Published in final edited form as:

J Pharmacol Toxicol Methods. 2012 November ; 66(3): 257–263. doi:10.1016/j.vascn.2012.08.166.

Comparison of the Agar Block and Lieber-DeCarli Diets to Study Chronic Alcohol Consumption in an Aging Model of Fischer 344 Female Rats

Daniel R. Sharda^{a,*}, Jennifer L. Miller-Lee^{a,*}, Gregory M. Kanski^a, J. Craig Hunter^a, Charles H. Lang^{b,c}, Mary J. Kennett^d, and Donna H. Korzick^{a,b}

^aDepartment of Kinesiology, The Pennsylvania State University, University Park, PA 16802

^bInter College Graduate Program in Physiology, The Pennsylvania State University, University Park, PA 16802

^cDepartment of Cellular and Molecular Physiology, The Pennsylvania State University, University Park, PA 16802

^dDepartment of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA 16802

Abstract

Introduction—Post-menopausal women have a greater risk of developing alcoholic complications compared to age-matched men. Unfortunately, animal models of chronic ethanol consumption with estrogen deficiency are lacking. Here, we characterize the ability of the agar block and Lieber-DeCarli models of chronic ethanol consumption to produce elevated blood alcohol content (BAC) and liver pathology in the F344 postmenopausal animal model of aging.

Methods—Adult (3 mo) and aged (18 mo) F344 ovary-intact or ovariectomized rats were administered ethanol for 14–20 weeks as follows: *diet 1*, standard chow access, 10% ethanol in drinking water, and 40% ethanol in agar blocks; *diet 2*, diet 1 plus low phytoestrogen chow (known to effect ethanol metabolism) for the final 4 weeks; *diet 3*, Lieber-DeCarli all liquid diet with 36% kcal ethanol. Control animals were matched isocalorically with dextrin.

Results—For the agar block diet, average BAC was 13 ± 4 mg/dL across groups. BAC was unaffected by reducing dietary phytoestrogen content (12 ± 4 mg/dL), which is known to interfere with ethanol metabolism. Liver pathology was unaffected by the agar block diet. In contrast, the Lieber-DeCarli diet resulted in BAC of 45 ± 5 mg/dL in conjunction with more severe hepatopathology.

Discussion—We conclude that the Lieber-DeCarli diet produces greater BAC and hepatopathology to study the effects of chronic ethanol administration in the F344 postmenopausal rodent model of aging when compared to an ethanol agar block diet.

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Correspondence: Donna H. Korzick, PhD, 106 Noll Laboratory, The Pennsylvania State University, University Park, PA 16802, Phone: 814-865-5679, FAX: 814-865-4602, dhk102@psu.edu.

*D.R.S. and J.L.M. contributed equally to this manuscript.

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Keywords

alcoholism; estrogen; methods; postmenopause; phytoestrogens; ovariectomy; senescence

Introduction

In humans, prolonged ingestion of excessive alcohol (>90 g/day for more than 5 years) results in increased susceptibility for developing a myriad of pathological sequelae, including alcoholic myopathy, cardiomyopathy, and liver disease (Becker, et al., 1996; Fauchier, et al., 2000; Laonigro, Correale, Di Biase, & Altomare, 2009; Maddrey, 2000; McKenna, Codd, McCann, & Sugrue, 1998; Urbano-Marquez, et al., 1995). Interestingly, post-menopausal women have a greater risk of developing alcoholic complications when compared to age-matched men (Fernandez-Sola, et al., 1997; Urbano-Marquez, et al., 1995). For instance, a lower total lifetime dose of ethanol (kg ethanol/kg body weight) is needed to induce alcoholic cardiomyopathy in women, indicating alcohol is more toxic to the myocardium of women as compared to men (Fernandez-Sola, et al., 1997; Urbano-Marquez, et al., 1995). Furthermore, alcohol consumption abrogates the cytoprotective effects of estrogen (E₂) and negatively regulates E₂ receptors (Chen, et al., 2009; Eagon, Elm, Tadic, & Nanji, 2001; Rajasingh, et al., 2007). However, studies using the aged Fischer 344 (F344) female rat, a standard aging rodent model (Dlugos, 2006; Dlugos & Pentney, 1997; Pentney & Quackenbush, 1990), to provide mechanistic insights on potential interactive effects of age-associated E₂ deficiency and chronic ethanol ingestion on pathophysiology have not been performed.

