals that receive alcohol by oral gavage Also, while the delayed-type hypersensitivity (DHT) response to 2, 4-dinitrofuorobenzene and the splenocyte proliferation response to concanavalin A stimulation remain unaffected in the IP alcohol injection model, there is a significant suppression in the DHT response and the splenocyte proliferation with the oral gavage [Plackett and Kovacs personal communication].

Overall, the effects of acute alcohol intoxication on the basal immune and other functions are minimal or nil with these models. Yet, when the intoxicated animals are subjected to an *in vivo* immune challenge or a traumatic injury, functions such as the expression and secretion of many early innate immune response cytokines, the recruitment of inflammatory cells to the insult site, and carbohydrate metabolism are mostly suppressed [D'Souza El-Guindy et al., 2007; Zhang et al., 1997; Goral et al., 2008; Molina et al., 1991]. *In vivo* alcohol administration also affects ex-vivo response of immune cells to immune stimuli [Szabo and Mandrekar, 2009]. Several other studies using the above described *in vivo* models of acute alcohol intoxication are referenced in Table 1.

Models of Chronic Alcohol Abuse

Chronic alcohol abuse models are relevant to decipher the mechanisms by which long-term alcohol abuse, most common in humans, facilitates the development and progression of a number of diseases. Most laboratory animals, due to their natural aversion, do not consume sufficient amounts of alcohol voluntarily. Therefore, it is difficult to replicate entirely the alcohol-related pathophysiological states seen in alcohol abusing humans. However, chronic alcohol abuse models are being refined constantly, and several rodent lines that drink pharmacologically significant amounts of alcohol have been developed and used to study ethanol drinking behavior and its consequences [Bell et al., 2006; Grahame et al., 1999].

Alcohol-induced organ damage in humans is multifactorial and observed mostly after many years of alcohol abuse. The earlier laboratory models of alcohol abuse were developed to explore the development and progression of human alcoholic liver disease, as it is one of the major causes of morbidity and mortality among alcoholics. Over the years it has become evident that the ill effects of alcohol are felt in almost every organ (e.g. lung, gut, brain and muscles) and system (e.g. immune, endocrine, and CNS) in our body, and that there is an inflammatory component to alcohol-induced organ damage [Bautista, 2002; Brown et al., 2006; Szabo and Mandrekar, 2009]. This has led to the development of new and/or variations of originally developed models of chronic alcohol abuse. When deciding on a laboratory model of chronic alcohol abuse, it is important to keep in mind that the investigational potential of a model is dictated by the ability of the model to 1) allow for administration of sufficient amount of alcohol to reproduce the histopathological changes seen in humans, 2) allow for control of alcohol and nutrients as both these contribute to alcohol-related disease in humans, and 3) allow the ability to test for genetic and environmental factors that contribute to the development of the diseases. Described below are most of the chronic alcohol abuse models used to date to study alcohol-induced organ damage and immune variations. Other models such as the ones used for behavioral and dependence studies are alluded to briefly in the Table 1.

In vitro models of chronic alcohol abuse

Exposure of cells to alcohol in culture—Exposure of cells (primary or transformed) to alcohol in a culture medium for greater than 24 hours is usually considered as *in vitro* chronic alcohol abuse model. The method of alcohol exposure is same as described under *in vitro* model of acute alcohol abuse. In published studies, cells have been exposed *in vitro* to alcohol for as long as 10 days [Szabo and Mandrekar, 2008]. For long-term exposure

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experiments, the alcohol concentration in the culture medium is maintained by replenishing the alcohol content in the culture medium and in the open Petri dish every 2–3 days.

Advantages: The advantages are similar to the ones previously mentioned under *in vitro* models of acute alcohol intoxication.

Disadvantages: In addition to those previously mentioned under the *in vitro* models of acute intoxication, the isolated cells (e.g. hepatocytes) may not retain their *in vivo* phenotype for extended periods, and the *in vitro* exposure of primary cells to alcohol concentrations of greater than 100 mM for more than 48 h may affect cell viability.

In vivo models of chronic alcohol abuse

In most commonly used animal models of chronic alcohol abuse, the animals receive alcohol orally (either in liquid diet or in drinking water), enterally (via feeding tube or surgically implanted gastric catheter), or via inhalation (exposure to alcohol vapors) for extended length of time.

The Liquid Diet Model—Lieber and DeCarli developed a liquid diet to which alcohol could be added and offered to laboratory animals as the only source of food and water [Lieber and DeCarli, 1982, 1986]. The diet in its original and modified forms can be prepared in the laboratory or purchased from Dyets Inc. (Bethlehem, PA), Bio-serv (Frenchtown, NJ), and Purina Mills (Richmond, IN). While in the standard rodent chow pellets (e.g. 2018 Teklad Global) 23% of the total calories are protein derived, 17% fat derived, and 60% carbohydrate derived, in the Lieber-DeCarli regular control diet 18% of the total calories are protein derived, 35% fat derived, and 47% are derived from carbohydrate, respectively. In the alcohol diet, ethanol constitutes 36% of the total calories, with protein, carbohydrate, and fat accounting for 18, 11, and 35% of the total calories. In addition to the Lieber-DeCarli formulations, other commercial or custom made liquid diet formulations are also being used in alcohol research [Thompson and Reitz, 1978; Weinberg, 1985; Weinberg and Bezio, 1987; Fisher et al., 2002; Baumgardner et al., 2007; Chen et al., 1995; Tipoe et al., 2008].

