Conclusions

Selection for mice resistant or susceptible to fescue toxicosis using the impact on post-weaning growth rate of a toxin-containing diet as a selection criterion was successful. Resistant line mice were also able to better withstand toxic challenge later in life, as measured by reproduction. However, the same mechanisms possibly responsible for less reduction in growth rate post-weaning, GST and UDPGT detoxification activity, may not be the mechanisms responsible for less reduction in reproductive measures, though GST enzyme activity was still higher in R than S mice. Further work may uncover additional physiological coping mechanisms in R mice.

Two other issues that should be examined are the choice of selection criteria and the definition of response. In this case, selection for changes in growth rate between lines also resulted in changes in reproductive abilities under toxic challenge. One would hope that these results of selection are correlated and changes in one would result in changes in another, but this may not be true in every case. Selection must change underlying physiological coping mechanisms common to all aspects of animal biology which we are seeking to improve.

In addition, selection for susceptibility may involve entirely different physiological variables than selection for resistance; the two responses may not be opposite extremes of the same trait. Instead, for example, selection for susceptibility to

toxic fescue may have resulted in mice with higher core body temperatures, which would cause a severe reduction in feed intake when exacerbated by the vasoconstrictive properties of toxic fescue.

Examining the true reason for susceptibility within our experiment may prove challenging. No control line was maintained, and purchasing mice from the original source may not allow a fair comparison. Our mice have been managed for the past five years under different environmental conditions than the original population. Slight inbreeding was unavoidable (Wagner and Hohenboken, 1997) and the population has endured at least one disease outbreak.

Implications

Fescue toxicosis is a result of the interaction among animal, grass, and endophyte genomes and the relationship among these three is still poorly understood. Selection for resistance to fescue toxicosis in cattle may prove effective in reducing the impact of the condition on the cattle industry. However, as these studies in mice have shown, selection for response in one clinical sign of fescue toxicosis may improve others, but not necessarily as a result of the same physiological factors. The impact of these changes must be studied at each phase of the animals' life cycle.

Literature Cited

Adcock, R. A., N. S. Hill, J. H. Bouton, H. R. Boerma, and G. O. Ware. 1997. Symbiont regulation and reducing ergot alkaloid concentration by breeding endophyte-infected tall fescue. J. Chem. Ecol. 23:691-704.

Aiken, G. E., D. I. Bransby, and C. A. McCall. 1993. Growth of yearling horses compared to steers on high- and low-endophyte infected tall fescue. J. Eq. Vet. Sci. 13:26-28.

Aiken, G. E., and M. A. Brown. 1994. Potential of cattle genetics for improving production on endophyte-infected tall fescue. In: Proc. Am. Forage Grassland Coun., March 6-10, Lancaster, PA. p 294.

Bacon, C. W., J. K. Porter, J. D. Robbins, and E. S. Luttrell. 1977. *Epichloe typhina* from tall fescue grasses. Appl. Environ. Microbiol. 34:576-581.

Bacon, C. W., P. C. Lyons, J. K. Porter, and J. D. Robbins. 1986. Ergot toxicity from endophyte-infected grasses: a review. Agron. J. 78:106-116.

Beconi, M. G., M. D. Howard, T. D. A. Forbes, R. B. Muntifering, N. W. Bradley, and M. J. Ford. 1995. Growth and subsequent feedlot performance of estradiol-implanted vs nonimplanted steers grazing fall-accumulated endophyte-infested or low-endophyte tall fescue. J. Anim. Sci. 73:1576-1584.

Bolt, D. J., and J. Bond. 1986. Effects in pregnant beef heifers grazing fungus-infected tall fescue on calf birth weight, milk yield, and calf growth. J. Anim. Sci. 63(Suppl. 1):297 (Abstr.).

Bolt, D. J., J. Bond, and T. Elsasser. 1983. Changes in plasma prolactin and grazing behavior in heifers treated with dopamine antagonist while grazing tall fescue and orchardgrass pasture. J. Anim. Sci. 57(Suppl. 1):48 (Abstr.).

Bond, J., G. P. Lynch, D. J. Bolt, H. W. Hawk, C. Jackson, Jr., and R. J. Wall. 1988. Reproductive performance and lamb weight gains for ewes grazing fungus-infected tall fescue. Nutr. Rep. Int. 37:1099-1115.

Bond, J., J. B. Powell, and B. T. Weinland. 1984. Behavior of steers grazing several varieties of tall fescue during summer conditions. Agron. J. 76:707-709.

Bransby, D. I. 1997. Steer weight gain responses to ivermectin when grazing fescue. Large Animal Practice 18:16-19.

Bransby, D. I., S. P. Schmidt, W. Griffey, and J. T. Eason. 1988. Heavy grazing is best for infected fescue. Highlights Agric. Res. 35:12.

Brown, M. A., A. H. Brown, Jr., W. G. Jackson, and J. R. Miesner. 1996. Milk production in Angus, Brahman, and reciprocal-cross cows grazing common bermudagrass or endophyte-infected tall fescue. J. Anim. Sci. 74:2058-2066.

Brown, M. A., A. H. Brown, Jr., W. G. Jackson, and J. R. Miesner. 1997. Genotype x environment interactions in Angus, Brahman, and reciprocal-cross cows and their calves grazing common bermudagrass and endophyte-infected tall fescue pastures. J. Anim. Sci. 75:920-925.

Bush, L., and R. C. Buckner. 1973. Tall fescue toxicity. In: A. G. Matches (Ed.) Anti-Quality Components of Forages. Spec. Publ. No. 4. Crop Science Society of America, Madison, WI.

Chestnut, A. B., H. A. Fribourg, J. B. McLaren, D. G. Keltner, B. B. Reddick, R. J. Carlisle, and M. C. Smith. 1991. Effects of *Acremonium coenophialum* infestation, bermudagrass, and nitrogen or clover on steers grazing tall fescue pastures. J. Prod. Agric. 4:208-212.