Animal models of chronic ethanol feeding include intragastric infusion, the Lieber-DeCarli all liquid diet, or an ethanol-containing agar block diet. Intragastric infusion requires surgical implantation of a cannula and daily infusions of diet that may not be viable for aged animals. The Lieber-DeCarli all liquid diet has been widely used since 1963 and has proven efficacious in producing alcoholic phenotypes in numerous rat strains including male and female F344, as well as in adult and aged male (Augustyniak, Waszkiewicz, & Skrzydlewska, 2005; Tajuddin & Druse, 1996), ovariectomized (Gavaler, et al., 1993), and diabetic (Jung, et al., 2010) rats. More recently, an agar block diet has been successfully implemented with adult male and female Sprague-Dawley rats (Bautista, 1997; Lang, Fan, Lipton, Potter, & McDonough, 1998), and a major advantage of this approach is that rats freely chose to consume ethanol, and thus may represent a more physiological model of alcoholism (Thomas C. Vary, Kimball, & Sumner, 2007). However, the efficacy of the agar block diet to increase blood alcohol content (BAC) in aged animals was unknown. Moreover, studies utilizing a postmenopausal animal model of aging such as the aged female F344 have not been performed.

Given mechanistic knowledge gaps in alcoholic phenotypes with E₂ deficiency and aging, we sought to determine the efficacy of different dietary routes for increasing BAC to pathological levels by administering a chronic alcohol diet to E₂-deficient aged female F344 rats. Here, three different feeding protocols were utilized including the agar block method (Bykov, Palmen, Piirainen, & Lindros, 2004; Fogle, et al., 2011; T. C. Vary, Lynch, & Lang, 2001), the agar block method coupled to a low phytoestrogen pellet diet to remove any interference from plant hormones which can increase ethanol metabolism and/or absorption (Kano, Ishikawa, Matsubara, Kikuchi-Hayakawa, & Shimakawa, 2002; Lin & Li, 1998), and the Lieber-DeCarli all liquid diet (Lieber & DeCarli, 1989; Lieber, Jones, & Decarli, 1965). Of the three protocols, only the Lieber-DeCarli all liquid diet was sufficient to produce elevated BAC and associated hepatopathology in aged F344 rats with E₂ deficiency.

Materials and Methods

Animal Care

Adult (3 mo) and aged (18 mo) female Fischer 344 (F344) rats were purchased from the NIA colony at Taconic (Hudson, NY). Rats were housed individually under a 12:12 hr light:dark cycle and initially received standard rat chow, LabDiet 5001 (PMI Nutrition International, St. Louis, MO) and water *ad libitum*. Bilateral ovariectomy (OVX) surgeries were performed in a subset of adult and aged rats to assure menopausal E₂ deficiency (Hunter & Korzick, 2005; Novotny, Simpson, Tomicek, Lancaster, & Korzick, 2009), given variable time to anestrus known to occur in aged rats (Savonenko & Markowska, 2003; Sone, et al., 2007). Animals were allowed to recover for 4 weeks prior to initiation of alcohol administration, and at the time of sacrifice the uterine weight was measured to confirm the ovary status. Animal protocols were reviewed and approved by the Penn State University Institutional Animal Care and Use Committee (IACUC) and conducted accordingly.

Ethanol Feeding Protocols

Agar block diet—Adult (3 mo, n=18) and aged (18 mo, n=19) F344 sham surgitized or OVX rats were given ethanol (or isocaloric maltose-dextrin control) containing agar blocks as described previously (T. C. Vary, et al., 2001). Briefly, for the first week, all animals received control agar blocks. Thereafter, rats were randomly assigned an ethanol or control diet. The ethanol group was provided ethanol via drinking water (10% vol/vol) and in the agar blocks, which contained 1% (w/v) peanut butter and 3% (v/v) chocolate syrup. The ethanol concentration in the agar blocks was initiated at 10% and was increased weekly by 10% over a 4 week period. Ethanol containing agar was subsequently maintained at 40% until the end of the study. Female rats in the control group received control agar blocks, which contained an isocalorically matched amount of maltose-dextrin in place of ethanol. All groups received free access to standard rat chow, LabDiet 5001 (PMI Nutrition International, St. Louis, MO). Agar and pellet consumption were measured twice weekly, while water consumption and rat weight were measured weekly. The ethanol feeding protocol was continued for 14-16 weeks according to sacrifice schedule.