In brief, age and weight matched rodents are housed in microisolater cages, and the animals assigned to the ethanol group are allowed free access to ethanol-containing liquid diet. The ethanol contained in the liquid diet is increased gradually from 1% to a final concentration of 5% over a 7 day period. Thereafter, the animals are maintained on the highest ethanol concentration throughout the feeding period. The animals assigned to the control group are pair-fed liquid diet containing maltose dextrin in amounts isocaloric to ethanol. Rats and mice generally dislike alcohol, but when provided with the alcohol-containing diet as the only source of food and water, they consume approximately 14–25 g alcohol/kg body weight per day after the first week on ethanol diet. In most studies designed to mimic chronic alcohol abuse in humans, the rodents are fed alcohol for 4–20 weeks. The model is also adaptable to baboons.

Published studies suggest that the composition of the liquid diet, in which the alcohol is administered, can influence significantly the intensity of alcohol effects. For example, the amount and the type of fat in the diet will influence the intensity of alcohol-induced organ damage. The liver fat and the rate of ethanol elimination is lower in rodents fed alcohol in high carbohydrate-low fat liquid diet compared to those fed identical amounts of alcohol in high fat-low carbohydrate diet [Fisher et al., 2002]. In a comparative study performed in C57Bl/6 female mice using Lieber-DeCarli regular ethanol diet and liquid AIN76A ethanol diet purchased from Dyets (Bethlehem, PA), ethanol was shown to affect liver antioxidant

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defenses. However, the mice fed the AIN76A ethanol liquid diet were able to maintain higher antioxidant capability than those on Lieber-DeCarli ethanol liquid diet [Chen, Xi, Cohen, 1995]. More recently, oral administration of ethanol in a liquid diet formulation rich in fish oil is reported to produce some of the pathological changes not observed in rodents fed alcohol in Lieber-DeCarli diet, and reproduce all pathological and biochemical changes observed with the intragastric intubation model [Tipoe et al., 2008]. These are among other studies suggesting that the choice of the diet may have significant impact on the results of the study, and support the contention for investigators to report the specific formulation used in their publications.

In rats and mice, the Lieber-DeCarli liquid diet treatment regimen induces the earliest form of damage to the liver and hepatocytes (i.e., fatty liver, characterized by fat deposits throughout the liver) [Lieber et al., 1965; Peng et al., 2009], but does not lead to the more serious forms of liver damage observed in humans (i.e., necrosis, inflammation and fibrosis) [DeCarli and Lieber, 1967; Lieber et al., 1967]. Yet, baboons fed a modified version of the aforementioned diet for several years were reported to develop cirrhosis [Rubin and Lieber, 1974]. Since its development, the Lieber-DeCarli diet in its original and/or modified form has been used widely in different laboratory animals to study alcohol effects not only on the liver but also on other organs, including the lung, heart, brain, and pancreas [Vander Top et al., 2005; Brown et al., 2007; Piano et al., 2007; Emanuele et al., 2005; Perides et al., 2005]. Using this model, the many biochemical consequences of alcohol abuse were identified. These include redox shift, induction of cytochrome P4502E1, acetaldehyde-protein adducts formation, enhanced lipid peroxidation, and depletion of mitochondrial glutathione, many of which are now viewed as potential mechanisms involved in alcohol-induced organ damage. Also, using this model, the role of gut derived flora or bacterial products in alcohol-induced activation of innate immunity via their binding to Toll-like receptors (TLRs) as well as the signaling pathways evoked in alcohol-induced inflammation have been characterized. Several of these findings are referenced in Table 1 and in other previously published review articles [Mandrekar and Szabo, 2009; Lau et al., 2009; Szabo and Mandrekar, 2009; Szabo et al., 2007; Boé et al., 2009; Lucrezio et al., 2008; Preedy et al., 1997]. An alcoholic pancreatitis model has been developed using Lieber-DeCarli diet in association with a second hit or a trigger factor such as lipopolysaccharide (LPS) to study the mechanisms of alcohol-induced pancreatitis [Vonlaufen et al., 2007b; Gukovsky et al., 2008]. In the model of alcoholic pancreatitis, *E. coli* LPS is administered in the systemic circulation via the tail vein once a week for the duration the animals are on the alcohol containing liquid diet. Using this model, investigators have identified an important role for LPS in the initiation and the progression of alcoholic pancreatitis [Vonlauffen et al., 2007a]. In addition, using this same laboratory animal model, it has been shown that alcohol and/or its metabolites as well as a necroinflammatory pathway (cytokines) may play an important role in the activation of pancreatic stellate cell, a key player in the pancreatic fibrogenesis process [Vonlauffen et al., 2007a; Vonlaufen et al., 2007b]. Similarly, the role for a trigger factor (second hit) in the development and progression of alcoholic pancreatitis was confirmed using this model of chronic alcohol abuse superimposed with viral (IP) infection [Jerrells et al., 2007].

Advantages: The diet is affordable and the contents can be manipulated with ease. The model allows for gradual increase of alcohol. Finally, there exists a large body of literature on the adverse effects of alcohol abuse that was generated using this model.

Disadvantages: The model does not mimic human drinking as animals are forced to consume alcohol every time they are hungry for food or thirst for water. The higher basal metabolic rates of rodents compared with humans may warrant the use of larger amounts of alcohol than the model allows. The switch from free access to regular solid chow and

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drinking water to a liquid diet high in fat, as the sole source of food and water, and the pair-feeding, may cause alterations, independent of alcohol, in the parameters investigated. For example, electron microscopy data on lung sections from mice pair-fed Lieber-DeCarli high fat liquid diet for varying lengths of time show lipid accumulation in the lungs, the intensity of which increases with the duration of feeding [D'Souza El-Guindy et al., unpublished observations]. Pair-feeding of control animals the liquid diet can also affect several aspects of the immune system [Meadows et al., 1992; Piano et al., 2001]. Finally, associated with the use of this model of alcohol feeding to non-human primates is the high cost of the species and maintenance during prolonged duration of ethanol feeding (2–5 years).