Coley, A. B., H. A. Fribourg, M. R. Pelton, and K. D. Gwinn. 1995. Effects of tall fescue endophyte on relative abundance of small mammals. J. Environ. Qual. 24:472-475.

Cornell, C. N., and G. B. Garner. 1983. Fescue foot. In: Proc. Tall Fescue Toxicosis Workshop, March 17-18, Atlanta, GA. p 11.

Cross, D. L. 1997. Fescue toxicosis in horses. In: C. W. Bacon and N. S. Hill (Eds.) *Neotyphodium*/Grass Interactions. p 289. Plenum Press, New York.

Daniels, L. B., A. Ahmed, T. S. Nelson, E. L. Piper, and J. N. Beasley. 1984. Physiological responses in pregnant white rabbits given a chemical extract of toxic tall fescue. Nutr. Rep. Int. 29:505-510.

Daniels, L. B., T. S. Nelson, and J. N. Beasley. 1981. Effects of extracts of toxic fescue given orally to rats. Can. J. Comp. Med. 45:173-176.

Danilson, D. A., S. P Schmidt, C. C. King, L. A. Smith, and W. B Webster. 1986. Fescue toxicity and reproduction in beef heifers. J. Anim. Sci. 63(Suppl. 1):296 (Abstr.).

Dawe, D. L., J. A. Stuedemann, N. S. Hill, and F. N. Thompson. 1997. Immunosuppression in cattle with fescue toxicosis. In: C. W. Bacon and N. S. Hill (Eds.) *Neotyphodium*/Grass Interactions. p 411. Plenum Press, New York.

deBethizy, J. D., and J. R. Hayes. 1994. Metabolism: a determinant of toxicity. In: A.W. Hayes (Ed.) Principles and Methods of Toxicology (3rd Ed.). p 59-100. Raven Press, New York.

Dew, R. K., G. A. Boissonneault, N. Gay, J. A. Bowling, R. J. Cross, and D. A. Cohen. 1990. The effect of the endophyte (*Acremonium coenophialum*) and associated toxin(s) of tall fescue on serum titer response to immunization of spleen cell flow cytometry analysis and response to mitogens. Vet. Immunology Immunopathology 26:285-295.

Dutton, G. J., J. E. A. Leakey, and M. R. Pollard. 1981. Assays for UDPglucuronyltransferase activities. Methods Enzymol. 77:383-391.

Filipov, N. M., F. N. Thompson, N. S. Hill, D. L. Dawe, J. A. Stuedemann, and J. C. Price. 1997. Effects of endophyte-infected fescue seed based diets upon rabbits vaccinated against ergot alkaloids. In: C. W. Bacon and N. S. Hill (Eds.) *Neotyphodium*/Grass Interactions. p 411. Plenum Press, New York.

Fribourg, H. A., A. B. Chestnut, R. W. Thompson, J. B. McLaren, R. J. Carlisle, K. D. Gwinn, M. C. Dixon, and M. C. Smith. 1991. Steer performance in fescue-clover pastures with different levels of endophyte infestation. Agron. J. 83:777-781.

Garner, G. B., G. E. Rottinghaus, C. N. Cornell, and H. Testereci. 1993. Chemistry of compounds associated with endophyte / grass interaction: ergovaline- and ergopeptine-related alkaloids. Agric. Ecosystems Environ. 44:65-80.

Gavora, J. S. 1984. Genetic aspects of selection for disease resistance. In: Proc. 2nd World Cong. on Sheep and Beef Cattle Breeding, April 16-19, Pretoria, South Africa. p 136.

Glenn, A. E., C. W. Bacon, R. Price, R. Hanlin. 1996. Molecular phylogeny of *Acremonium* and its taxonomic implications. Mycologia 88:369-383.

Godfrey, V. B., S. P. Washburn, E. J. Eisen, and B. H. Johnson. 1994. Effects of consuming endophyte-infected tall fescue on growth, reproduction, and lactation in mice selected for high fecundity. Theriogenology 41:1393-1409.

Gould, L. S., and W. D. Hohenboken. 1993. Differences between progeny of beef sires in susceptibility to fescue toxicosis. J. Anim. Sci. 71:3025-3032.

Hannah, S. M., J. A. Paterson, J. E. Williams, M. S. Kerley, and J. L. Miner. 1990. Effects of increasing dietary levels of endophyte-infected tall fescue seed on diet digestibility and ruminal kinetics in sheep. J. Anim. Sci. 68:1693-1701.

Hemken, R. W., J. A. Bowling, L. S. Bull, R. H. Halton, R. C. Buckner, and L. P. Bush. 1981. Interaction of environmental temperature and anti-quality factors on the severity of summer fescue toxicosis. J. Anim. Sci. 52:710-714.

Hill, N. S., F. N. Thompson, D. L. Dawe, and J. A. Stuedemann. 1994. Antibody binding of circulating ergot alkaloids in cattle grazing tall fescue. Am. J. Vet. Res. 55:419-424.

Hinton, D. M., and C. W. Bacon. 1985. The distribution and ultrastructure of the endophyte of toxic tall fescue. Can. J. Bot. 63:36-42.

Hohenboken, W. D., P. L. Berggren-Thomas, W. E. Beal, and W. H. McClure. 1991. Variation among Angus cows in response to endophyte-infected fescue seed in the diet, as related to their past calf production. J. Anim. Sci. 69:85-90.

Hohenboken, W. D., and D. J. Blodgett. 1997. Growth and physiological responses to toxicosis in lines of mice selected for resistance or susceptibility to endophyte-infected tall fescue in the diet. J. Anim. Sci. 75:2165-2173.

Hohenboken, W. D., C. R. Wagner, and D. J. Blodgett. 1998. Inbreeding and reproduction in mice divergently selected for response to a dietary toxin. Theriogenology 49:1397-1407.

Hoveland, C. S. 1993. Importance and economic significance of the *Acremonium* endophytes to performance of animals and grass plant. Agric. Ecosystems Environ. 44:3-12.