Phytoestrogen diet—Adult (3 mo, n=10) and aged (18 mo, n=9) F344 ovary-intact or OVX rats received control agar blocks for one week as above. Thereafter, the female animals were randomly assigned to one of two pellet diets with reduced phytoestrogen content compared to the standard rat chow, LabDiet 5001 (PMI Nutrition International, St. Louis, MO), which contains ~500 ppm isoflavones (daidzein, genistein, and glycitein). The female rats were given either the 2018 Teklad Global 18% Protein Rodent Diet (Harlan Laboratories, Inc., Madison, WI) containing ~150-250 ppm isoflavones (daidzein and genistein aglycone) or the Advanced Protocol Verified 75 IF from Lab Diet (PMI Nutrition International, St. Louis, MO) containing less than 75 ppm isoflavones (daidzein, genistein, and glycitein). This modification was made in conjunction with administering ethanol by agar blocks as described above for the final month of study. Agar and pellet consumption were measured twice weekly, while water consumption and rat weight were measured weekly.

Lieber-DeCarli diet—Adult (3 mo, n=22) and aged (18 mo, n=17) F344 ovary-intact or OVX rats were given the Lieber-DeCarli control liquid diet (Bio-Serv, Frenchtown, NJ) for one week. Thereafter, rats were randomly assigned the ethanol or control diet. Female rats consuming the ethanol liquid diet, initially obtained 12% of the calories from ethanol and this was gradually increased each week by 12% over a 3 week period. Once 36% of the calories were obtained from ethanol, this was maintained until the end of the study. Control

rats received liquid diet containing isocaloric dextrin in place of the ethanol. Consumption of the liquid diets was assessed daily and the animals were weighed weekly. Because alcohol is an appetite suppressant, animals were pair-fed to more closely match consumption (see Table 3). The study was performed for 19-20 weeks.

Blood Alcohol Assessment

Plasma was collected from the tail vein of each rat at either 11pm (five hours into 12 h dark cycle) or 8am (onset of 12 h light cycle). BAC was assessed by two different enzymatic assays. Plasma BAC was assayed following the directions of the NAD-ADH diagnostic kit (Sigma-Aldrich Co., St. Louis, MO). Briefly, in this reaction, ethanol is oxidized to acetaldehyde, while NAD is reduced to NADH by the enzyme alcohol dehydrogenase. In addition, the Analox GL5 analyzer (Analox Instruments, Lunenburg, MA) was used to assess BAC according to the manufacturer's instructions. Here, ethanol is oxidized to acetaldehyde, while oxygen is reduced to hydrogen peroxide by the enzyme alcohol oxidase.

Liver Histology

Livers from control and ethanol fed rats were initially perfused with a 0.9% saline solution followed by a 10% formalin solution. Livers were dissected into one centimeter cubes, fixed in 10% formalin, paraffin embedded, sectioned, stained with hematoxylin-eosin, and examined microscopically for the presence of hepatic lipidosis, inflammation, and other signs of alcoholic liver disease. Sections were examined by a laboratory animal veterinarian with training in rodent pathology (MJK), who was blinded to the experimental groups. The sections were examined microscopically for the presence of hepatic lipidosis, inflammation, necrosis, and/or other lesions. In sections where lesions were observed, the primary lesions observed in all cases were hepatic lipidosis and inflammation. The overall lesion score was estimated as the percent area of the section affected by examination of several (>10) 200x fields for each section. The lesions were scored as follows: 0 = no lesions/normal, 1 = minimal lesions i.e. less than 20% of the section was affected, 2 = mild lesions, or approximately 21-40% affected, 3 = moderate lesions, or approximately 41-60% affected, 4 = marked lesions, or approximately 61-80% affected, and 5 = severe or extensive lesions or 80-100% affected.