Cautionary note: When using liquid diet, it is best to prepare the diet using autoclaved water. Feed should be provided to the animals in sterilized feeders and feed changed daily. Prepared food should be stored at 4° C and not used beyond 2 days. Avoid the possibility of the feeders being clogged with bedding and feces by hanging the feeders sufficiently high, without impairing easy access of the animals to the diet. It is important to monitor the cages several times a day to ensure that the feeders are not clogged and/or have leaked. Some of the concerns that the liquid diet and pair-feeding may affect the basal levels of parameters investigated can be addressed by introducing additional groups in the experimental design (e.g. ad libitum chow-fed control animals, ad libitum liquid diet-fed control animals, and providing ad libitum water along with the liquid diet). On occasions, mostly during the winter months, our laboratory has observed [D'Souza El-Guindy et al., unpublished observations] a sudden and substantial deterioration in ethanol containing liquid diet intake by batches of mice (strain, gender, and age constant) purchased from the same vendor. There could be several reasons for this observation, including mice shipments coming from different breeding colonies and seasonal variations.

Other Liquid Diet Models—Sustacal (Mead Johnson, Evansville, IN) and Carnation Slender (Nestlé, Vevey, Switzerland) are two other liquid diets that have been used by investigators to maintain rodents on alcohol long-term [Bautista, 1995]. It is important to note that a comparative study performed using Lieber-DeCarli, Sustacal and Carnation Slender diets suggest that bioavailability of ethanol may not be identical in all liquid diets [de Fiebre et al., 1994].

The Intragastric Ethanol Infusion (IEI) Model—As previously mentioned, alcohol-induced liver damage in rodents using the Lieber-DeCarli liquid diet model does not go beyond the fatty liver stage. Therefore, Tsukamoto and coworkers [Tsukamoto et al., 1986, 2008] developed the rodent intragastric ethanol infusion (IEI) model. The model was developed based on the hypothesis that rats have a higher rate of alcohol breakdown (i.e., metabolism) than do humans and may require sustained higher blood alcohol levels than humans to induce liver damage. In this model, a catheter is implanted into the stomach under aseptic conditions. Alcohol added to the liquid diet (Lieber-DeCarli formulation) is infused via the implanted catheter directly into the stomach with the aid of an infusion pump. The rats can be maintained on the diet for several months without any complications, and blood alcohol levels of 250–500 mg/dl can be achieved and sustained. Using the IEI rodent model, alcohol along with other dietary manipulations is reported to produce fatty liver, localized necrosis, inflammation, and mild portal fibrosis (i.e., fibrosis in the vicinity of small bile ducts) [Tsukamoto et al. 1990], but not cirrhosis or other irreversible changes. The IEI model has also been used successfully to report alcohol-induced injury in other organs including the kidneys and pancreas (Table 1) [Tsukamoto et al., 1988; Razani-Boroujerdi et al., 1993]. Some novel findings that were added to the alcohol literature using this model include the cyclical pattern for alcohol clearance, the triggering of a second mechanism of alcohol clearance at blood alcohol levels above the threshold level of 250 mg/dl, the

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development of physical and metabolic tolerance as well as induction of CYP2E1 following chronic alcohol abuse [Tsukamoto et al., 1985; Ingelman-Sundberg et al., 1993]. The cyclical pattern of alcohol clearance was also reported in the self-administration monkey model, developed to mimic a binge drinking pattern of human alcoholics [Altshuler et al., 1975]. In general, the IEI model has advanced our understanding on the pathogeneses of alcoholic liver injury by providing valuable insights into possible mechanisms underlying alcohol's damaging effects at the cellular level, including the role secondary factors play in augmenting alcohol-induced organ damage [Tsukamoto, 1998].

Advantages: Blood alcohol concentration can be sustained at desired level for long durations. The dietary factors can be manipulated and a second hit such as LPS administered enterally with ease [Yeligar et al., 2009]. It is feasible to administer alcohol in amounts (as much as 43% of the total calories) that produce significant liver injury, elevated serum alanine aminotransferase levels, and lead to the development of severe steatohepatitis.

Disadvantages: The model is not physiological. It is expensive, labor intensive, requires constant monitoring of animals, and highly trained technical personnel to perform the catheter implantation surgery. The model does not produce diffuse liver necrosis and fibrosis without added oxidative stress nor does it reproduce alcoholic hepatitis, a clinically important feature of human alcoholic liver disease. For example, the formulation of the Lieber-DeCarli diet used by Tsukamoto-French in the IEI model [Tsukamoto et al., 1986, 1995] has high content of unsaturated fats and iron, and a low content of carbohydrates; a combination that generates substantial oxidative stress and exacerbates alcohol-induced oxidative stress.

