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Project Summary

Microinjection of Fish Embryos as a Laboratory Assay for Chemical Carcinogens

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During this project, techniques were developed for microinjection of chemicals into fish eggs, lesions were described in fish reared from injected eggs, and eggs of various species were compared to determine which were best suited for use in carcinogenicity assays. Eggs of the following species were injected: gulf killifish, sheepshead minnow, rivulus, inland silverside, gulf toadfish, and channel catfish. Chemicals injected into eggs were diethylnitrosamine, N-methyl-N'-nitro-N-nitroso-guanidine (MNNG), aflatoxin B1, and trichloroethylene.

Quantification of carcinogen dose in the egg immediately after injection indicated that variation of the dose retained was a major problem. During the project, improvements in procedures resulted in increased mean percentage of the dose remaining in the egg, but variation between eggs remained high.

The incidence of all lesions, and especially neoplasms, was low, probably because of the low doses retained in the eggs. The most important lesion found during this project was a pancreatic acinar cell carcinoma in a gulf killifish injected with MNNG.

Gulf killifish, sheepshead minnow, and rivulus eggs have potential for use in carcinogenicity assays. However, improvements in microinjection techniques are needed before an assay system involving injection of test chemicals into embryos of these species can be used reliably. Our results suggest that channel catfish are less susceptible than other species to the pathologic effects of the chemicals that we tested.

This Project Summary was developed by EPA's Environmental Research Laboratory, Gulf Breeze, FL, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Several species of fish are potentially valuable as laboratory animals in oncology Numerous studies have indicated that fish develop neoplasms after exposure to a variety of known carcinogens. For some fish models, the neoplastic responses of fish to chemical carcinogens resemble those of rodents. Two major advantages of fish as assay animals are evident from previous studies. The length of time required for neoplasms to develop in fish is often less than 6 months and has been reported as 5 to 8 weeks in some experiments. In addition, spontaneous neoplasms are rarely found in non-exposed fish used in laboratory assays. These features of fish assay systems indicate that they could be useful as alternative or complementary tests for carcinogens.

Rainbow trout, *Salmo gairdneri*, embryos are highly sensitive to chemically induced neoplasia, embryo exposure of Shasta strain rainbow trout appears to be the most sensitive model

available for hepatocarcinogenicity of aflatoxin B1 (AFB). Initially, embryo exposures involved dipping whole eggs into the test solution, a technique that worked well with slightly water-soluble carcinogens such as AFB. However, this model is not suitable for exposing the embryos to highly water-insoluble chemicals.

Microinjection of carcinogens inside fish eggs ensures exposure of the embryo to the test chemical. This technique has been used to induce neoplasms in rainbow trout and coho salmon, *Oncorhynchus kisutch*, exposed to a variety of carcinogens. Microinjection using acetone or dimethylsulfoxide (DMSO) as carriers for test chemicals allows embryo exposure to highly water-insoluble compounds such as benzo[a]pyrene and 7,12-dimethylbenz(a)anthracene. An additional advantage of the microinjection method is the diminutive amount of test chemical needed.

Although fish embryo exposures appear to be a promising method to test new chemicals for carcinogenicity, the use of salmonids presents limitations to this model. These limitations include exacting rearing conditions needed for these species, long generation time, and difficulty in obtaining eggs outside of the short, natural spawning season. The relative merits of non-salmonid species for use in embryo exposure models have not been adequately tested. There are many fish species that would eliminate all of the limitations listed above for the salmonid species.

The objectives of this project were (1) to develop techniques needed for microinjection of chemicals into eggs of several fish species, (2) to compare eggs of several fish species to determine which are best suited for use in carcinogenicity assays, and (3) to determine the types of lesions resulting from exposure of eggs to known carcinogens. Ultimately this could result in a method for rapid, reproducible, and sensitive screening of chemicals relevant to humans.

Six species of fish were selected for this project: gulf killifish (*Fundulus grandis*), sheepshead minnow (*Cyprinodon variegatus*), rivulus (*Rivulus ocellatus*), inland silverside (*Menidia beryllina*), gulf toadfish (*Opsanus beta*), and channel catfish (*Ictalurus punctatus*). Chemicals injected into eggs were AFB, diethylnitrosamine (DEN), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and trichloroethylene (TCE).

Results

Hatching percentages of some groups of injected channel catfish and gulf killifish eggs were nearly as high as for uninjected eggs. The highest survival of sheepshead minnow eggs was less than one-half of the percentage for uninjected eggs. Eggs that were not disinfected with antibiotics had a lower hatching percentage that disinfected eggs. There was only a small difference between groups of sheepshead minnow eggs injected in the perivitelline space or into the yolk sac. Only two groups of inland silversides eggs were injected because hatching percentage of injected eggs was low [9% for DMSO injected (control) and 8% for DEN injected]. Hatching percentages of many groups of eggs injected with test chemicals were as high or higher than eggs injected with DMSO or saline. Except for inland silversides, the lowest hatching percentages were above 12%.

Long-term survival of test fish was highly variable among groups of fish, but survival did not seem related to treatment. Many of the test fish and controls died of infectious diseases. Enteric septicemia of catfish caused by the bacterium *Edwardsiella ictaluri* killed most of the fish in some of the channel catfish treatments, including the DMSO-injected controls. Posthatching survival of injected toadfish was low, apparently because of fungal infections. These fish could have been predisposed to this disease because of injury caused by the injection. An intestinal protozoan was noted in sections of sheepshead minnows.

