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Research Article

Detection of Mutagenic-Carcinogenic Pollutants in Aquatic Systems Using Cytogenetic Methods in Fish

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Abstract: In this study, the detection of mutagenic-carcinogenic pollutants in water by using cytogenetic methods in fish was examined along with the necessity of sister chromatid exchange (SCE), anaphase aberrations (AA) and micronucleus (MN) tests for chemical analysis in aquatic systems. It has been reported that central mudminnow (*Umbra limi*) appear to be the most suitable species for such analysis because of its large and fewer chromosomes (2n=22) and high cell division ratio. This species also has a wide distribution, and can be easily captured and held for study. In such analysis, intestines, stomach, kidney and gill tissues stand out as giving superior numbers of usable metaphase and have been widely used.

Key Words: Mutagenic - carcinogenic pollutants, Cytogenetic, Sister chromatid exchange (SCE), Anaphase aberration, (AA), Micronucleus (MN), Central mudminnow, *Umbra limi*

Balıklarda Sitogenetik Yöntemlerle Su Ortamlarındaki Mutajenik ve Kanserojenik Kirleticilerin Belirlenmesi

Özet: Bu çalışmada, balıklarda sitogenetik metotlar kullanmak suretiyle, sularda mutajenik - kanserojenik kirleticilerin belirlenmesi irdelenmiş, suların kimyasal analizlerinin yanında, "Kardeş Kromatid Değişimi (SCE), Anafaz Hatası (AA) ve Mikroçekirdek (MN)" testlerinin de yapılması gerektiği ortaya konulmuştur. Bu tip çalışmalar için, kromozom sayısının az (2n=22), kromozomlarının büyük ve yüksek hücre bölünmesi oranına sahip olması nedeniyle en uygun tür olarak çamur balığı (*Umbra limi*) tavsiye edilmektedir. Ayrıca bu türün oldukça geniş bir yayılıma sahip olup, balıkların elde edilmesi ve laboratuvarda muhafazası da kolaydır. Bu tip çalışmalarda en iyi sonuçlar bağırsak, mide, böbrek ve solungaç dokuları yüksek sayıda kaliteli metafaz yayılımları sağlayabilmekte ve yaygın olarak kullanılabilmektedir.

Anahtar Sözcükler: Kanserojenik ve mutajenik kirleticiler, Sitogenetik, Kardeş kromatid değişimi (SCE), Anafaz hatası (AA), Mikroçekirdek (MN), Çamur balığı, *Umbra limi*

Introduction

As a result of rapid industrial development programmes, about 50,000 chemical substances have been produced within the last decade, and recently more than 1,000 new chemicals have been introduced (1). Rapidly developing industry has caused considerable environmental pollution. In addition, the life of almost all organisms on the planet appears to be under threat from various radiation sources (such as ultraviolet and ionizing radiation). Increases in cancer rates in almost all age groups have been observed due to radiation exposure following the Chernobyl accident. In particular, increments in dicentric chromosome number have been determined. For example, Celep (2) proved that high-level radiation was absorbed through tea and nut consumption by the people of the eastern Black Sea region of Turkey.

Heavy metals (namely Fe, Mn, Cu, Zn, Ni, Cd, Pb, Cr) and aromatic hydrocarbons (naphthalene, anthracene, phenanthrene, fluoranthene, pyrene, triphenylene, chrizene, perylene, 3-4 benzoapyrene) also cause an enhanced frequency of chromosomal aberrations in molluscs (3).

It is the aquatic environment and its resources that are much more affected by environmental pollution, since pollutants that contaminate the air and soil reach the aquatic environment ultimately and accumulate there. It is well known that the disposal of pollutants into aqueous

ecosystems can lead to their accumulation both in sediments and the benthic and pelagic food chains (including fish). Nevertheless, numerous fish and shellfish species (e.g. anchovy, trout, whiting, carp, mullet, shrimp, crayfish, mussels etc.) provide an important source of protein and other nutrients in the diet of humans and certain animals raised for human consumption. For this reason, humans are exposed throughout their lifetime to low levels of toxicants present in both the water and aquatic food. It is essential to know, therefore, what effects, if any, water-borne pollutants have on the genetic material of aquatic organisms, particularly fish (4, 5).