Ethanol Agar Block Model—This model of chronic alcohol abuse was developed by Bautista [1997]. It consists in maintaining rodents on rodent chow, agar block containing alcohol 40% (v/v) plus peanut butter (0.5 g/kg) and alcohol 10% (v/v) supplemented water. The agar blocks are provided to the animals in Petri dishes. The alcohol concentration in the agar block is increased gradually to 40%. The pair-fed animals receive isocaloric chow, similar amount of agar without alcohol, and alcohol-free water. Using this model, Bautista [1997; 2002] and Bautista and Spitzer [1999] reported the presence of significant amounts of endotoxin in plasma, a 6-fold increase in serum aspartate aminotransferase, polymorphonuclear neutrophils (PMN) infiltration, and a mild fat accumulation in the livers of Sprague-Dawley rats fed alcohol for 16 weeks. Also, reported in these studies is an alcohol-induced increase in macrophage inflammatory protein-2 (MIP-2), superoxide release by Kupffer cells, and an up-regulation in the expression of CD18 and intracellular adhesion molecule-1 (ICAM-1) on PMN and hepatic cells. Furthermore, using this same model, long-term alcohol abuse is reported to prime rat Kupffer and endothelial cells for enhanced chemokine production and at the same time suppress phagocytic and chemotactic functions [Bautista 2002]. Gentry-Nielsen et al., [2001] have reported that rats fed alcohol for 8 weeks using this model mimic alcohol-induced disturbances in iron homeostasis seen in human alcoholics. The model has also been used in C57BL/6 mice by other investigators [Sepúlyeda et al., 2002] with a higher concentration of peanut butter (20 gm/kg) added to the 5% agar containing the 40% (v/v) alcohol and 40% alcohol supplemented drinking water. The alcohol in the drinking water is increased gradually starting at 10%. Using this model, the group has reported that alcohol exacerbates cytokine dysregulation during murine acquired immunodeficiency syndrome (AIDS) and exacerbates coxsackievirus B3 cardiomyopathy by heightening the cytokine imbalance to favor a Th2 response. These are some notable findings that have advanced our understanding significantly of the possible mechanisms by which alcohol abuse may induce organ damage and immune variations.

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Advantages: The model is easy to handle, affordable and has leeway for dietary and cofactors manipulation.

Disadvantages: From the reported blood alcohol concentrations of 125 ± 28 mg/dl, this model may not be ideal to attain and sustain very high blood alcohol levels. This most likely is due to significant loss of ethanol from the agar blocks by evaporation.

Agar Gel Diet Model—According to Bykov et al., [2004], 50% of the alcohol in the alcohol agar block is lost if the agar blocks are left in Petri dish for 6 hours, and at the end of 24 hours less than 20% of alcohol is left in the agar block. This finding led to modification of the original alcohol agar block model. The model consists of giving rodents free access to either the original or modified Lieber-DeCarli liquid diet prepared into a gel by addition of 0.5% agar. The alcohol in the diet accounts for 34.5 % of the total calories. In the control diet, these calories are accounted for by addition of 40% carbohydrate. The agar gel diet is provided to the mice in Falcon tubes equipped with 2 × 2 cm opening and mounted in a tilted position inside the pellet grid of the cage using metal strings. The animals are also allowed free access to a water bottle. According to the authors, the loss of alcohol to evaporation in this model is significantly less than in the original ethanol agar block model (26% at 6 hours and no additional loss at 24 hours). The gel consumption in this model is high enough to attain sustained high blood alcohol levels and within 6 weeks produce significant liver steatosis and elevation in plasma alanine aminotransferase levels.

Advantages: The model is simple compared to the oral liquid diet feeding or IEI model and can reproduce most of the hepatic changes observed in rats on the IEI model [Bykov et al., 2004; Ronis et al., 2004]. It does not require daily diet preparation and contains enough water in the gel to satisfy the animal's daily fluid requirement.

Disadvantages: There is still a significant loss of alcohol to evaporation.

Alcohol in Drinking Water Model—This model is a more practical solution for long-term ethanol exposure, and the model has been used in several species, including mice, rats and guinea pigs. Age and sex-matched animals are allowed free access to rodent chow and alcohol in drinking water (Single bottle - no choice). The alcohol concentration is increased gradually, and thereafter, the animals are kept on the highest concentration throughout the study. Alcohol preferring mice usually consume alcohol concentrations up to 20% w/v or 25.32% (v/v) and rats as high as 31.6% w/v or 40% (v/v). Control mice are allowed free access to rodent chow and drinking water [Coleman et al., 2008]. Depending on the research question, the model can be modified from single bottle – no choice, to two bottles – choice between water and alcohol, multiple bottles – choice between water and alcohol of varying concentrations, and drinking in the dark (DID). The two and multiple bottles choice variations are used mostly for behavioral and dependence studies (Table 1). In the DID variation, the experimental animals are allowed free access to water until such time when the water bottle is replaced with alcohol for 2–4 hours during the dark cycle. This variation has been used in studies such as ones designed to study the effects of human binge drinking on behavior and developing fetus. Though not very commonly used to study alcohol-induced organ damage (e.g. liver and pancreas), histological changes of fatty liver, inflammation, focal necrosis and perivenular fibrosis have been reported in rats maintained on 32.40% w/v alcohol in drinking water (single bottle-no choice) for 29 weeks [Keegan et al.,1995]. The effects of long-term alcohol use on the immune system have been well characterized using this model. The model was originally developed in the late 1970s, but has since been refined [Li et al.,1979; Juggi and Prathap, 1979; Blank et al., 1991; Blank et al., 1992; Blank et al., 1993; Meadows et al., 1989]. In this model, serum corticosterone levels are unaffected and