Jackson, J. A., R. W. Hemken, L. P. Bush, J. A. Boling, M. R. Siegel, and P. M. Zavos. 1987. Physiologic response in rats fed extracts of endophyte infected tall fescue seed. Drug Chem. Toxicol. 10:369-379.

Jackson, J. A., D. R. Varney, R. J. Petroski, R. G. Powell, L. P. Bush, M. R. Siegel, R. W. Hemken, and P. M. Zavos. 1996. Physiological responses of rats fed loline and ergot alkaloids from endophyte-infected tall fescue. Drug Chem. Toxicol. 19:85-96.

Jackson, J. A., S. G. Yates, R. G. Powell, R. W. Hemken, L. P. Bush, A. Boling, P. M. Zavos, and M. R. Siegel. 1989. Physiological responses in rates fed extracts of endophyte-free and endophyte-infected tall fescue seed relative to some known ergot alkaloids. Drug Chem. Toxicol. 12:147-164.

Jernigan, A. D., N. H. Booth, J. D. Robbins, J. M. Zahner. 1986. Effect of yohombine hydrochloride on serum prolactin concentration in the rat: possible antagonist for fescue toxicosis. Am. J. Vet. Res. 47:949-952.

Johnson, M. C., T. P. Pirone, M. R. Siegel, and D. R. Varney. 1982. Detection of *Epichlöe typhina* in tall fescue by means of enzyme-linked immunosorbent assay. Phytopathology 72:647-650.

Kaplowitz, N., J. Kuhlenkamp, and G. Clifton. 1975. Drug induction of hepatic glutathione S-transferases in male and female rats. Biochem. J. 146:351-356.

Larson, B. T., D. M. Sullivan, M. A. Yordy, J. T. Turner, M. S. Kerley, and J. A. Paterson. 1992. Dopamine D2 receptor density and mRNA are reduced by ingestion of endophyte-infected tall fescue and increased by D2 antagonist in rat. J. Anim. Sci. 70(Suppl. 1):188 (Abstr.).

Latch, G. C. M. 1993. Physiological interactions of endophytic fungi and their hosts. Biotic stress tolerance imparted to grasses by endophytes. Agric. Ecosystems Environ. 44:143-156.

Lipham, L. B., F. N. Thompson, J. A. Stuedemann, and J. L. Sartin. 1989. Effects of metoclopramide on steers grazing endophyte-infected fescue. J. Anim. Sci. 67:1090-1097.

Lipesy, R. J., D. W. Vogt, G. B. Garner, L. L. Miles, and C. N. Cornell. 1992. Rectal temperature changes of heat and endophyte stressed calves produced by tolerant or susceptible sires. J Anim. Sci. 70(Suppl. 1):188 (Abstr.).

Lundqvist, G., and R. Morgenstern. 1995. Ontogenesis of rat liver microsomal glutathione transferase. Biochem. Pharmacol. 50:421-423.

Lusby, K. S., W. E. McMurphy, C. A. Strasia, S. C. Smith, and S. H. Muntz. 1990. Effects of fescue endophyte and interseeded clovers on subsequent finishing performance of steers. J. Prod. Agric. 3:103-105.

Mahmood, T., R. S. Ott, G. L. Foley, G. M. Zinn, D. J. Schaeffer, and D. J. Kesler. 1994. Growth and ovarian function of weanling and yearling beef heifers grazing endophyte-infected tall fescue pastures. Theriogenology 42:1149-1158.

McMurphy, W. E., K. S. Lusby, S. C. Smith, S. H. Muntz, and C. A. Strasia. 1990. Steer performance on tall fescue pasture. J. Prod. Agric. 3:100-102.

Miller, B. F., K. L. Armstrong, L. A. Wilson, W. D. Hohenboken, and R. G. Saacke. 1994. Variation among inbred and linecross mice in response to fescue toxicosis. J. Anim. Sci. 72:2896-2904.

Monroe, J. L., D. L. Cross, L. W. Hudson, D. M. Hendricks, S. W. Kennedy, and W. C. Bridges, Jr. 1988. Effect of selenium and endophyte-contaminated fescue on performance and reproduction in mares. Eq. Vet. Sci. 8:148-153.

Morgan-Jones, G., and W. Gams. 1982. An endophyte of *Festuca arundinacea* and the anamorph of *Epichlöe typhina*, new taxa in one of two new sections of *Acremonium*. Mycotaxon 15:311-318.

Morris, C. A. 1998. Genetics of disease resistance in *Bos taurus* cattle. Animal Genetic Resources Information 23:1-11.

Neal, W. D., and S. P. Schmidt. 1985. Effects of feeding Kentucky 31 tall fescue seed infected with *Acremonium coenophialum* to laboratory rats. J. Anim. Sci. 61:603-611.

Oliver, J. W., A. J. Robinson, L. K. Abney, and R. D. Linnabary. 1992. Effects of phenothiazine and thiabendazole on bovine dorsal pedal vein contractility induced by ergonovine and seratonin; potential for alleviation of fescue toxicity. J. Vet. Pharmacol. 15:247-251.

Paterson, J., C. Forcherio, B. Larson, M. Samford, and M. Kerley. 1995. The effects of fescue toxicosis on beef cattle production. J. Anim. Sci. 73:889-898.

Pederson, J. F., J. A. McGuire, S. P. Schmidt, C. C. King, Jr., C. S. Hoveland, and L. A. Smith. 1986. Steer performance as affected by tall fescue cultivar and level of

Acremonium coenophialum infection. N. Z. J. Exp. Agric. 14:307-312.

Peters, C. W., K. N. Grigsby, C. G. Aldrich, J. A. Paterson, R. J. Lipsey, M. S. Kerley, and G. B. Garner. 1992. Performance, forage utilization, and ergovaline consumption by beef cows grazing endophyte fungus-infected tall fescue, endophyte fungus-free tall fescue, or orchardgrass pastures. J. Anim. Sci. 70:1550-1561.

