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TOPIC HIGHLIGHT

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Alcoholic liver injury: Influence of gender and hormones

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Abstract

This article discusses several subjects pertinent to a consideration of the role of gender and hormones in alcoholic liver injury (ALI). Beginning with an overview of factors involved in the pathogenesis of ALI, we review changes in sex hormone metabolism resulting from alcohol ingestion, summarize research that points to estrogen as a cofactor in ALI, consider evidence that gut injury is linked to liver injury in the setting of alcohol, and briefly review the limited evidence regarding sex hormones and gut barrier function. In both women and female animals, most studies reveal a propensity toward greater alcohol-induced liver injury due to female gender, although exact hormonal influences are not yet understood. Thus, women and their physicians should be alert to the dangers of excess alcohol consumption and the increased potential for liver injury in females.

Key words: Alcohol; Liver injury; Female; Estrogen; Oxidative injury

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INTRODUCTION

Much work has been done in recent years to provide insight into the pathogenesis of alcoholic liver injury (ALI). Liver injury from alcohol has many components, including intrahepatic events [such as hepatocyte alcohol metabolism, generation of reactive oxygen species (ROS) that result in cellular oxidative stress, loss of protective enzymes and transporters] and extrahepatic stimuli, such as gut-derived endotoxin, induced by ethanol exposure. These factors can act separately or in concert to trigger common pathways involving an inflammatory cytokine cascade. These cytokines bring about interplay between different functional cell types in the liver, i.e. hepatocytes, Kupffer cells, stellate or Ito cells, mononuclear cells, and neutrophils. The outcome of such interplay between biochemical alterations and changes in stimuli is the induction of early metabolic dysfunction in the form of fatty liver, followed possibly by an inflammatory response (alcoholic hepatitis), and progression to a more progressive liver disease (fibrosis and cirrhosis). The current model of ALI pathogenesis involves roles of different cytokines that induce various transcription factors, which in turn modulate expression of different genes (reviewed)[1-9]. Contributing to this injury is mitochondrial dysfunction, with resultant reduced cellular ATP

levels, increased oxidative stress brought about by mitochondrial injury and altered activities of enzymes such as CYP2E1 and NADPH oxidase, along with a decrease in protective antioxidant enzymes such as superoxide dismutase and glutathione peroxidase[8,9].

The aims of this review are to examine the relationship between sex hormones, changes in hormone levels due to alcohol exposure, and potential roles for sex hormones in the pathogenesis of ALI. To explore such relationships, evidence from both human studies and animal models will be considered. Human studies include collection of serum for hormone analysis under a variety of patient populations, including long-term heavy drinkers and alcoholics, and normal subjects challenged with acute doses of alcohol.

Most of the biochemical mechanisms of alcohol-related injury have been discovered using animal models, particularly rodent models. While studies in human populations are critical to determine demographics, risks, and outcomes, human studies have a number of drawbacks, such as the difficulty of reliable reporting of alcohol intake by subjects, genetic differences in ability to metabolize alcohol, coexisting disease, nutritional deficiencies, and individual timelines in progression of disease. In animal models, factors such as alcohol dose, age, sex, and dietary composition can be controlled by the investigator to limit differences among experimental and control groups. From such studies, it is becoming clear that certain dietary factors are critical in the development of ALI. Further, use of animal models allows testing of potential therapeutic agents prior to testing in human populations.