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animals do not become dehydrated [Blank et al., 1991; Cook et al., 2007; Sipp et al., 1993]. Blood alcohol levels detected can range from 0 to 400 mg/dl (87mM) depending on the amount of alcohol consumed and the time at which the blood is drawn for alcohol determination. For example, mice fed 20% alcohol ad libitum (Single bottle – no choice) appear to be visibly intoxicated early in the morning with blood alcohol levels as high as 400 mg/dl, with much lower levels later in the day. Many of the progressive changes in innate and acquired immunity that accompany short-term and long-term administration of alcohol using the single bottle-no choice model are described [Blank et al., 1991, 1992, 1993; Coleman et al., 2008; Cook et al., 2007; Edsen-Moore et al., 2008; Meadows et al., 1989; Song et al., 2002; Zhang and Meadows, 2005, 2008]. These include decreases in spleen cellularity and T, B and NK cell numbers, increased activation of T cells, decreases in peripheral NK and Langerhans cell numbers, compromised NK cell activity, delays in migration of dermal dendritic and Langerhans cells, and immune variations in other organs including the lung, liver, and heart in response to an immune insult [Edsen-Moore et al., 2008; Zhang, Meadows 2008; 2009; Xu et al., 2007; Ness et al., 2008]. Additional studies utilizing the different variations of the model are cited in Table 1.

Advantages: The model is physiological, inexpensive, and can be manipulated to mimic human drinking. The animal husbandry is not as cumbersome as is with some of the other models. Unlike some of the other models, it is easy to maintain sterility of the food and water provided to the animals. The model allows for manipulation of the diet and to superimpose other cofactors into the experimental design. Since the model works for mice, it allows building on existing literature on the immunology of mice and utilizing genetically modified strains of mice to explore the mechanisms by which alcohol impairs immunity and induces organ damage. Specific modeling of immunodeficiency of the chronic human alcoholic requires ethanol to be administered to the laboratory animal for a significant portion of its life span. In mice, it has proven to be necessary to administer ethanol daily for up to 32 wk (8 months) or longer to observe all the immune abnormalities that occur in middle-aged alcoholic humans. Such time spans that are usually problematic with many of the other common protocols for ethanol administration can be accomplished using this most practical model. The available variations of this model make it one of the best suited chronic alcohol abuse models for a wide range of studies including genetic, dependence and behavioral.

Disadvantages: It may be difficult to control for and maximize alcohol intake using any of three available variations of the model. Single bottle-no choice model is not suitable for use in non alcohol preferring, or low alcohol preferring animals, as their aversion to alcohol will affect the fluid intake. The model may not be feasible to mimic advanced stages of human alcoholic diseases.

Cautionary Note: When using this model for immunological studies care has to be taken to maintain a barrier protection against infectious agents (e.g. housing the animals in cages with filter tops or under other pathogen free conditions). The source of the water and alcohol used, especially for long-term studies, is particularly important since the presence of any extraneous substances can potentially influence the immune system of the animals. Sterilized distilled water that is passed through a filter to remove organic substances is ideal for administration to control animals and for preparing alcohol dilutions. It is necessary to use an undenatured analytical grade 190 proof (95%) ethanol and avoid the use of absolute (anhydrous) ethanol. Absolute ethanol may contain remnants of agents used to remove water during azetropic distillation. Since only 70% alcohol exhibits disinfectant properties, it is important to ensure that the alcohol administered to mice is germ-free. The alcohol should be sterilized through a 0.2 micron filter. When administering alcohol to low alcohol preferring rodents such as BALB/c, it is generally necessary to phase alcohol in by starting

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with lower concentrations (5–10%) of alcohol and gradually increasing the concentrations over several days to the desired level. One method for accomplishing this is described by Song et al., [2002]. Finally, although previously mentioned that blood alcohol levels as high as 400 mg/dl can be achieved, it may not be the case with all strains of mice and gender. Most of the published data using this model have been generated using female mice.

Exposure to Alcohol Vapors (Pulmonary Alcoholization) Model—Attaining high blood alcohol concentration continues to remain a challenge with laboratory models of chronic alcohol abuse and, in addition, the available models fail to induce a clear stable dependence on alcohol for longer than a month. De Witte has developed an alcohol inhalation model termed "pulmonary alcoholization". It is an efficient and reliable model in which constant blood alcohol levels of 200mg/dl can be achieved night and day with clear signs of dependence, as estimated in a double choice alcohol *versus* water drinking bottle. The De Witte model is a modification of the earlier models of Goldstein [1971], Roach et al., [1973], and Le Bourhis [1975]. The model can be applied to mice, rats and guinea pigs housed individually or in groups under standard conditions of 12 hour light dark cycle, 22°–23°C temperature, and 55% humidity. The animals are maintained in an isolated plastic chamber (160 × 60 × 60 cm) in which a mixture of alcohol and air is pulsed *via* a mixing system allowing the quantity of alcohol to be increased every two days during the experimental period to avoid tolerance. Blood alcohol levels are determined using blood collected from the caudal portion of the tail twice every week throughout the period of alcohol exposure. At the end of the alcohol exposure period, i.e. when blood alcohol levels have reached 200 mg/dl, animals are subjected to three successive periods: first to a full beverage deprivation period (the last 18 h of alcohol intoxication and the first 6 hours of withdrawal period), the presentation of a 10% v/v alcohol in water as the sole beverage during the following 18 h, and finally, a free choice of either water or 10% alcohol in water. During the free choice period, fluid consumption is recorded and the respective positions of both water and alcohol solutions are maintained during the entire period.

Advantages: There is no diet manipulation or pair-feeding involved. The model allows the possibility of inducing repeated cycles of pulmonary alcohol exposure interrupted by brief or long withdrawal periods. Using this model, physical dependence can be induced fairly rapidly with relatively minimal health-related problems. The dose and duration of exposure can be precisely controlled, and the level of intoxication can be maintained relatively stable during entire course of exposure as well as from one cycle to another [Griffin WC 3rd et al., 2009b]. Finally, the model allows animals to be treated simultaneously with other drugs such as Acamprosate or Rimonabant dissolved in drinking water [De Witte et al., 1990; 1996].