Statistical Analysis

All data are presented as means $\pm$ SE and analyzed using the Statistical Analysis System (SAS). Data were compared with a three way ANOVA (age $\times$ ovary status $\times$ diet) using the SAS general linear models procedure. Higher order terms were employed to reveal significant interaction effects with respect to age, ovary status, and diet, and the Tukey-Kramer method was used for all post hoc comparisons. An alpha level of $P < 0.05$ was considered statistically significant.

Results

Body weight characteristics

As expected, end of study rat weight was significantly greater in aged vs adult rats (Tables 1 and 2). For the agar block diet, final rat weight was significantly reduced in alcohol vs control-fed rats (Table 1; $P < 0.01$). An effect of diet and age on reducing weight gain from baseline to end of study was also observed (age effect, $P < 0.03$; diet effect, $P < 0.001$; Table 1). For the Lieber-DeCarli diet, end of study rat weight was reduced for ethanol-fed vs control-fed animals, reaching significance in aged (age $\times$ diet interaction, $P < 0.01$; Table 2). Weight gain was also less in ethanol-fed vs control fed-rats, and an age $\times$ diet interaction revealed that this reduction was greatest in aged ($P < 0.01$; Table 2). For both the agar block

($P = 0.001$) and Lieber-DeCarli ($P < 0.01$) diets, an age $\times$ ovary interaction revealed that end of study rat weight was significantly increased in adult but not aged OVX ($P < 0.001$; Tables 1 and 2). As anticipated, uterine weights were significantly less in OVX vs non-OVX animals and were independent of ethanol treatment or diet ($P < 0.001$; Tables 1 and 2).

Alcohol consumption and BAC

Dietary consumption of the agar block and Lieber-DeCarli diets is presented in Table 3. In general, adult and aged alcohol-fed animals on the agar block diet consumed less agar and greater amounts of traditional chow (expressed relative to body weight) compared with control fed animals. Specifically, chow consumption was increased in ethanol consuming adult and aged, but not aged non-alcoholic rats (age $\times$ diet $P = 0.001$). Chow consumption was also significantly reduced in OVX ($P < 0.01$). A concomitant reduction in agar consumption was observed in ethanol consuming rats, reaching significance in adult (age $\times$ diet interaction; $P < 0.001$). Adult animals on the agar block diet also consumed more alcohol in their drinking water than aged ($P < 0.001$). With regard to the ethanol containing Lieber-DeCarli diet, normalized consumption was significantly increased in all rats when compared to animals consuming the control diet ($P < 0.0001$; Table 3). Consumption was also reduced by OVX in adult but not aged (age $\times$ ovary interaction, $P < 0.01$). In this regard, it is important to note that intake of the non-alcoholic containing Lieber-DeCarli diet was restricted in adult and aged rats to match intake in alcohol consuming counterparts.

Average values for plasma BAC across all groups assessed by the NAD-ADH enzymatic assay were 13 ± 4 mg/dL in rats consuming the agar block diet with standard 5001 chow (group values are presented in Table 4). Replacing standard chow with a low phytoestrogen chow, either Teklad Global 2018 or LabDiet 75 IF, did not alter mean BAC across groups (9 ± 3 mg/dL and 16 ± 8 mg/dL, respectively; Table 4). In contrast, plasma BAC increased when the rats were given the Lieber-DeCarli diet (45 ± 5 mg/dL) ($P < 0.05$; Table 4), and group differences were not observed ($P > 0.05$). Interestingly, BAC assessed by the NAD-ADH enzymatic assay (adult 58 ± 6 mg/dL, adult OVX 45 ± 9 mg/dL, aged 39 ± 6 mg/dL, aged OVX 37 ± 11 mg/dL) was higher than values tested with the alcohol oxidase enzymatic reaction (adult 31 ± 7 mg/dL, adult OVX 19 ± 4 mg/dL, aged 8 ± 1 mg/dL, aged OVX 17 ± 9 mg/dL).