By April of 1986, more than 20,000 chemicals had been evaluated for genotoxicity, and in excess of 10,000 of these had been evaluated by a single test, on one occasion only (6). It is well known that the effects of pollutants are usually displayed first at the biochemical and molecular levels. Then, they lead to genetic changes which become cytologically visible, especially in the tissues of organisms which are good pollutant bioaccumulators, e.g. fish and molluscs (3). Mutations can be grouped as chromosome mutations, comprising changes in chromosome number and structure (i.e. chromosomal changes or anomalies), and gene mutations which can not be observed at chromosomal level, but can be determined from phenotypic changes in individual organisms (7, 8).

Chromosomal aberrations in some animals (including fish) in the wild could serve as useful indicators of the presence and action of clastogenic chemicals in areas known to be polluted with petrochemical products (9, 10). Chemicals that cause structural alterations in fish chromosomes are known as clastogens. Polynuclear aromatic hydrocarbons (PAH) can be metabolized into derivatives that are highly carcinogenic. A well known example is benzo(a)pyrene, a procarcinogen that can be activated into a carcinogen through the metabolic addition of oxygen at specific sites on the molecule. This PAH usually enters nature via discharges from steel mills with cooking ovens and from petrochemical industries, and natural forest fires. PAH pollution is correlated with an increase of tumours in aquatic biota. Many carcinogenic chemicals must be metabolically activated, so we have to know the ability of fish to convert a procarcinogen and/or promutagen into its active form (11). Many types of DNA damage caused by mutagens present in water induce an alteration in chromosomes, so the measurement of chromosomal aberrations offers an acceptable parameter for monitoring mutagenic substances in water. Moreover, chromosomal aberrations selectively count only the primary DNA lesions that are not repaired by the machinery of the cell (12, 13).

The genetic material of fish can be also be affected by ionising radiation in different ways. Ionising radiation, at least in high doses, is known to cause chromosomal damage. The investigation of the consequences of radiation accidents like that of Chernobyl should not be restricted only to physical measurements, since biological methods can also be useful.

Advances in studies with mammalian, and particularly human, cytogenetic materials have led to similar studies on fish, and considerable achievements have been obtained in determining the effects of various genotoxic factors using cytogenetic techniques. However, because of the low public, governmental and even scientific interest in environmental mutagenesis and carcinogenesis, many wild and cultivated aquatic organisms, including fish, are already being exposed to relatively high levels of these uncontrolled carcinogenic-mutagenic chemicals such as industrial waste products, which can affect human health. For the assessment of toxic effects of different pollutants in the aquatic environment, fish are proven to be one of the most important indicator organisms, due both to their top position in the food chain and their requirements for large volumes of water in respiration, making their exposure to pollutants intensive. However, it has to be noted that not all in vitro mutagens prove carcinogenic, and that not all fish carcinogens are mutagens (11). Fish have been proven to be important animals in experimental laboratory work not only for cytotoxicological and different genetic studies, but also for biochemical and physiological research (14).

In spite of the fact that the fish make up the largest and most diverse group of vertebrates, the use of fish chromosomes in genotoxic investigations has been little practised (11, 15).

Unfortunately, only a small number of fish species are suited for cytogenetic investigation because of their large number of chromosomes and/or small size. Moreover, the mitotic index in fish is too low when compared to that of mammals (16).

About the genotoxicity of an investigated body of water, the fish species chosen for chromosomal aberration studies should be easy to handle and inexpensive, require limited amounts of space and equipment, be sensitive, and have a suitable karyotype consisting of a small number of large chromosomes. According to Kligerman (17) the most suitable fish species with karyotypes suitable for clastogenic studies are: *Ameca splendens* (2n=26), *Aphyosemion celia* (2n=20), *Aphyosemion christyi* (2n=18), *Aphyosemion franzwerneri* (2n=22), *Apteronotus albifrons* (2n=24), *Characodon lateralis* (2n=24), *Galaxias maculates* (2n=22), *Nothobranchius rachowi* (2n=16), *Spharicthys osphoromoides* (2n=16), *Umbra limi* (2n=22) and *Umbra pygmaea* (2n=22) (8,17).