Disadvantages: The model may not be ideal to study advanced alcohol-induced organ damage such liver cirrhosis.

Cautionary Note: Parameters for chronic intermittent alcohol exposure that are optimal to producing escalation of drinking have not been well established.

The focus of the symposium entitled "Methods of Ethanol Application in Alcohol Model – How Long is Long Enough," was to summarize the laboratory animal models available and most frequently used to study the adverse effects of drinking on various organs and systems including the liver, lung, and the immune system and to highlight the pros and cons of each. These have been discussed in the review.

From the available literature describing alcohol effects on various organs and the immune system, it is apparent that despite comparable alcohol metabolism, the potential exists for

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differences in end-organ response. Therefore, choosing the most appropriate animal model to study the effects of alcohol on a specific organ or a system is a daunting task. From the wealth of information that is available and emerging on the pivotal role of the immune system in health and diseases, including alcohol-related diseases, it is an equally important task to select the right alcohol model to adequately address the research question asked. From the various models that are currently available for alcohol research, it is clear that there is no definitive model that is considered standard. Indeed, there are many factors to consider in this selection, and it is important to understand the advantages and disadvantages (limitations) of each model. Of paramount importance is the investigator's research question. This, in large part, will dictate the choice of the alcohol model and other interacting factors imposed such as an infectious disease, cancer, carcinogen, other drugs or a chemical toxin. The immune system is dynamic and influenced by factors such as the age of the animal, hormonal status, stress, and duration of alcohol exposure. While it is important to consider these influences and to minimize and/or control exogenous and extraneous influences that could complicate interpretation of the study's results, it is virtually impossible to design the perfect experiment. Lastly, the results in animal models might or might not reflect those of the human situation; however, to the extent possible, the human situation/condition should take center stage in selection of the appropriate animal model for the research question at hand.

Summary and Conclusions

Selecting an appropriate animal model for research related to the effects of alcohol on various organs and systems is not only necessary, but can also influence the outcome of the proposed experiment. Alcohol is different from other drugs that work by modulating a defined receptor system. Since it is a drug that diffuses widely throughout the body, it can affect multiple organs. This is especially true of the immune system, where alcohol can have dissimilar effects in different immune compartments [Zhang and Meadows, 2008]. Investigators can control many variables that influence the outcome of their study by choosing an appropriate animal model and experimental design. However, it is virtually impossible to control the secondary effects associated with alcohol intake such as nutrient availability or metabolism. Independent from the model utilized for an alcohol study, it is crucial that investigators make conclusions about their findings in the context of the experimental design and not generalize their results.

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Several of the research papers cited in this review are from Dr Ron Thurman and Dr Charles Lieber. Although they did not always agree, both contributed in a major way to the development and use of animal models for alcoholism. Both will be sorely missed by family, friends and colleagues following their recent deaths.

The views expressed in this manuscript are those of the authors and do not reflect the official policy of the Department of the Army, Department of Defense, or the U.S. Government.