Effects of dietary alcohol consumption on liver pathology

Histopathological analysis revealed an effect of age ($P < 0.001$), but not diet in liver pathology of animals on the agar block diet (Figure 1). In addition to an age effect, hepatopathology was worsened by OVX in Lieber-DeCarli fed animals ($P < 0.01$; Figure 2). Importantly, chronic ethanol exposure exacerbated pathology in all groups ($P = 0.006$; Figure 2).

Discussion

The salient finding of the current study is that the Lieber-DeCarli diet increased BAC and hepatopathology in the aged female Fischer 344 rat model of postmenopausal aging. We observed marginal changes in BAC (<15 mg/dL) in all experimental groups on the agar diet, which was consistent with a complete absence of hepatopathology. Low phytoestrogen diets, which have been previously shown to reduce BAC in rats by interfering with ethanol absorption and/or metabolism (Kano, et al., 2002), and voluntary consumption (Lin & Li, 1998), were unable to further elevate BAC. Here, in direct comparison, F344 rats on the Lieber-DeCarli all liquid diet yielded BAC of 45 ± 5 mg/dL and, notably, ethanol consuming rats developed hepatopathology. Importantly, this is the first report

demonstrating the efficacy of chronic ethanol ingestion in a postmenopausal animal model of aging.

The ethanol agar block diet has been successfully used for more than a decade with Sprague-Dawley rats (Bautista, 1997; Lang, et al., 1998). In contrast to results shown here, both male and female Sprague-Dawley rats routinely exhibit BAC in excess of 80 mg/dL on the agar block diet (Fogle, et al., 2010; Thomas C. Vary, et al., 2007). Thus our current findings of BAC well below values of 80 mg/dL on the agar block diet do not appear to be gender specific, but rather specific to the F344 strain. Indeed, when given a choice, F344 rats consume less ethanol than Long-Evans, Sprague-Dawley, and Wistar rats (Khanna, Kalant, Shah, & Sharma, 1990; Li & Lumeng, 1984). This aversion may help explain why in the current study, a diet involving voluntary consumption of ethanol by F344 produced marginal BAC compared to that reported in Sprague-Dawley rats.

Strain specific differences in metabolism of ethanol have also been documented. Both Long-Evans and Wistar-Kyoto (WKY) rats have yielded higher BAC compared with the F344 rats in studies where direct comparisons were made with the Lieber-DeCarli diet, suggesting increased ethanol elimination rates (EER) in the F344 strain (Denucci, et al., 2010; Pentney, Mullan, Felong, & Dlugos, 2002). Moreover, BAC in F344 is further compounded when age is considered. Studies from acute ethanol administration in aged F344 rats reveal increased EER compared with adult (Ott, Hunter, & Walker, 1985; Rikans & Kling, 1987). In male F344 rats, alcohol dehydrogenase (ADH) activity increases significantly with age (5 vs 24 month), and is hypothesized to account for enhanced EER in aged males (Ott, et al., 1985; Rikans & Kling, 1987). Moreover, testosterone was found to be a potent inhibitor of alcohol dehydrogenase in the spontaneously hypertensive rat (Rachamin, et al., 1980), and decreased ADH and decreased EER observed in the adult F344 is dependent on testosterone (Erickson, 1984; Ott, et al., 1985; Rikans & Kling, 1987). Thus decreased testosterone levels observed in aged males may account for the increased ADH activity and EER. In contrast to males, female F344 have persistently elevated ADH activity that does not change with age (Rikans & Kling, 1987). Thus, the mechanism for enhanced ethanol clearance in aged female F344 rats observed herein do not appear to be explained by differences in ADH activity, and the elucidation of this mechanism remains an important direction of future studies.