ANIMAL MODELS AND DIETARY FACTORS

The first diet protocol used widely to study ALI was that of Lieber and DeCarli[10]. This liquid diet is easy to administer and provides nutritional equivalence by pair-feeding, in which the control animal of the pair receives the exact number of calories consumed by the experimental animal the previous day. This model typically results in alcohol-induced fatty liver, but not the degree of fibrosis and/or inflammation observed in human liver disease. Thus, this model is not appropriate for studying factors involved in generation of cirrhosis. More recently, an enteral feeding model, that of Tsukamoto and French (T/F)[11,12], has been used to induce inflammation and fibrogenic changes more like that seen in human disease. However, major concerns regarding this model are that alcohol is administered 24 h a day, surgery is required for gastric tube placement, infection is a risk, and the equipment to maintain diet administration is expensive. The original T/F diet has been modified by several groups to produce more or less liver injury, based on the type of fat present. For example, Nanji *et al*[13-15] showed that addition of medium-chain saturated fatty acids result in prevention or reversal of ALI, presumably due to decreases in lipid peroxidation and CYP2E1 induction. Other studies showed that polyunsaturated fats result in more severe injury[16]. Of interest with respect to sex, these diets result in an increase of fat, inflammation, and necrosis in females but not males on the control diets containing no ethanol, suggesting that diet alone may have sex-specific effects[17,18]. Tipoe *et al*[19] have now refined this dietary approach by developing a diet that results in significant liver injury particularly in females; the details of this fish oil-containing diet have recently been published. Our studies with this diet have shown that female rats fed alcohol have significant elevations of serum markers such as aspartate aminotransferase, alanine aminotransferase, and endotoxin, and much more fatty change and inflammatory foci in their livers than male rats fed the same diet (manuscript in preparation). Thus, the roles of dietary components, along with differences in response to ethanol between the sexes, have only begun to be elucidated.

ALCOHOL INGESTION CHANGES SEX HORMONE LEVELS IN BOTH SEXES

Clinical observations indicate that females are at greater risk than males for liver injury due to several different causes[20]. One of these causes is ALI; women develop severe ALI and cirrhosis at lesser intake of alcohol and fewer years of exposure[1]. While exact mechanisms remain obscure, these findings strongly suggest an involvement of sex hormones in the pathophysiology of alcohol-induced liver disease. Much research has shown that chronic alcohol ingestion changes dramatically the hormonal milieu of blood and the liver in both sexes. The liver is a key player in this interplay, because in addition to being the site of steroid hormone metabolism, the liver is responsive to sex hormones. Our laboratory has studied the effect of sex hormones on the liver for more than 25 years; we performed much of the characterization of sex steroid receptors in human and rat liver, and our work has examined the mechanism(s) of alcohol-induced alterations in hepatic sex hormone function and metabolism.

Because the liver itself is an end organ for sex hormone actions, an alcohol-induced disruption in sex hormone status can result in a self-perpetuating and detrimental pattern of sex hormone metabolism. Our studies and those of others have identified specific mechanisms by which sex hormone homeostasis changes in both male and female rats, reviewed in this section and the next. Newer information on the effect of alcohol on the gut barrier function also enhances our understanding of the relationships between sex hormones and injury to the liver.

Chronic alcohol ingestion results in significant alterations in sex hormone levels and function; the spectrum of sexual dysfunction has been reviewed[21-25]. Alcoholic men often display phenotypic changes due to an inability to maintain appropriate hormone balance, displaying low

serum testosterone and elevated estrogen levels. Such changes have been reproduced in rat models as well. Our studies reveal that in male rats with chronic alcohol exposure, key hepatic enzymes involved in hormone homeostasis are profoundly affected. Alcohol-fed male rats demonstrated a significant reduction in serum testosterone, and reduced hepatic activity of two androgen-dependent estrogen-clearing enzymes [estrogen sulfotransferase (EST) and estrogen 2-hydroxylase (E2-OHase)], resulting in increased serum estradiol levels[26-29]. Decreases in serum testosterone, due to alcohol's toxicity to the testes, occurs early in alcohol exposure, whereas increases in estrogen levels are evident upon longer periods of feeding, thus hypogonadism precedes liver feminization. The studies emphasize that the disruption of the androgen-estrogen balance resulting from chronic alcohol ingestion in male rats can drastically affect the hepatic route and rapidity of sex steroid metabolism[26].