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Table 1

Animal models of alcohol abuse

Model	Species	Application
Gavage (I/G)		
Animals are allowed free access to rodent chow and water. Alcohol or the vehicle is delivered orally into the stomach either via curved blunted tipped feeding needle attached to a syringe or a pediatric feeding tube [Plackett and Kovacs 2008; Majchrowicz 1975].	Rodents and rabbits	The model has been used extensively to study adverse effects of acute and chronic alcohol abuse alone (First Hit) and in association with other insults ("Second Hit" e.g. burn injury, bacterial challenge, viruses, hemorrhagic shock and smoking) on various organs and systems including the liver [McCuskey et al., 2005; Ito et al., 2004; Gong et al., 2008; Carmiel-Haggai et al., 2003; Wheeler and Thurman 2003; Bautista and Wang 2002], brain [Tiwari et al., 2009], blood [Pruett et al., 2004; Schwab et al., 2005], esophagus[Bor et al., 1998; Bor and Capanoglu 2009], myocardium [Kannan et al, 2004], skeletal muscle [Frost et al., 2005], lung [Zhang et al., 2007], gut [Kavanaugh et al., 2005; Choudhry and Chaudry, 2008; Li et al., 2009; Keshavarzian et al., 2009], immune system [Pruett et al., 2004; Bautista and Wang 2002], pancreas [Letko et al., 1991] and on fetus development [Maier et al., 1996; Gibson et al., 2000]. The model has also been used to study alcohol dependence and alcohol-induced epigenetic changes in a variety of organs [Faingold 2008; Kelly and Lawrence 2008; Kim and Shukla, 2006).
Intravenous (IV) infusion		
Animals have free access to rodent chow and water. Alcohol or the vehicle is administered IV as a bolus injection followed by a continuous infusion via a catheter implanted in jugular, femoral or cephalic vein [D'Souza et al., 1989; Zink et al., 2001; Gillmian 1989; Wilkinson and Rheingold, 1981].	Rodents, pigs, dogs, and sheep	The model has been used to study hypothermic, tolerance [Gilliam 1989], metabolic [Molina et al., 1989, 1991; D'Souza et al., 1991,1992] hemodynamic [Zink et al., 2001; Molina et al., 2004], immunological [Bautista et al., 1991; Bautista 2002], and neuroendocrine [Molina et al., 2004] changes induced by acute alcohol intoxication alone and in association with a Second Hit in numerous organs including liver [Bautista 2002; Spitzer and Zhang 1996], muscle [Pagala et al., 1995; Vary and Lang 2008], blood [Spitzer and Zhang 1996], brain [Pawlosky et al., 2010], and the lung [Spitzer et al., 2002].
Intraperitoneal (IP) injection		
Animals have free access to rodent chow and water. Alcohol or the vehicle is administered as an IP injection [D'Souza et al., 2007].	Rodents and guinea pigs	The model has been used extensively, alone and in association with a Second Hit, to study the adverse effects of acute alcohol intoxication on a variety of organs including lung [D'Souza et al., 2007; Bagby et al., 1998; Kolls et al., 1998; Walker et al., 2008; Lanzke et al., 2007; Happel et al., 2006], liver [Blanco et al., 2005; Emanuele et al., 2007], muscle [Paice et al., 2002; Lang et al., 2004; Vary and Lang, 2008; Salem et al., 2002], male reproductive axis [Emanuele et al., 2008], the cardiovascular system [McDonough et al., 2002], brain [Zhao et al., 1997; Guaza et al., 1983], the immune system [Bird and Kovacs, 2008; Emanuele et al., 2009], and on developing fetus [Da Lee et al., 2004] and the fetal lung [Wang et al., 2007]. In addition, the model has also been used to show that a single episode or binge drinking may augment the transition of pancreatic edema to acute pancreatitis [Letko et al., 1991].
Alcohol in liquid diet (Oral)		
A. Lieber DeCarli liquid diet The animals have free access to alcohol in Lieber DeCarli liquid diet. The model involves pair-feeding of control animals isocaloric diet [Lieber and DeCarli 1982].	Rodents and non human primates (baboons and rhesus macaques)	Variation **A** of the liquid diet model has been used widely, alone and in association with another insult, to study biochemical, physiological, morphological, immunological and pathological consequences of chronic alcohol abuse on numerous organs. These include the liver [Lieber et al., 1989; Earnest et al., 1993; Bhopale et al., 2006; Deaciuc et al., 2001], pancreas [Perides et al., 2005; Kubisch et al., 2006; Vonlaufen et al., 2007a; Gukovsky et al., 2008], gastrointestinal tract [Fleming et al., 2001], lung [Mason et al., 2004; Brown et al., 2001; Vander Top et al., 2005; Brown et al., 2007], bone [Sampson et al., 1996], muscle [Kim et al., 2001], brain [Herrera et al., 2003] as well as the immune [Jerrells et al., 1990; Worrall and Wilce, 1994] and neuroendocrine [Emanuele et al., 2005] systems. The model has also been used in identifying mechanisms involved in pathogenesis of alcoholic diseases [Roychowdhury et al., 2009; Hritz et al., 2008], in the development of tolerance

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Model	Species	Application
Gavage (I/G)		
		and physical dependency on alcohol, fetal alcohol syndrome [Parnell et al., 2006], and in identifying novel markers of alcoholic disease [Bradford et al., 2008].
B. Fish oil rich liquid diet formulation The animals have free access to alcohol in a liquid diet formulation rich in fish oil. The model involves pair-feeding of control animals isocaloric diet [Tipoe et al., 2008].	Rodents	Variation **B** of the liquid diet model is reported to produce some of the pathological changes not observed with the Lieber-DeCarli liquid diet and also reproduce all pathological changes observed with the intragastric ethanol infusion model [Tipoe et al., 2008].
C. Sustacal or Carnation Slender liquid diet These are commercially available flavored and sweetened liquid diets to which alcohol is added. Animals in the alcohol group are allowed free access to the alcohol containing diet while those in the control group are pair-fed isocaloric diet [de Fiebre et al., 1994].	Rodents	Variation **C** of the liquid diet model has been used to study the effects of chronic alcohol abuse on developing brain (prenatal exposure) [Zhou et al., 2001], the hypothalamic-pituitary-thyroid axis [Zoeller et al., 1996], and the immune system [Bautista 1995].
Tsukamoto- French (IEI) Model (Enteral)		
The diet of desired composition is prepared in the liquid form with or without alcohol and administered continuously into the stomach via a permanent gastric cannula (enteral). The control animals are pair-fed isocaloric diet [Tsukamoto et al., 2008].	Rodents, guinea pigs, and non human primates	The model in association with a second hit [Tsukamoto et al., 2009] has been used primarily to elucidate mechanisms involved in the development and progression of alcoholic liver disease [Tsukamoto 1998; Wheeler et al., 2003; Hines and Wheeler, 2004; Uesugi et al., 2001, 2002; Deaciuc et al., 2004; Baumgardner et al., 2007; Oliva et al., 2009]. The model has also been used in studies such as those designed to study ethanol-induced bone loss during pregnancy, to explore the implications of nutrition ethanol interaction during gestation on the fetus [Shankar et al., 2006a, 2006b}, and to study the effects of chronic alcohol abuse on immunity [Siggins et al., 2009]. The enteral protocol of this model has been used to develop a rodent model of chronic alcohol-induced pancreatitis and also an intragastric model suited for use in genetically manipulated mice [Kono et al., 2000 and 2001].
Alcohol in drinking water		
A. Single bottle-no choice The animals have unrestricted access to rodent chow and either water or alcohol in drinking water [Coleman et al., 2008].	Rodents and non-human primates	Variation **A** of the model is used in association with a Second Hit to explore the mechanisms by which chronic alcohol abuse induces metabolic [Seiva et al., 2009; Kudo et al., 2009; Macho et al., 2003], morphological [Evrard et al., 2006; Andersson et al., 1995; Sarphie et al., 1996], immunological, and functional [Cook et al., 2004; Zhu et al., 2004; Song et al., 2002; Abdallah et al., 1988; Blank et al., 1991; Meyerholz et al., 2008; Edsen-Moore et al., 2008] changes in organs and cells and increases susceptibility to infections [Jerrells et al., 2007], [Masui et al., 2002]. The model has also been used to study effects of (uterine) chronic alcohol abuse on fetal lung development [Gauthier et al., 2005], on bone mineral content [Broulik et al., 2009], and in the exercebation of liver damage induced by other hepatotic agents [Nadkarni and D'Souza 1988; Nadkarni et al., 1983].
B. Two bottle- free choice The animals have free access to rodent chow and an unrestricted choice between water and alcohol [Rhodes et al., 2005; Ehringer et al., 2009]. **C.** Multiple bottles – free choice The animals have free access to rodent chow, and unrestricted choice between water and alcohol of varying concentrations [Yoneyama et al., 2008]. **D.** Drinking in the dark (DID) The animals have free access to rodent chow and unrestricted access to water except when alcohol is substituted for water for 2–4 h per day, usually 3 h into the dark cycle) [Rhodes et al., 2007; Ehringer et al., 2009].	Rodents and rhesus macaques	Variations **B C** and **D** are very valuable for studies designed to explore neurochemical and molecular pathways that contribute to alcohol abuse. These variations have also been used in studies such as ones designed to identify the genetic basis for alcohol preference and dependence [Samson et al., 1998; Rhodes et al., 2007; Yoneyama et al., 2008; Dyr and Kostowski, 2008; Blednov et al., 2009; Tabakoff et al., 2009], to study the effects of alcohol abuse alone or in association with other drugs of abuse on behavior and growth [Knackstedt et al., 2006; Zou et al., 2009; Farook et al., 2009], and to identify the role of delta opioid receptors in alcohol-drinking behavior [Roberts et al., 2001]. The DID model is used quite commonly to mimic prenatal human binge drinking [Boehm et al., 2008].