Porter, J. K., J. A. Stuedemann, F. N. Thompson, Jr., and L. B. Lipham. 1990. Neuroendocrine measurements in steers grazed on endophyte-infected fescue. J. Anim. Sci. 68:3285-3292.

Rankins, D. L. 1996. Metoclopramide and (or) cimetidine administration to sheep fed tall fescue. Vet. Human Toxicol. 38:173-176.

Redmond, L. M., D. L. Cross, T. C. Jenkins, and S. W. Kennedy. 1991. The effect of Acremonium coenophialum on intake and digestibility of tall fescue hay in horses. Eq. Vet. Sci. 11:215-219.

Redmond, L. M., D. L. Cross, J. R. Strickland, and S. W. Kennedy. 1994. Efficacy of domperidone and sulpride as treatments for fescue toxicosis in horses. Am. J. Vet. Res. 55:722-729.

Rice, R. L., D. J. Blodgett, G. G. Schurig, W. S. Swecker, J. P. Fontenot, V. G. Allen, and R. M. Akers. 1997. Evaluation of humoral immune responses in cattle grazing endophyte-infected or endophyte-free fescue. Vet. Immunol. Immunopathol. 59:285-291.

Rice, R. L., G. G. Schurig, and D. J. Blodgett. 1998. Evaluation of physiologic indicies in mice vaccinated with protein-ergotamine conjugates and fed an endophyte-infected fescue diet. Am. J. Vet. Res. 59:1258-1262.

Rottinghaus, G. E., G. B. Garner, C. N. Cornell, and J. B. Ellis. 1991. HPLC method for quantitating ergovaline in endophyte-infested tall fescue: seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades, and seed heads. J. Agric. Food Chem. 39:112-115.

Samford-Grigsby, M. D., B. T. Larson, J. C. Forcherio, D. M. Lucas, J. A. Patterson, and M. S. Kerley. 1997. Injection of a dopamine antagonist into Holstein steers to relieve symptoms of fescue toxicosis. J. Anim. Sci. 75:1026-1031.

SAS. 1985. SAS User's Guide: Statistics (Version 5 Ed.). SAS Inst. Inc., Cary, NC.

Schmidt, S. P., and T. G. Osborn. 1993. Effects of endophyte-infected tall fescue on animal performance. Ag. Ecosystems Environ. 44:233-262.

Simeone, A., M. L. Westendorf, R. E. Tucker, L. P. Bush, and G. E. Mitchell, Jr. 1998. Ammoniation to reduce the toxicity of endophyte-infected tall fescue seed fed to rats. Drug Chem. Toxicol. 21:67-78.

Sipes, I. G., and A. J. Gandolfi. 1991. Biotransformation of toxicants. In: M. O. Amdur, J. Doull and C. D. Klaassen (Eds.) Casarett and Doull's Toxicology: The Basic Science of Poisons (4^{th} Ed.). p 88-126. Pergamon Press, New York.

Smith, W. L., N. Gay, J. A. Bowling, and M. W. Crowe. 1986. Post-grazing response of steers previously consuming high and low-endophyte fescue. J. Anim. Sci. 63(Suppl. 1):296 (Abstr.).

Strickland, J. R., J. W. Oliver, and D. L. Cross. 1993. Fescue toxicosis and its impact on animal agriculture. Vet. Human Toxicol. 35:454-464.

Studemann, J. A., and C. S. Hoveland. 1988. Fescue endophyte: history and impact on animal agriculture. J. Prod. Agric. 1:39-44.

Tannenbaum, M. G., S. A. Seematter, and D. M. Zimmerman. 1998. Endophyte-infected and uninfected fescue seeds suppress white-footed mouse *(Peromyscus leucopus)* reproduction. Am. Midl. Nat. 139:114-124.

Thompson, R. W., H. A. Fribourg, J. C. Waller, W. L. Sanders, J. H. Reynolds, J. M. Phillips, S. P. Schmidt, R. J. Crawford, Jr., V. G. Allen, D. B. Faulkner, C. S. Hoveland, J. P. Fontenot, R. J. Carlisle, and P. P. Hunter. 1993. Combined analysis of tall fescue steer grazing studies in the eastern United States. J. Anim. Sci. 71:1940-1946.

Thompson, F. N., and J. A. Stuedemann. 1993. Pathophysiology of fescue toxicosis. Agric. Ecosystems Environ. 44:263-281.

U. S. Meat Animal Research Center (USMARC). 1990. Germ plasm evaluation reports. Progress Reports:1-12.

Varney, D. R., C. J. Kappes, S. L. Jones, R. Newsome, M. R. Siegel, and P. M. Zavos.

1988. The effect of feeding tall fescue seed infected by *Acremonium coenophialum* on pregnancy and parturition in female rats. Comp. Biochem. Physiol. 89C:315-320.

Varney, D. R., M. Ndefru, S. L. Jones, R. Newsome, M. R. Siegel, and P. M. Zavos. 1987. The effect of feeding endophyte infected tall fescue seed on reproductive performance in female rats. Comp. Biochem. Physiol. 87C:171-175.

Varney, D. R., L. A. Varney, R. W. Hemken, P. M. Zavos, and M. R. Siegel. 1991. Onset of puberty in CD-1 mouse pups exposed prenatally through weaning to endophyte-infected tall fescue seed. Theriogenology 35:883-892.

Westendorf, M. L., G. E. Mitchell, Jr., and R. E. Tucker. 1992. Influence of rumen fermentation on response to endophyte-infected tall fescue seed measured by rat bioassay. Drug Chem. Toxicol. 15:351-362

White, J. F., Jr., G. Morgan-Jones, and A. C. Morrow. 1993. Taxonomy, life cycle, reproduction and detection of *Acremonium* endophytes. Agric. Ecosys. Environ. 44:13-37.

Wilkinson, S. R., J. A. Stuedemann, and D. J. Williams. 1983. Animal performance on tall fescue: fat necrosis. Proc. Tall Fescue Toxicosis Workshop, March 17-18, Atlanta, GA.