Endocrine changes in chronic alcoholic females have been less well studied. Alcoholic females in their reproductive years also display profound abnormalities in hormone homeostasis and reproductive potential. Chronic alcoholic women may have menstrual cycle disorders, including amenorrhea, anovulatory or irregular cycles, and luteal phase dysfunction[21,23,24,30-33]. Fertility is often (but not always) impaired[31]. Onset of menopause is earlier in these women[32]. Hypothalamic and pituitary dysfunction is also observed, with luteinizing hormone (LH) and follicle stimulating hormone (FSH) release suppressed, although hyperprolactinemia is common. Estrogen levels in the amenorrheic women may be reduced or normal[30-33], and may differ among ethnic groups[34]. In contrast, other studies showed that acute alcohol ingestion in nonalcoholic women, especially when administered simultaneously with hypothalamic/pituitary stimulation, results in increased estrogen levels[35]. Whether this increase is due to increased synthesis or decreased hepatic clearance is not known, but the latter is suggested by the results of a study showing that women taking oral contraceptives had higher estrogen levels than control subjects after an acute dose of alcohol[36]. Rat studies have shown that chronic alcohol ingestion leads to lower estrogen levels, and in anovulatory cycles characterized by reduced progesterone[37], similar to findings demonstrated in premenopausal alcoholic women. Not all chronic studies in rats show a change in estrogen levels, however; after 18 mo of alcohol treatment, estradiol levels in one study remained normal[38]. In postmenopausal chronic alcoholic women, however, estrogen levels appear to be higher (approximately 2-fold) than in controls, and even higher when cirrhosis is present[32,33]. In these women, pituitary hormones (LH, FSH) are lower than in controls, although prolactin is elevated[33]. In postmenopausal women on hormone replacement therapy, an acute dose of alcohol results in dramatically increased estrogen levels, presumably as a result of decreased hepatic metabolism of estrogens[39]. Beyond this study, the effect of chronic alcohol ingestion on hepatic metabolism of sex steroids in fertile or postmenopausal women does not appear to have been studied in any detail. All of these results taken together suggest that if estrogen (and progesterone) status is important in development of ALI, then the impact of hormones can differ, depending on menopausal/postmenopausal status and hormone replacement therapies, when alcohol ingestion is part of the equation.

IS ESTROGEN A PATHOGENIC COFACTOR IN ALI?

Some evidence suggests that estrogen may be an important pathogenic cofactor in development of alcohol liver injury. For example, females are more susceptible to ALI than males, both in human studies[40-42] and in certain animal models of ALI[43]. Further, female sex is considered as a risk factor for development of more profound and irreversible types of ALI[42]. Estrogens are also implicated in other types of liver disease. In acute fatty liver of pregnancy, estrogen results in of mitochondrial injury[44] by decreasing β-oxidation of fatty acids, altering the function of mitochondrial proteins and enzymes in mitochondrial uptake, and causing ultrastructural changes such as mitochondrial enlargement. These changes lead to fat accumulation in the liver, in the form of micro/macrovesicular steatosis[45]. Of interest, estrogen also mediates mitochondrial ROS generation in breast cancer cells, resulting in increases in proliferation signals[46]. It is known that mitochondrial damage is a major apoptosis-triggering event, which is necessary for further development of ALI[47-49]. In an enteral feeding rat model (T/F), Thurman[8] documents an increased susceptibility in female rats, showing that females had an increased pathology score, neutrophil infiltration, and increased levels of circulating endotoxin; the authors concluded that increased endotoxin, LPS, and Kupffer cell activation are responsible for the increased ALI in these rats. Further, it has been shown that the sensitivity of Kupffer cells to endotoxin is increased by very high doses of estriol (a weak estrogen with high levels in pregnancy)[50]. In a recent study in humans, estrogen treatment was shown to increase inflammatory cytokine production[51]. Other reports stated that estrogen treatment enhances ALI in ovariectomized females[52]; however, no data comparing control rats treated with estrogen were presented, so it is difficult to factor out the effects of estrogen alone from the effects of the combined treatment (alcohol plus estrogen). None of these studies determined whether the effect of estrogen is at the level of the liver, the gut, or both. This factor is important, since it is clear that sterilizing the gut with antibiotics prevents much of the ALI in male rats so treated[53]. In a Lieber/DeCarli model, post-pubertal females have been shown to incur more alcohol-induced oxidative injury than males, as evidenced by increased levels of malonaldehyde[54]. There is also evidence that estrogens induce potentially more damaging immunological responses[55], due to estrogen's effects on macrophages[56], although such effects may be dose-dependent[57,58]. Estrogen receptors (ER) have been