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Model	Species	Application
Gavage (I/G)		
Operant Models		
In these models animals are trained to perform a certain task in order to self- administer alcohol orally, intravenously or directly into the stomach through surgically implanted catheters. The consumption of alcohol is initially initiated by providing a 10% solution of alcohol as the only available fluid several days before operant training, adulterants such sucrose or saccharin may be added to the alcohol solution to induce robust reliable self-administration [Green and Grahame, 2008].	Rodents and rhesus macaques	The Operant Models are mostly suited to study neurobiology of craving and reinforcing effects of alcohol [Koob 2000; Meyer 2000; Chu et al., 2007; Schwandt et al., 2010].
Alcohol in Agar		
The animals have free access to rodent chow, alcohol containing agar and drinking water containing alcohol. Involves pair-feeding isocaloric amounts of rodent chow and agar containing dextrin/maltose [Bautista et al., 1995].	Rodents	The model has been used to study chronic alcohol abuse-induced disturbances in iron homeostasis [Gentry-Nielsen et al., 2001], cellular immune responses during retroviral infection [Sepúlveda et al., 2002], and to explore the mechanisms of alcohol-induced liver [Bautista et al., 1995, 1999, 1997, 2002] and muscle damage [Vary et al., 2002; 2008; Lang et al., 1999, 2004].
Alcohol in Agar diet		
In this model the animals have free access to jellified liquid diet containing alcohol. Involves pair-feeding jellified isocaloric diet [Bykov et al., 2004].	Rodents	The model has been used to study mechanisms of alcohol-induced liver injury [Bykov et al., 2004; Ronis et al., 2004]
Alcohol vapor inhalation		
The animals are maintained in special chambers in which a mixture of alcohol and air is pulsed via a mixing system [Le Bourhis, 1975, Gilpin et al., 2008].	Rodents	This model is suited for testing of somatic and motivational aspects of alcohol dependence and neurobiological mechanisms underlying chronic alcohol drinking, alcoholism, and abnormal alcohol-seeking behavior [Gilpin et al., 2008, 2009, Becker and Lopez 2004, Lopez and Becker 2005, Rimondini et al., 2003, Griffin WC III et al., 2009a, Dhaher et al., 2008, Finn et al., 2007].
Models of alcohol dependence		
These models are usually a combination of two or more of the above described models of chronic alcohol abuse.	Rodents	These models have mostly been used for alcohol preference and dependence studies and for exploring the neurochemical, biochemical and immunological changes induced by binge drinking or chronic alcohol abuse. [Crews and Nixon 2009; Ward, 1987; Ward et al., 2009, Griffin WC 3rd et al., 2009b]
Second Hit Models for Alcoholic Disease		
Published research suggests that gene- environment interactions [Whitcomb 2006; Stacey et al., 2009] play a major role in the pathogenesis and individual predisposition to alcoholic disease. Since laboratory models of alcohol abuse (First Hit) alone may not mimic all aspects of human alcoholic diseases, various "Second or Multiple Hit" models have been developed. These include superimposing nutritional, bacterial, viral, and hemodynamic factors, and/or using genetically manipulated rodents with the above models of alcohol abuse [Tsukamoto et al., 2009; Vonlaufen et al., 2007b; Gukovsky et al., 2008; Kono et al., 2001].	Rodents, guinea pigs, rabbits, pigs, and non-human primates	These models are used extensively to elucidate the mechanisms by which alcoholic diseases develop and progress.