Nevertheless, it has been well documented that alcohol must account for at least 36% of caloric intake for rats to obtain elevated BAC, while levels less than 36% result in a precipitous decline in BAC (Lieber & DeCarli, 1989). We encountered three challenges to accurately estimating the caloric percentage from ethanol in administration of the agar block diet. First, we determined that overnight evaporation accounted for ~35% loss in agar weight in empty cages. Second, confounding the loss from evaporation is that the outer agar surface loses ethanol rapidly as determined by the NAD-ADH enzymatic assay (data not shown). As early as five and 24 hours after placing the agar in an empty cage, ~50% and 100% of the ethanol, respectively, was lost to evaporation at the surface of the agar block while the core retained its concentration (five hours coinciding with the start of their dark cycle). Daily observations further indicated that rats would wait for the alcohol to evaporate from the outer regions before any further ingestion. These findings are in accordance with the F344 strain exhibiting a strong ethanol aversion when given a choice (Li & Lumeng, 1984). Our third challenge was accurate estimation of ethanol consumption from water. While glass water bottles exhibited little leakage, they were not entirely leak proof. Thus, if we were to calculate the ethanol consumption assuming no evaporation, ethanol loss, or leakage from bottles, ethanol consumption would have been 70% of total calories per day (or 34g/kg body weight), a level well beyond the daily limit of metabolism, which is 14-18g/kg body weight in the rat (Lindros, et al., 1983). Estimates taking these factors into account, however, range

from 15-20% of calories per day (or ~4.5 g/kg body weight) and are in line with moderate alcohol consumption studies (Zhu, Zhou, & Gray, 2000).

The Lieber-DeCarli diet has been widely used since 1963 as the gold standard for chronic alcohol administration in rodents. In contrast to the agar-based diet that has only been used on Sprague-Dawley rats, the Lieber-DeCarli diet has been used successfully on numerous rat strains including F344 (Denucci, et al., 2010). Not only are concerns of evaporation and accurate measurement minimized, but the Lieber-DeCarli diet also guarantees a 36% caloric content derived from ethanol. The ability to accurately measure consumption allows caloric matching of control fed animals as well as estimation of ethanol intake. Importantly, in this study female F344 animals consumed 12 g/kg of ethanol per day on the Lieber-DeCarli diet, and elevated BAC was achieved regardless of age or ovary status. Furthermore, hepatopathology was more severe in Lieber-DeCarli ethanol than control fed animals. Interestingly, liver scores were also influenced by ovary status suggesting a protective role of estrogen.

The role of estrogen in alcoholic liver disease (ALD) remains controversial. Women require less alcohol than men to develop alcoholic liver disease suggesting that the presence of estrogen may be deleterious when consuming alcohol (Maddrey, 2000). Not only does reducing estrogen in female rats reduce ALD (Järveläinen, Lukkari, Heinaro, Sippel, & Lindros, 2001; Yin, et al., 2000), but microarray analysis of livers from alcohol fed female rats also suggests attenuated upregulation of hepatoprotective genes when compared with males (Tadic, et al., 2002). Despite these findings, our understanding of age, menopause status and ALD are unclear (Eagon, 2010). That non-alcoholic fatty liver disease (NAFLD) is increased two fold in postmenopausal women suggests an increased susceptibility to ALD (Clark, Brancati, & Diehl, 2002; Gutierrez-Grobe, Ponciano-Rodriguez, Ramos, Uribe, & Mendez-Sanchez, 2010). In female Sprague-Dawley rats, NAFLD is induced by both age and OVX with either low or high fat diets (Paquette, Shinoda, Lhoret, Prud'homme, & Lavoie, 2007). Our findings are in agreement with a protective role for estrogen, as increasing liver pathology was observed with OVX in both adult and aged rats. Furthermore, liver injury was exacerbated by ethanol ingestion in all groups. Apparent discrepancies between studies in females may be related to the model of estrogen deficiency utilized. In contrast to studies using OVX in 1 mo rats (Yin, et al., 2000) (a time well before maturation), we performed OVX at 3 mo and 18 mo, respectively, which was followed by ~5 mo of ethanol administration in combination with estrogen deficiency to more clearly define age-associated hepatopathology. Furthermore, studies using the Wistar rat and the estrogen blocker toremifene used a restricted carbohydrate diet which influenced BAC (Järveläinen, et al., 2001). Additional studies using true models of female aging are indicated to better characterize effects of chronic ethanol.