Zavos, P. M., A. H. Cantor, R. W. Hemken, R. J. Grove, D. R. Varney, and M. R. Siegel. 1993. Reproductive performance of Japanese quail fed tall fescue seed infected with *Acremonium coenophialum*. Theriogenology 39:1257-1266.

Zavos, P. M., B. Salim, J. A. Jackson, Jr., D. R. Varney, M. R. Siegel, and R. W. Hemken. 1986. Effect of feeding tall fescue seed infected by endophytic fungus *(Acremonium coenophialum)* on reproductive performance in male rats. Theriogenology 25:281-290.

Zavos, P. M., M. R. Siegel, R. J. Grove, R. W. Hemken, and D. R. Varney. 1990. Effects of feeding endophyte-infected tall fescue seed on reproductive performance in male CD-1 mice by competitive breeding. Theriogenology 33:653-660.

Zavos, P. M., D. R. Varney, L. P. Bush, R. W. Hemken, J. A. Jackson, and M. R. Siegel. 1988a. Reproductive responses in male rats fed extracts of fescue seed infected by the endophytic fungus *Acremonium coenophialum.* Drug and Chem. Toxicol. 11:113-133.

Zavos, P. M., D. R. Varney, R. M. Hemken, M. R. Siegel, and L. P. Bush. 1988b. Fertilization rates and embryonic development in CD-1 mice fed fungal endophyte-infected tall fescue seed. Theriogenology 30:461-468.

Zavos, P. M., D. R. Varney, J. A. Jackson, R. W. Hemken, M. R. Siegel, and L. P. Bush. 1988c. Lactation in mice fed endophyte-infected tall fescue seed. Theriogenology 30:865-875.

Zavos, P. M., D. R. Varney, J. A. Jackson, M. R. Siegel, L. P. Bush, and R. W. Hemken. 1987. Effect of feeding fungal endophyte (*Acremonium coenophialum*)-infected tall fescue seed on reproductive performance in CD-1 mice through continuous breeding. Theriogenology 27:549-559.

Zhu, B. T., L. A. Suchar, M. Gwynne, and M. Huang. 1995. Effect of age and sex on the inducability of hepatic microsomal 4-nitrophenol UDP-glucuronyltransferase activity in rats. Life Sci. 57:19PL-24PL.

Appendix I

Laboratory Procedure for Determining Glutathione-S-Epoxytransferase and Uridine Diphosphate Glucuronosyltransferase Activities in Mouse Livers

by

Catherine Ann R. Wagner
Linda Correll

Completed
May 1, 1999

Liver collection

Purpose

To collect and preserve mouse livers for determination of glutathione-S-epoxytransferase and uridine diphosphate glucuronosyltransferase activities.

Procedure

Kill mice by CO_2 asphyxiation or other approved, humane method.

Extract liver, seal in labeled plastic biological sample bag, and place immediately on ice or in liquid nitrogen. Do not keep sample on ice for longer than two hours. Store at –70°C until needed.

Materials

CO_2 tank

Dissection kit, including scalpel or scissors

Plastic storage bags

Ice or liquid nitrogen

Cytosolic and Microsomal Extractions

Purpose

To obtain cytosol and microsome fractions from mouse liver for glutathione-S-transferase and UDP-glucuronyltransferase assays

Reagents and Buffers

Prepare stock solutions prior to procedure; expiration 3 mo.

Liver Homogenizing Buffer (pH 7.4)

K_2HPO_4 (MW 174) and NaH_2PO_4 (MW 120) in 0.1 M 1.15% KCl buffer

Prepare 1.15% KCl solution.

11.5 gm KCl per 1 L ddH_2O
or
5.75 gm KCl per 500 mL ddH_2O

Prepare K_2HPO_4 and NaH_2PO_4 in KCl solution.

B	K_2HPO_4	17.4 gm / 1 L KCl or 8.7 gm / 500 mL KCl
A	NaH_2PO_4	12.0 gm / 1 L KCl or 6.0 gm / 500 mL KCl

Add A to B and check pH. Store at -4 °C; bring to room temperature before use.

Microsome Resuspension Buffer (pH 7.4)

10 mM Tris-Acetate, 0.1 mM EDTA, 20% wt/vol glycerol

Tris-Acetate	1.81 gm / 1 L
EDTA	29.2 mg / 1 L
Glycerin	200 gm / 1 L

Add Tris-Acetate and EDTA to 750 mL ddH_2O. Adjust pH to 7.4 using 2.5 M NADH. Add 200 gm glycerin. After mixing, bring to 1 L volume with ddH_2O. Check pH. Store in -4 °C; bring to room temperature before use.

Procedure

Divide livers into manageable groups for processing and record date and group number

Prepare 25% homogenates in liver homogenizing buffer.

Weigh approximately 1.75 gm mouse liver and place in glass test tube.

Add KCl buffer.
mL of buffer = liver weight in gm x 3. Example: use 1.75 gm liver and 5.25 mL KCl buffer.

Homogenize mechanically for approximately 10 sec using Polytron homogenizer with speed set at 5.

Centrifuge 10,000 rpm at 4° C for 10 min after visually balancing tubes. Aspirate supernatant into ultracentrifuge tubes, carefully avoiding fat layer on surface and "junk" tissue layer on bottom of tube.

Centrifuge 40,000 rpm at 5° C for 1 hr after carefully balancing pairs to within 0.05 gm. Do not overfill ultracentrafuge tubes- maximum weight should not exceed 7.45 gm.

Mark volume of supernatant on centrifuge tube with marker or thumbnail, then pour off supernatant (cytosol), leaving pellet (microsomes), into three 1.5 mL microcentrifuge tubes (Eppendorf) and freeze at -70° C.

Bring pellet to marked volume using microsome resuspension buffer. Resuspend pellet by dislodging with a spatula, then homogenize with a glass tissue homogenizer. Aliquot into three 1.5 mL microcentrifuge tubes (Eppendorf) and freeze at -70° C.