Recently, increasing environmental pollution and public awareness, especially in developed countries, have forced scientists to study the direct and indirect effects of the disposal of industrial and other wastes on the aquatic environment. In this review, we have evaluated previous studies and suggested that fish can provide an excellent source of material for the study of the mutagenic and/or carcinogenic potential of water samples under laboratory and field conditions since they are aquatic vertebrate organisms that can metabolise, concentrate and store waterborne pollutants.

Cytogenetic Methods for the Detection of Mutagenic-Carcinogenic Pollutants: Sister Chromatid Exchange is one of the most widely used methods for detecting genotoxic effects. The other well accepted methods on cytogenetic damage are Micronucleus and Anaphase Aberrations.

Sister Chromatid Exchange (SCE) Method: SCE analysis is a far more sensitive indicator of chemically-induced chromosome damage than traditional chromosome breakage studies (8). Many mutagenic-carcinogenic pollutants can induce a significant increase in SCE frequencies (11). Significant increases in SCE have been induced in cultured cells with a number of chemicals (mitomycin C, EMS, MMS, HN_2, etc.) at levels that produce little or no gross chromosome damage (18).

Most of the systems now employed to detect SCE involve cell cultures. These in vitro systems have valuable experimental features, but can only roughly approximate what happens when chemical substances enter a living animal. However, there are two in vivo systems where detection of SCE with a bromodeoxyuridine (Brdu) probe has been achieved: Vicia faba root tips (19, 20), chicks (21) and mouse embryos (22, 23). With this technique, the visualisation of the exchange of genetic material between the two sister chromatids of the same chromosome is easy. This can be done by exposing dividing fish cells to the thymidine analogue 5-bromodeoxyuridine (Brdu) for at least two rounds of DNA replication (Figure 1) (11, 24). Different researchers (e.g. 8, 11, 18) investigated the SCE method that had been described by Kligerman (17).

However, there is no universally established concentration of Brdu that can be used. The optimum could vary from species to species, depending on different factors such as whether they are warm-or cold-water fish, sea or freshwater, carnivorous or omnivorous. Laboratories should experiment to determine a satisfactory level of Brdu concentration in the medium for cell culture or by i.p. or i.m. injection. The SCE method is more sensitive than the analysis of chromosome breakage. The chromosomal aberration test is useful for detecting the effects of carcinogenic-mutagenic chemicals and ionising radiation in high doses where they can induce different types of aberrations, but at low doses they can be detected more easily by SCE.

Anaphase Aberrations (AA) Method: The anaphase aberrations test is one of chromosome aberrations test. The purpose of the *in vitro* and *in vivo* aberration test is to identify agents that cause structural chromosome aberrations in cells (25). Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals (26).

The principle behind the *in vivo* chromosome aberrations test method is that animals are exposed to the test substance by an appropriate route of exposure and are sacrificed at appropriate times after treatment. Prior to sacrifice, animals are treated with a metaphase – arresting agent (e.g. colchicine or colcemid). Chromosome preparations are then made from cells and stained, and metaphase cells are analysed for chromosome aberrations. As for the principle behind the *in vitro* chromosome aberrations test method, cell cultures are exposed to the test substance both with and without metabolic activation. At predetermined intervals