We set out to validate a method of chronic alcohol administration to be used in a postmenopausal animal model of aging. Chronic alcohol consumption in humans is not defined as a level of legal intoxication (>80 mg/dL), but rather as greater than 90 grams of ethanol per day (100 grams of ethanol is 36% of a 2,000 kcal diet). Therefore, delineation of chronic alcohol effects within the rat should not be given solely on the basis of BAC, but daily ethanol intake in g/kg and hepatopathology should also be considered in total. Based on these indices the Lieber-DeCarli diet is sufficient to produce significantly elevated BAC and liver pathology to study the effects of chronic alcohol consumption in the aged estrogen deficient female F344 animal model of postmenopausal aging.

Acknowledgments

This project was supported by NIH RC2 AA019403 and RO1 HL091097 to DHK; NIH AA11290 to CHL.

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Fig. 1. Liver pathology in response to feeding the agar block diet

The agar block diet was described previously in Table 1. (A) Representative images (100X magnification) of hematoxylin-eosin stained livers from the control and alcohol groups. (B) Average liver scores as described in the Materials and Methods section. Adult control (n=3), Adult OvX control (n=4), Aged control (n=5), Aged OvX control (n=4), Adult alcohol (n=4), Adult OvX alcohol (n=5), Aged alcohol (n=4), and Aged OvX alcohol (n=4). OvX, ovariectomized. †Effect of alcohol vs control diet, ‡effect of aged vs adult, *effect of ovariectomy within age group, $P < 0.05$.

Fig. 2. Liver pathology in response to the Lieber-DeCarli diet

The Lieber-DeCarli all liquid ethanol diet was described previously in Table 2. (A) Representative images (100X magnification) of hematoxylin-eosin stained livers from the control and alcohol groups. (B) Average liver scores as described in the Materials and Methods section. Adult control (n=5), Adult Ovx control (n=5), Aged control (n=1), Aged Ovx control (n=3), Adult alcohol (n=6), Adult Ovx alcohol (n=6), Aged alcohol (n=5), and Aged Ovx alcohol (n=7). Ovx, ovariectomized. †Effect of alcohol vs control diet, ‡effect of aged vs adult, *effect of ovariectomy within age group, $P < 0.05$.

Table 1

Baseline morphology of female F344 fed agar block diet

The ethanol group was provided ethanol via drinking water (10% vol/vol) and in the agar blocks (40%), while the control group received isocalorically matched maltose-dextrin in agar blocks. OVX, ovariectomized; BW, body weight; and UW, uterine weight. ^aDiet main effect; ^bage main effect; ^covary main effect; ^dage*ovary interaction. Analysis of variance (ANOVA) effects were at $P < 0.05$.

	Control Diet				Alcohol Diet				Statistical Effects
	Adult	Adult Ovx	Aged	Aged Ovx	Adult	Adult Ovx	Aged	Aged Ovx	
n	4	5	5	4	4	5	4	6	
Final BW (g)	218 ± 7	262 ± 6	303 ± 4	304 ± 7	207 ± 5	246 ± 4	276 ± 5	297 ± 8	a,d
Weight Gain (ΔBW)	38 ± 7	46 ± 5	25 ± 7	44 ± 3	26 ± 8	27 ± 2	12 ± 12	13 ± 5	a,b
UW (g)	0.58 ± 0.04	0.07 ± 0.02	0.54 ± 0.04	0.17 ± 0.02	0.59 ± 0.09	0.10 ± 0.01	0.61 ± 0.07	0.18 ± 0.02	c

Table 2

Baseline morphology of female F344 fed Lieber-DeCarli diet

The ethanol group consuming the liquid diet received 36% of their total calories from ethanol, while the control group received liquid diet containing an isocalorically matched amount of dextrin. For uterine weight adult control (n=4). OVX, ovariectomized; BW, body weight; and UW, uterine weight. ^cOvary main effect; ^dage*ovary interaction ^eage*diet interaction; . Analysis of variance (ANOVA) effects were at $P < 0.05$.