Quantification of carcinogen dose retained in the egg immediately after injection indicated that variation in the percentage of the dose retained was a problem in our methodology. Rivulus eggs injected in the perivitelline space retained less than 7% of the nominal dose and those injected in the yolk sac retained 6 to 27%. One attempt to inject 0.25 µl instead of the usual 0.5 µl dose did not improve the results. Injection of sheepshead minnow eggs was the most successful, 35 to 47% of the dose was retained. Standard deviations were approximately equal to the mean for most injections of labeled compounds. The syringe system was not the source of error because the mean dose delivered was 106% of the nominal dose with a standard deviation of 6%.

The incidence of all lesions, and especially neoplasms, was low, probably because of the low doses retained in the embryos. Two neoplastic lesions and another lesion that further study may determine to be neoplastic were found in carcinogen-exposed fish. Other lesions were preneoplastic, teratogenic, toxic reactions, or pathogen related. Lesions caused by pathogens were not considered in this report, although the occurrence of infectious diseases could have been influenced by the chemical exposures.

The most important lesion found during this study was a pancreatic acinar cell carcinoma in a gulf killifish necropsied 139 days after an injection of 2 µg MNNG. This neoplasm had invaded the posterior liver. The tumor cells resembled pancreatic acinar cells by having basophilic cytoplasm except for the strongly eosinophilic zymogen granules. The cells tended to form acini and had a high mitotic index. This is only the third report of an experimentally induced neoplasm of the exocrine pancreas in fish.

Sheepshead minnows injected with DEN or AFB tended to have increased numbers of rodlet cells in the mesentery or mesenteries. Nuclear pleomorphism was observed in livers of AFB injected sheepshead minnows. Lesions in rivulus injected with DEN included hepatic adenoma, megalocytic hepatosis, nuclear pleomorphism, thyroid hyperplasia, and a convoluted retina embedded in the brain. Nuclear pleomorphism was also observed in the liver of rivulus injected with AFB. A mass that has not been identified yet was found in the posterior body cavity of a rivulus injected with MNNG. No lesions related to carcinogen exposure were observed in the 72 exposed channel catfish examined. Only one gulf toadfish exposed to a test chemical survived until necropsy; this fish had no lesions.

Conclusions and Recommendations

Survival of channel catfish and gulf toadfish embryos injected with stainless-steel needles (31 gauge) was satisfactory as was the survival of gulf killifish, sheepshead minnow, and rivulus injected with sharpened glass needles (48 to 112 µm outside diameter). Mortality of injected inland silverside embryos was too high for this species to be useful for this technique. Survival of sheepshead minnows was similar for injection of test chemicals into the yolk sac or into the perivitelline space. A 5-minute rinse in a combination of penicillin, streptomycin, and fungizone

reduced the number of embryos killed by bacterial and fungal infections. Although these antimicrobial drugs reduced mortality of injected embryos, future research should consider their possible role as promotors or inhibitors of neoplastic responses.

Quantification of carcinogen dose retained in the egg immediately after injection indicated that variation in the percentage of the dose retained was a problem. During this project, improvements in procedures resulted in increased mean percentage of the dose remaining in the egg, but variation between eggs remained high. Additional research is needed to complete development of techniques for accurate delivery and adequate retention of carcinogens in small eggs of fish.

The incidence of lesions, and especially neoplasms, was probably low because of the low doses retained in the embryos The variation in retention of carcinogen could have resulted in survival of fish that retained little of the dose while other fish, injected with the same nominal dose, died because they retained a higher percentage. This would have biased the lesion-incidence data because it was not possible to determine which fish retained high doses.

The use of fish embryo exposures seems promising as a method to induce pancreatic tumors that could be used to resolve questions about histogenesis of exocrine pancreatic carcinomas There are only two previous reports of chemical carcinogens inducing pancreatic neoplasms in fish and both of these previous studies involved exposure of young fish Our results support the hypothesis that the types of neoplasms developing in carcinogen-exposed fish depend on the age of the fish when exposed.

Gulf killifish, sheepshead minnow, and rivulus eggs have potential for use in carcinogenicity assays. Eggs of these species can be easily obtained at any time of the year, and these species develop neoplasms after exposure to carcinogens. However, improvements in microinjection techniques are needed before an assay system involving injection of test chemicals into embryos of these species can be used reliably. Gulf toadfish and channel catfish have relatively large eggs, an advantage for a microinjection assay, but eggs of these species are available only during a relatively short spawning season. In addition, our results suggest that channel catfish are less susceptible than other species to the pathologic effects of the chemicals that we tested. The biologic basis for the low susceptibility of channel catfish to carcinogens should be investigated.

With some further development, microinjection of fish eggs appears to be a promising method for testing chemical carcinogens. The problem of inconsistent delivery of test chemicals to fish eggs must be solved before embryos of sheepshead minnow, gulf killifish, and rivulus can be used in a microinjection test procedure. Reasonable approaches for resolving this experimental problem include injection of smaller volumes of the test chemical, use of smaller diameter needles, a partial enzymatic digestion of the chorion of the egg, or use of other fish species such as brown bullhead (*Ictalurus nebulosus)* and black bullhead (*Ictalurus melas*).

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***John A. Couch** is the EPA Project Officer (see below).*
The complete report, entitled "Microinjection of Fish Embryos as a Laboratory Assay for Chemical Carcinogens," (Order No. PB 88-124 623/AS; Cost: $12.95; subject to change) will be available only from:
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