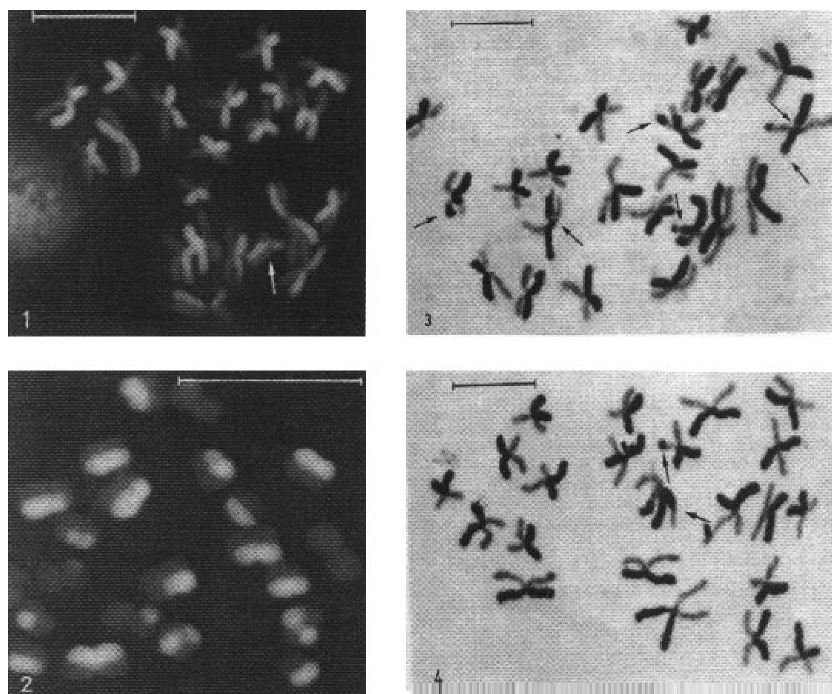

Figure 1. Sister chromatid exchanges (SCEs) in intestinal metaphases of ***Umbra limi*** obtained after i.p. injection of Brdu. 1) Fluorescence preparation showing one SCE at arrow after two rounds of replications. 2) Cell showing SCE after three rounds of replication. 3) Fluorescence plus Giemsa (FPG) preparation showing six SCEs (arrows). 4) FPG preparation with only two SCEs (arrows). Bars indicate 10 microns (11).

after the exposure of cell cultures to the test substance, they are treated with a metaphase – arresting agent.

The anaphase aberration test system in fish has proven to be a relatively simpler procedure than that of chromosomal aberration analysis at metaphase. It allows one to visualise chromosomal macrolesions in mitotic cells during the anaphase, when exposed to very low levels of genotoxicants, in *in vitro* cell cultures (27), and in *in vivo* (28). Anaphase aberrations could occur as a result of a delay in the movement of one or more chromosomes from the metaphase plate during anaphase (anaphase lagging), or by disjunction. In this instance, the centromere is not divided, but is still attached to the spindles of both opposite poles. Trailing and attached fragments are the most frequent in anaphase aberrations and can be seen as a deep-stained structure between the separated chromosomes during anaphase. The anaphase aberrations test on the cells of *Oncorhynchus mykiss* is improved by Liguori and Landolt (29) and its details are presented by Al-Sabti (1).

Micronucleus (MN) Method: The micronucleus test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts by the analysis of arythrocytes as sampled from the peripheral blood cells of animals (usually rodents).

Micronuclei are formed by the condensation of the acentric chromosomal fragments or by whole chromosomes that are not included (18, 30). A micronucleus is a supernumerary nucleus visible under light microscopy in the cytoplasm of a cell (31, 32). Al-Sabti (14) suggested that the micronuclei could be about 1/10 to 1/30 smaller than the principal nucleus (Figure 2 a-d) (11).

Carrasco et al. (33) have applied the micronucleus test to fish, amphibians, or invertebrates that have in common one or more of the following factors which have prohibited a rigorous appraisal of the technique as a biological indicator of chemical contamination: (1) lack of consensus as to which irregularities of nuclear morphology may be considered genotoxic analogues of micronuclei; (2) inadequate laboratory verification that the nuclear lesions actually do result from genotoxicity or, indeed if they result from exposure to any exogeneous agent; (3) inadequate descriptions or few photographs of the putatively genotoxic nuclear lesions that are being enumerated; (4) inconsistent methods of chemical exposure in the laboratory studies; (5) inadequate chemical analysis to confirm the presence of chemical contaminants at field sites where biological samples are collected; and (6) a paucity of samples collected from areas of intermediate pollution severity where the