identified in both Kupffer cells and sinusoidal endothelial cells[59] as well as in hepatocytes[27], although the levels of ERs are about 10-fold lower in the nonparenchymal cell types. These latter cells, along with hepatocytes, are major producers of those cytokines shown to have crucial roles in pathogenesis of ALI[60,61], tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and interleukins, and they also attract PMNs, which produce additional cytokines and ROS, all of which are contributing pathogenic factors in ALD[1]. One report notes that injury in a low-carbohydrate alcohol feeding model is greater in females[62], and that such injury is partially prevented by administration of an antiestrogen toremifene[63]. The antiestrogen primarily reduced inflammation and necrosis, reduced TNF-α release from isolated Kupffer cells, enhanced selenium-glutathione peroxidase activity, and reduced CYP2E1 activity, as compared to the control groups. Lastly, there is known cross-signaling between estrogen and growth factor (EGF, TGF-β, HGF) intracellular pathways. The estrogen appears to act through nuclear and possibly cell-surface ERs, and post-transcriptional regulation of signaling elements, with impact on cell growth, regeneration and fibrosis[64-67]. Since similar changes are observed in alcohol-induced liver injury, the conclusion was reached that estrogen must play a role in this injury. It is also possible that males might also be susceptible to estrogenic influences, since hyperestrogenization is common with chronic alcohol ingestion, noted above. Our studies[28] showed that androgen-responsive functions in male rat liver are altered during chronic alcohol exposure, with feminization of male rat liver as a consequence of lowered testosterone levels and elevation of estrogen in liver and serum. The latter appears to be due to a time-dependent reduction in activity of androgen-controlled estrogen metabolizing liver enzymes E2-OHase and EST[26]. In males, and in both the Lieber/DeCarli[26] and the intragastric models[29], the greatest liver injury is observed under circumstances of high estrogen and low testosterone. However, the serum estradiol level in male rats, regardless of alcohol intake, is always several-fold (7-10 ×) lower than in females. Thus, if estrogen is a cofactor in ALI, then it stands to reason that females should be more affected by this hormone than males. In addition, in both female rats and women, progesterone, the "counter-hormone" to estrogen, is reduced with alcohol consumption, since ovulation is less likely to occur. Thus, the estrogen present is less opposed by progesterone. It should also be noted that no studies have been done to determine if the other female sex hormone, progesterone, has any role in causing, or preventing, liver injury.

DIFFERENTIAL GENE EXPRESSION IN FEMALE RATS FED ETHANOL

Several studies have shown that the livers of female rats fed alcohol express different gene sets than males in response to injury. Tadic *et al*[68] showed that a microarray analysis in the Lieber/DeCarli model of alcohol exposure, female rats fail to up-regulate a number of hepatoprotective genes in response to alcohol, and that those genes that regulate various compensatory metabolic pathways also do not respond appropriately. Using the same model and with addition of lipopolysaccharide treatment, Banerjee *et al*[69,70] also showed differential expression of genes involved in oxidative stress and inflammation. Sharma *et al*[71] found similar changes in genes involving oxidative stress and metabolic adaption. Donohue *et al*[72] demonstrated a lack of induction of hepatoprotective enzymes and increased lysosomal leakage in female rats fed an ethanol- and fish-oil containing diet. While these studies reveal differences in response to metabolic stress in females caused by alcohol, it is not clear how many of these changes are due directly to hormone influences.