Glutathione-S-Epoxytransferase Activity Assay

Purpose

To provide a microassay for glutathione-S-epoxytransferase activity in mouse liver using the substrate 1,2 epoxy(p-nitrophenoxy)propane

Reagents and Buffers

0.1 M Sodium Phosphate Buffer (pH 6.5)

A NaH_2PO_4 (Sigma # S-0751; mw 120) 6.0 g / 500 mL ddH_20 (sodium phosphate monobasic)

Swirl before adding full volume to prevent clumping.

B Na_2HPO_4 (Sigma # S-0876; mw 142) 7.1 g / 500 mL ddH_20 (sodium phosphate dibasic)

Swirl before adding full volume to prevent clumping.

Add B (~270 mL) to A (500 mL) until pH equals 6.5. Store in -4°C; bring to room temperature before use. Prepare stock solution prior to assay; expiration 3 mo.

10 mM Glutathione Solution

Glutathione (Sigma # G-4251; mw 307)

18.42 mg / 1 mL sodium phosphate buffer

Prepare on day of assay. Store at -4°C until immediately before use; discard unused portion.

0.5 mM 1,2 Epoxy(p-nitrophenoxy)propane Solution

2X: Epoxy (Acros # 40944.0050)

11.70 mg / 10 mL 100% ETOH

Epoxy is difficult to soluablize in ddH_20.

1X: Above epoxy/ETOH solution is diluted 1:2 in sodium phosphate buffer just prior to assay.

Want final concentration of 0.585 mg epoxy / 1 mL solution and 8.33% ETOH in system for assay. Prepare on day of assay. May store at -4°C until needed; bring to room temperature prior to use.

Procedure

Prior to assay

Thaw cytosol samples at -4° C.

Prepare 10 mM glutathione solution and refrigerate.

Bring epoxy solution and sodium phosphate buffer to room temperature.

Warm microplate reader (Spectramax 250, Molecular Devices Corp., Sunnyvale, CA) to 37° C.

Set microplate reader parameters using SoftmaxPro program (Version 1.2.0, Molecular Devices Corp., Sunnyvale, CA, 1994).

Choose kinetic run.

Read at 360 nm wavelength.

Run at 37° C.

Include a 3 minute lag time prior to start.

Set run time of 11 min with 23 readings of 30 seconds each.

Mix one time prior to the run but do not mix in between readings.

Diagram template. Use 3 replicates of each sample, a tissue blank for each sample, and one reagent blank per tray.

Microplate preparation

In flat bottom plate (ninety-six 300 ul wells), pipette into each well:

25 ul vortexed cytosol

175 ul sodium phosphate buffer

Do not mix in microplate reader.

Pipette into each well:

50 ul vortexed glutathione solution

QUICKLY ADD 50 ul 1X vortexed epoxy solution

Mix in microplate reader for 10 seconds.

Choose Read in SoftmaxPro and run for 11 min.

Protein assay

See Protein Determination Using the Bio-Rad Method (SOP 71-M-05)

Use a 1:100 dilution for this assay.

Sample Calculations

Twenty-three samples can be run in triplicate per plate with a tissue blank for each sample and up to 8 reagent blanks (these calculations include 3).

4 wells x 25 ul cytosol = 100 ul cytosol from each sample.

For two plates, only need 0.2 mL from the 1.5 mL microcentrifuge tubes.

91 wells x 50 ul / well glutathione solution = 4550 ul glutathione / plate.

1 formulation (10 mL) of glutathione solution can be used for 2 plates.

72 wells x 50 ul / well epoxy solution = 3600 ul 1X epoxy / plate.

Formulate 8 mL of 1X epoxy solution for two plates: 4 mL 2X epoxy solution + 4 mL sodium phosphate buffer.

Tissue blank- cytosol, glutathione solution, sodium phosphate buffer

Reagent blank- epoxy solution, glutathione solution, sodium phosphate buffer

Epoxy (for two plates):

2x: make 6 mL

$$\frac{11.70 \text{ mg epoxy}}{10 \text{ mL ETOH}} = \frac{7.02 \text{ mg epoxy}}{6 \text{ mL ETOH}}$$

1x: make 10 mL

use 5 mL 2x + 5 mL sodium phosphate buffer

Glutathione (for two plates):

make 12 mL

$$\frac{18.42 \text{ mg GST}}{1 \text{ mL sodium phosphate buffer}} = \frac{.22104 \text{ g GST}}{12 \text{ mL sodium phosphate buffer}}$$

References

Kaplowitz, N., J. Kuhlenkamp, and G. Clifton. 1975. Drug induction of hepatic glutathione S-transferases in male and female rats. Biochem. J. 146:351-356.

Uridine Diphosphate Glucuronosyltransferase Activity Assay

Purpose

To provide an assay for UDP-Glucuronyltransferase activity in mouse liver

Reagents and Buffers

75 mM Tris-Maleate buffer (pH 7.4)

Prepare 17.79 gm trizma maleate (Sigma # T-3128, mw 237.2) in 800 mL ddH_20.

Adjust pH using 1N or 5N sodium hydroxide.

Bring to 1 L and check pH.

Store at -4° C; bring to room temperature before use. Prepare stock solution prior to assay; expiration 3 mo.

1.2 M Sodium Phosphate Buffer (pH 2.35 - 2.45)

Prepare 144.0 g NaH_2PO_4 (Sigma # S-0751; mw 120) in 800 mL dd_H20.

Adjust pH using (approx. 100 mL) 85% phosphoric acid.

Bring to 1 L and check pH.

Store at -4° C; bring to room temperature before use. Prepare stock solution prior to assay; expiration 3 mo.

Substrate Solution

0.5 mM 2-aminophenol (Aldrich # A7,130-1; mw 109.13), 1.0 mM ascorbic acid (Sigma # A-7631; mw 195.1), and 10 mM magnesium chloride (Sigma # M-0250; mw 406.6) in 75 mM tris-maleate buffer

Prepare 10.9 mg 2-aminophenol, 39.6 mg ascorbic acid, and 406.6 mg magnesium chloride in 100 mL tris-maleate buffer.