	Control Diet				Alcohol Diet				Statistical Effects
	Adult	Adult Ovx	Aged	Aged Ovx	Adult	Adult Ovx	Aged	Aged Ovx	
n	13	5	10	7	14	6	7	13	
Final BW (g)	257 ± 3	298 ± 6	416 ± 6	418 ± 14	231 ± 4	295 ± 5	353 ± 10	372 ± 6	d,e
Weight Gain (ΔBW)	63 ± 3	84 ± 3	122 ± 2	136 ± 5	40 ± 2	76 ± 4	69 ± 6	90 ± 4	c,e
UW (g)	0.68 ± 0.06	0.08 ± 0.01	0.57 ± 0.03	0.19 ± 0.01	0.64 ± 0.03	0.07 ± 0.01	0.68 ± 0.03	0.19 ± 0.01	c,d

Table 3
Diet and water consumption with the agar block and Lieber-DeCarli ethanol feeding protocols

Assessment of diet consumption was carried out on agar block and Lieber-DeCarli fed animals as described in Tables 1 and 2. OVX, ovariectomized; BW, body weight; and n.d., not determined. ^adiet main effect; ^bovary main effect; ^cage*ovary interaction, ^dage*ovary interaction, ^eage*diet interaction. Analysis of variance (ANOVA) effects were at $P < 0.05$.

	Control Diet				Alcohol Diet				Statistical Effects
	Adult	Adult OvX	Aged	Aged OvX	Adult	Adult OvX	Aged	Aged OvX	
n	4	5	5	4	4	5	4	6	
Chow (g)/ 100g BW	2.9 ± 0.1	2.4 ± 0.1	2.2 ± 0.1	2.1 ± 0.2	4.0 ± 0.1	3.6 ± 0.1	2.9 ± 0.2	2.5 ± 0.2	c,e
Agar with 5001 Chow	10.4 ± 0.3	8.7 ± 0.2	7.5 ± 0.1	7.5 ± 0.1	7.4 ± 0.4	6.4 ± 0.2	6.9 ± 0.2	6.7 ± 0.3	d,e
Water (mL) /day	n.d.	n.d.	n.d.	n.d.	9.8 ± 0.7	9.1 ± 0.4	5.6 ± 0.7	6.5 ± 1.0	
n	13	5	10	7	14	6	12	13	
Lieber- DeCarli	18.1 ± 0.2	15.4 ± 0.3	15.5 ± 0.3	15.3 ± 0.4	18.9 ± 0.2	17.5 ± 0.4	15.9 ± 0.2	15.9 ± 0.2	d,e

Table 4
Assessment of blood alcohol content in female F344 rats in response to different ethanol feeding protocols

The agar block ethanol diet coupled to the 5001 chow (~500 ppm isoflavones) was described in Table 1. In addition, the agar block ethanol diet was administered as before except for the substitution of a chow diet with a lower phytoestrogen content, either the 2018 chow (<250 ppm isoflavones) or the 75 IF chow (<75 ppm isoflavones) for one month. The Lieber-DeCarli all liquid ethanol diet is described in Table 2. Blood alcohol content was measured using the NAD-ADH enzymatic assay. OVX, ovariectomized; BAC, blood alcohol content; n.d., not determined. *Effect of Lieber-DeCarli vs Agar Diet, $P < 0.05$.

	Adult		Adult OvX		Aged		Aged OvX	
	n	6	4	4	5	5	4	4
Agar with 5001 Chow	BAC (mg/dL)	3 ± 3*	6 ± 2*		33 ± 9		11 ± 4*	
	Range (mg/dL)	0-11	0-11		9-51		0-20	
	n	2	3	2	3			
Agar with 2018 Chow	BAC (mg/dL)	9 ± 10*	8 ± 2*		13 ± 17*		6 ± 4*	
	Range (mg/dL)	0-18	6-11		0-30		0-12	
	n	3	2	0	3			
Agar with 75 IF Chow	BAC (mg/dL)	25 ± 16*	0*		n.d.		19 ± 14*	
	Range (mg/dL)	0-55	0		n.d.		4-47	
	n	6	6	4	6			
Lieber-DeCarli	BAC (mg/dL)	58 ± 6	45 ± 9		39 ± 6		37 ± 11	
	Range (mg/dL)	43-76	5-65		22-53		18-89	
	n	6	6	4	6			