RELATIONSHIP OF GUT INJURY TO ALI

The intestinal barrier to antigens, food materials, bacteria, invasive organisms, such as viruses, is a gatekeeper that is critical in maintaining the health of the host. The barrier is a continuous epithelial cell layer, connected by tight junctions that may permit passage of small amounts of materials under normal circumstances, but in pathological conditions, become leaky, permitting the inappropriate passage of particles, bacteria, macromolecules, and immunogenic substances. Stress, infection, chemical insult, surgery, and burns are among the conditions that result in gut injury. The response of the intestine is to secrete fluid and ions to wash away the causative agent, and an immune response involving recruitment of inflammatory cells, and mucus secretion. Key to host protection, however, is maintenance of tight junction integrity in the gut. The tight junctions are highly specialized complexes at the apical end of epithelial cells, and form the actual barrier between the intestine and the blood. Tight junctions are characterized by complex interactions among a group of transmembrane proteins including claudins, occludins, and junction adhesion molecules that interact with other proteins, such as the family of zonula occludens proteins. Complex interactions among these proteins involve anchoring the protein complexes with the actin cytoskeleton. Loss of the appropriate interactions among members of this complex can lead to loss of integrity, and result in a gut that is leaky to bacteria, bacterial products such as endotoxin and other toxins, immunogenic food particles, and other substances[73,74].

The evidence linking gut-derived endotoxin in the blood as a factor in ALI is compelling. Endotoxins, for example, lipopolysaccharide originating from the cell wall of gram-negative bacteria that populate the gut, are maintained almost exclusively in the lumen as a result of the intestinal barrier afforded by a healthy gut mucosa. However, in circumstances such as stress, trauma, or other gut injury (such as chronic alcohol ingestion),

the mucosal barrier becomes more permeable, allowing endotoxin to cross the mucosa and enter the portal circulation. In the healthy liver, Kupffer cells maintain a constant surveillance and will sequester and degrade endotoxin. However, if Kupffer cell function is impaired, or if levels of endotoxin are excessive, an inflammatory response can result. In addition, endotoxin will be taken up by the resident macrophage population within the intestinal mucosa, triggering an inflammatory response that can lead to further impairment of mucosal barrier function. Endotoxin is linked to ALI in that clinical observations show that endotoxin levels are high in patients with ALI[1,73,74]. Animal studies show that circulating endotoxin and subsequent liver injury can be eliminated by sterilization of the bowel with antibiotics[53] and reduced by dietary strategies such as oat fiber[75] and zinc supplementation[76] that presumably help maintain barrier function. While the appearance of endotoxin in the circulation implies the possibility of gut injury, very few studies address directly the mechanism of alcohol injury to the gut beyond nutrient uptake considerations. Several studies in Caco-2 cell monolayers, which are derived from human epithelial colorectal adenocarcinoma, show that alcohol[77] and its first metabolic product, acetaldehyde[78] can result in disrupted tight junctions and increased paracellular permeability in these cells. Acetaldehyde treatment results in loss of tight junctions because it results in redistribution of tight junction proteins[78], an effect that may be mediated by a tyrosine kinase-dependent mechanism. Increased endotoxin in rat alcohol feeding models have been shown, and reports document a greater endotoxin level in female rats fed alcohol, and more liver injury as well[17,43]. Our recent studies using the Nanji rat alcohol feeding model have indicated that young female rats show significantly more gut inflammation, injury, and loss of barrier function due to alcohol ingestion than do male rats (submitted for publication).