Aliquot 8-10 mL into freezable tubes (polyethylene) and store at -70° C. Solution should remain colorless- yellow color indicates oxidation and solution must be freshly prepared. Expiration 3 mo.

1.0% w/v Ammonium Sulfamate Solution

Prepare 3.0 gm $NH_4SO_3NH_2$ (Sigma # A-8670; mw 114.1) in 300 mL ddH_20.

Store at -4° C; bring to room temperature before use. Expiration 3 mo.

1.0 M Trichloroacetic Acid Solution (TCA)

Prepare 81.7 gm TCA (Mallinckrodt # 2928; mw 163.4) in 500 mL ddH_20.

Store at -4° C; bring to room temperature before use. Expiration 3 mo.

2-Aminophenol-b-glucuronide [or O-Aminophenyl b-D-glucuronide] Standard Solutions

Prepare from 1 mg / 1 mL stock solution (Sigma # A-4667; mw 285.3)).

See sample calculations below. Expiration 3 mo.

0.2% w/v Sodium Nitrite Solution

Prepare 0.60 gm $NaNO_2$ (Sigma # S-2252; mw 69.0) in 300 mL ddH_20.

Store at -4° C; bring to room temperature before use. Expiration 1 wk

52 mM Uridine 5'-diphosphoglucuronic acid (UDP-GA) Solution

Prepare 342.9 mg UDP-glucuronic acid (Sigma # U-6751; mw 646.2; purity 98%) in 10 mL tris-maleate buffer.

If purity or mw differ, prepare using the equation:

(52 mM / L) x (0.01 L) x (mw) x (100 / % purity) = ___ mg / 10 mL

Prepare fresh on day of use.

0.2% w/v N-(1-napthyl)ethylenediamine, dihydrochloride Solution

Prepare 0.60 gm N-(1-napthyl)ethylenediamine (Sigma # N-5889; mw 259.2) in 300 mL ddH_20.

Leave at room temperature. Prepare fresh on day of use.

Procedure

Prior to assay

Thaw microsomes, return to -4° C.

Thaw substrate solution, return to -4° C.

Set water bath to 37° C.

Prepare UDP-GA and ethylenediamine solutions daily and sodium nitrite solution weekly.

Sample analysis

Prior to initiating sample assays, analyze standards (calculations below) to provide standard curve and to determine technician proficiency. During all following sample analyses, use one standard (40 ug/mL) as a positive control.

During each run, analyze microsome samples, a tissue blank (negative control), a reagent blank, and a standard.

Add the following to 50 mL beakers:

Sample beakers:	Standard beaker:
325 ul substrate solution	325 ul substrate solution
150 ul tris-maleate buffer	150 ul tris-maleate buffer
50 ul UDP-GA solution	50 ul UDP-GA solution
125 ul liver microsomes	125 ul (40 ug/mL) standard solution

Tissue blank beaker:	Reagent blank beaker:
no substrate solution	325 ul substrate solution
475 ul tris-maleate buffer	275 ul tris-maleate buffer
50 ul UDP-GA solution	50 ul UDP-GA solution
125 ul liver microsomes	no liver microsomes

Swirl beakers vigorously and incubate in a 37°C oscillating water bath (7-8 opm) (Precision # 668800) for 20 min.

Stop the reaction by adding 650 ul TCA to beakers.

Pour solutions into test tubes and centrifuge for 5 min. in a tabletop centrifuge (Fisher Centrific) set at speed 7.

Transfer ~1.0 mL of supernatant to new test tubes. Due to evaporation during incubation, supernatant volume may range from 600 ul to 1 mL. The available supernatant volume must be recorded for later calculations, but the following reagent additions do not have to be reduced accordingly; they are added in excess anyway.

Add 1.0 mL sodium phosphate buffer. Vortex vigorously.

Add 500 ul sodium nitrite solution. Vortex vigorously. Wait at least 3 min.

Add 500 ul ammonium sulfamate solution. Vortex vigorously. Wait at least 3 min.

Add 500 ul N-(1-napthyl)ethylenediamine solution. Vortex vigorously.

Incubate in the dark at room temperature for 90 min.

Read absorbance at 550 nm in a spectrophotometer (Beckman DU640B) by vortexing the solution and pouring it into a 3 mL cuvette. Read immediately to prevent bubble formation on sides of cuvette. Rinse the cuvette with ddH_20 prior to reading each sample.

Note: Normally standards, especially concentrated ones, will turn purple before the 90 min. incubation. The pH of standards after incubation should be 2.2-2.3. After incubation, tissue samples will become various shades of purple, the reagent blank will be faintly purple, and the tissue blank will remain clear. However, if the tissue samples turn purple before incubation the solutions may be too acidic. If the solutions are too basic, they will turn cloudy yellow-brown or yellow-pink.

Protein analysis

Measure the protein concentration following the protocol titled "Protein Determination Using the Bio-Rad Method" (SOP 71-M-05). Use a 1:50 dilution.

Sample Calculations

Standards

final concentration (ug/mL)	vol. of 500 ug/mL solution (ul)	vol. tris-maleate buffer (ul)	
200	400	600	= 1000 ul
100	200	800	
80	160	840	
60	120	880	
40	80	920	
20	40	960	
10	20	980	
5	10	990	
	1030 ul		

Make 1.0 mL of each standard. Stock solution is 1 mg/mL. Prepare 500 ug/mL solution to create standards.

1050 ul of 500 ug/mL sln = $\frac{\text{525 ul 1 mg/mL stock sln}}{\text{525 ul 1 mg/mL stock sln + 525 ul tris-maleate buffer}}$

1000 ul of 200 ug/mL sln = $\frac{\text{400 ul 500 ug/mL prepared sln}}{\text{400 ul 500 ug/mL prepared sln + 600 ul tris-maleate buffer}}$

References

Dutton, G. J., J. E. A. Leakey, and M. R. Pollard. 1981. Assays for UDPglucuronyltransferase activities. Methods Enzymol. 77:383-391.