Once endotoxin is released by the intestine to the blood, it is bound with high affinity to lipopolysaccharide binding protein (LBP), an acute phase protein synthesized in the liver. The LBP binds endotoxin to its N-terminus through its lipid A moiety, whereas its carboxy terminus is a ligand for the CD14 receptor on the Kupffer cell membrane. Binding of the LBP-LPS to CD14 triggers the activation of Toll-like receptor 4, with subsequent induction of pro-inflammatory transcription factors (NF-κB), the production of pro-inflammatory mediators such as cytokines [TNFα, interleukin (IL)-6, IL-18], and the production of COX-2-derived prostaglandins[79]. The crucial role of the Kupffer cell in this process is illustrated by experiments in an alcohol feeding model where treatment with GdCl3, an inactivator of cells of macrophage lineage, significantly reduced liver injury[8].

EFFECT OF SEX HORMONES ON GUT PERMEABILITY AND ENDOTOXIN LEVELS

Very little is known about possible direct effects of female sex hormones on gut permeability. A recent report shows that increased intestinal permeability is a factor in intrahepatic cholestasis of pregnancy[80]; this finding is relevant in that pregnancy is characterized by high levels of estrogen and progesterone. A few studies have examined the effects of hormones on liver-enterotoxin interaction. When female rats are treated with a sublethal dose of endotoxin *via* tail vein, those rats also treated with a combination of synthetic estrogen and progesterone identical to that in oral contraceptives showed pronounced gut permeability and liver injury, with increased TNFα production by Kupffer cells[81]. However, these studies used very high doses of hormones, and the hormones were only used as a combination, and not separately. In an alcohol model, Enomoto *et al*[50] showed that treatment of female rats with very high doses of estriol, a very weak estrogen, resulted in increased endotoxin levels and increases in Kupffer cell TNFα and CD14 content. Because these effects were blocked with oral nonabsorbable antibiotics, the authors concluded that the estriol treatment resulted in increased gut permeability. In another study using burn and trauma/hemorrhagic models, it was shown that estrogen decreased markers of gut injury, whereas testosterone increased susceptibility[82]. It should be noted that the mechanisms in these models may differ from that in ours, since in gut trauma models injury to the mucosal barrier is acute and severe, whereas in alcohol models the injury is chronic. Thus, hormonal changes in the acute gut trauma models may be entirely different than in the chronic alcohol models. In Caco-2 cells, Asai *et al*[83] showed that estradiol treatment potentiated alcohol-induced apoptosis, leading to speculation that estrogen might increase gut permeability *in vivo*. With respect to the liver, an interesting study using cultured hepatic stellate cells showed that estradiol treatment reduced ROS generation through the NADPH oxidase system, and also attenuated TGFβ1 expression, cell activation, MAPK pathways, as well as fibrogenic responses. In contrast, progesterone treatment increased ROS generation, TGFβ1 expression, and stellate cell proliferation[84]. Thus, a potential role of progesterone in ALI should be considered.

FUTURE RESEARCH AND CLINICAL IMPLICATIONS

Future work will foster further understanding of the roles of sex in susceptibility to and in the pathogenesis of ALI. The role of sex hormones, both estrogen and progesterone, remain to be elucidated in this injury. Furthermore, the influences of age and menopausal status are not clear. Are postmenopausal women who are not using hormone replacement less susceptible to ALI? Another emerging area of interest is whether dietary supplements such as fiber and probiotics can alter the course of gut injury and thus liver injury. Obviously, much work needs to be done to understand the interplay of these factors. However, it remains prudent to advise

women that they are at greater risk for alcohol-induced injury, and that intake should be limited to one drink per day. Further, caution should be exercised with heavy use of alcohol in the setting of oral contraceptives or hormone replacement therapy. Since heavy alcohol use interferes with hepatic metabolism of hormones, circulating levels of hormones may increase, thus also placing women at risk for hormone-responsive tumors of the breast and reproductive tract.

CONCLUSION

The evidence outlined herein strongly points to greater risk for women for developing severe ALI and cirrhosis than men. Although the exact roles of female hormones have not yet been proven or elucidated, women should exert caution by limiting their alcohol intake to one drink or less per day to avoid the complications of alcohol-induced liver injury.

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