Protein Determinations

Purpose

To determine protein content of liver tissue using the Bio-Rad protein method.

Procedure

A. Preparation of the standard curve

Use Bovine Serum Albumin (BSA) (Bio-Rad Chemical Division, South Richmond, CA) to prepare 1 mL of a .500 mg/mL solution using $V_1C_1 = V_2C_2$

For example, if stock solution = 1.42 mg/mL protein:

$V_1 = X$ $V_2 = 1.00$ mL

$C_1 = 1.42$ mg/mL $C_2 = .50$ mg

$$(X)\ (1.42) = (1.00)\ (.50)$$

$$(X) = \underline{.35 \text{ mL BSA}}$$

Adjust to 1.00 mL with .65 mL distilled water.

Use this solution to prepare a range of standards.

Storage at -70° C for ≤6 mo.

Standard	Final Concentration (mg/mL)	BSA (.5 mg/mL) (ul)	Volume H_2O (ul)
S8	.50	500	0
S7	.40	400	100
S6	.30	300	200
S5	.20	200	300
S4	.10	100	400
S3	.05	50	450
S2	.025	25	475
S1	0	0	500

B. Preparation of Bio-Rad dye reagent

Dilute Bio-Rad protein dye reagent (Bio-Rad Chemical Division, South Richmond, CA):

1 part Bio-Rad protein dye and 4 parts water (Ex: 25 mL Bio-Rad + 100 mL H_2O)

Filter through #1 Whatman paper

Protect from light; can be stored at room temperature for 12 d.

C. Read samples in microplate reader (Spectramax 250, Molecular Devices Corp., Sunnyvale, CA)

Samples:

Add 120 ul BioRad solution to microplate wells

Add 10 ul sample* to microplate wells

Mix in microplate reader

Add 130 ul BioRad solution to microplate wells

Mix in microplate reader

* Samples may be diluted prior to analysis to fall on absorbance curve defined by standards. A 1:100 dilution was generally best (25 ul sample + 2475 ul PBS buffer)

Blank:

Add 250 ul Bio-Rad solution to microplate wells

Add 10 ul buffer to microplate wells

Mix in microplate reader

A correlation above .97 is acceptable

Sample Calculations

Example:

Absorbance:	.566
Dilution Factor:	100
Slope:	2.02
Protein in mg/mL:	((.566) / (2.02)) (100) = 28.01 mg/mL

On microplate reader printout, multiply mean result by dilution factor for same answer.

References

Procedure modified from SOP 71-M-05, Toxicology Laboratory, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Glutathione-S-Epoxytransferase Activity Calculations

Definitions

$\Delta A = \varepsilon\ b\ c$
ΔA: change in absorbance, measured in OD/minute
ε = absorbtivity, a constant for GST measured in millimolar
b = cell length, a constant for GST measured in centimeters
c: concentration, measured in nanomoles/mL/minute

ΔA:	.008015 OD/minute
protein:	13.0 milligrams/milliliter
total well volume:	.3 milliliters
cytosolic well volume:	.025 mL
ε:	.5 /millimolar
b:	.86 cm

Example

To find the change in absorbance per minute:

$\Delta A = \varepsilon\ b\ c$
$(.008015) = (.5)\ (.86)\ (c)$
c = 18.6 nanomoles/mL/minute

To find the activity per incubation:

(18.6 nanomoles/mL/minute) (.3 milliliters/incubation)
= 5.59 nanomoles/minute/milliliter

To find the enzyme activity per milliliter:

5.59 nanomoles/minute/milliliter / (.025 milliliter) (13.3 milligram/incubation)
= 16.8 nanomoles per minute per milligram protein

References

Kaplowitz, N., J. Kuhlenkamp, and G. Clifton. 1975. Drug induction of hepatic glutathione S-transferases in male and female rats. Biochem. J. 146:351-356.

Laboratory notebook of Diane M. Flaherty, Toxicology Laboratory, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Uridine Diphosphate Glucuronosyltransferase Activity Calculations

Definitions

$\Delta A = \varepsilon\ b\ c$

ΔA: change in absorbance, measured in OD/minute

ε = absorbtivity, a constant for UDPGT measured in millimolar

b = cell length, a constant for UDPGT measured in centimeters

c: concentration, measured in nanomoles/mL/minute

ΔA:	.1349 OD/minute
protein:	5.9 milligrams/milliliter
total tube volume:	650 microliters
microsomal tube volume:	125 microliters
TCA volume:	650 microliters
supernatant volume:	750 microliters
post water-bath volume:	4 milliliters
ε:	39 /micromolar
b:	1 cm

Example

$$\text{Absorbance} = \frac{(\Delta A)\ (\text{Factor})}{(\varepsilon)\ (\text{protein})}$$

Factor = (Volume of tubes into water bath / Volume of microsomes) ((Volume of tubes into water bath + Volume of TCA)/Volume of tubes into water bath)) (Volume of supernatant + volume of post-water bath additions / Volume of supernatant)

Factor = (650 ul / 125 ul) (650 ul + 650 ul/ 650 ul) (750 ul + 4000 ul / 750 ul)

Factor = 32.9

Absorbance = $\frac{(.1556 \text{ OD/minute}) (32.9 \text{ ul})}{(39 \text{ micromolar}) (5.9 \text{ milligrams/milliliter})}$

= 2.2 nanomoles per minute per milliliter

References

Dutton, G. J., J. E. A. Leakey, and M. R. Pollard. 1981. Assays for UDPglucuronyltransferase activities. Methods Enzymol. 77:383-391.

Laboratory notebook of Diane M. Flaherty, Toxicology Laboratory, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Appendix II

Data Listing

Entered By

Catherine Ann R. Wagner
William D. Hohenboken

Completed
April 10, 1999