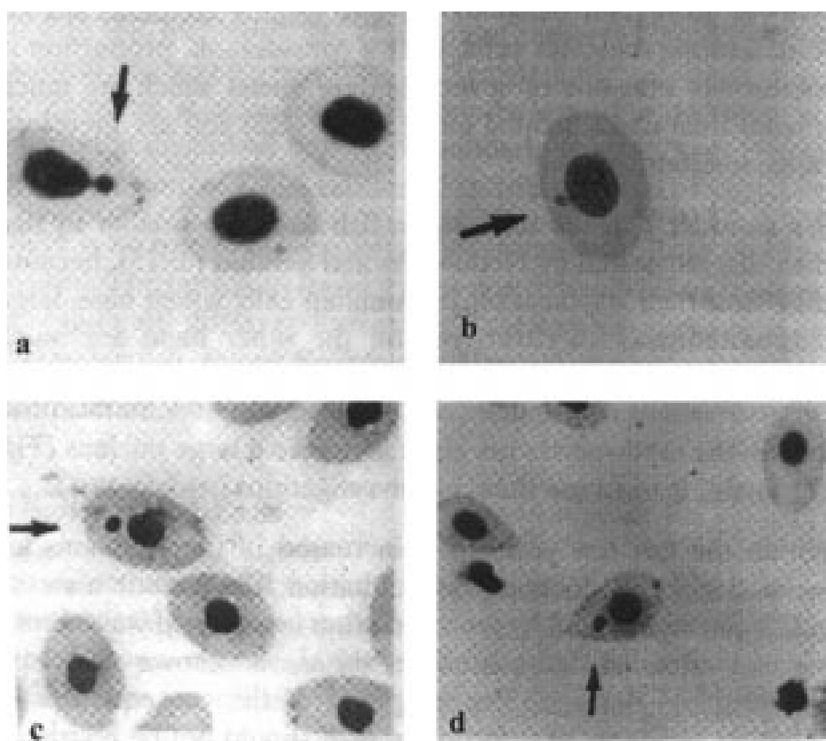

Figure 2. Micronucleated erythrocyte induction (arrowed): a) in common carp (*Cyprinus carpio*); b) chub (*Leuciscus cephalus*); c) tench (*Tinca tinca*); and d) grass carp (*Ctenopharyngodon idella*) (11).

sensitivity of a biological indicator would be of critical importance (33).

In this method, animals are exposed to the test substance by an appropriate route. The peripheral blood is collected at appropriate times after treatment and smear preparations are made and stained. For studies with peripheral blood, as little time as possible should elapse between the last exposure and cell harvest. Preparations are analysed for the presence of micronuclei (34).

For analysis, the proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 1,000 erythrocytes. All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. At least 2,000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes. Additional information may be obtained by scoring mature erythrocytes for micronuclei. When analysing slides, the proportion of immature erythrocytes among total erythrocytes should not be less than 20% of the control value. When animals are treated continuously for 4 weeks or more, at least 2,000 mature erythrocytes per animal can also be scored for the incidence of micronuclei. Systems for automated analysis (image analysis and cell suspensions flow cytometry) are acceptable alternatives to manual evaluation if appropriately justified and validated.

Although originally developed for use with mice (35), the technique was subsequently modified by Hooftman and de Raat (36) for its application to fish in the laboratory. This modification, known as the piscine micronucleus test, has been recently proposed as a potentially rapid and inexpensive *in situ* biological indicator of chemical contamination in fish (37) and invertebrates (38). Investigators of piscine cytogenetics became interested in the micronucleus test, because it had the potential of avoiding the problems of other cytogenetic techniques such as the chromosome aberration and the sister chromatid exchange tests. The latter procedures can effectively detect the genotoxic effects of chemical pollutants in fish, but the tests are time consuming and do not seem to be very effective for the many fish species which have relatively large numbers of small chromosomes (39).

The micronucleus test provides estimates of the frequencies of mutational damage, and also gives insights into the direct and/or indirect risk to human health of environmental pollutants (37, 40-43). The micronucleus assay for any type of cell requires that a substantial fraction of the population treated should undergo mitosis so that the centric fragments induced by treatment during the first cell cycle manifest themselves as micronuclei in the cytoplasm at any stage of the second or subsequent cell cycles (11, 44).

Micronuclei measurements in fish were shown to be a better parameter than chromosomal aberrations in environmental studies under laboratory and field conditions (45, 46). Two other end-points for cytogenetic damage, namely micronuclei and anaphase aberrations, have proven to be very fast and inexpensive tests, when compared to chromosomal aberrations. Aberration analysis is difficult because fish chromosomes are small in size and large in number. In contrast, micronuclei and anaphase test systems are technically simpler and easier to apply. Micronuclei in fish erythrocytes are quite distinct, easily scored, and persist in the cytoplasm. Their recognition is technically much easier and the technique is fifteen times more rapid than the direct scoring of chromosomes during the metaphase. Thus, they are particularly useful for fish cultivators and biologists who are in need of rapid results about the quality of the water (47). This assay system also proved to be successful in field conditions on fish cells from various polluted Baltic Sea areas contaminated by industries in Sweden (48), and Swedish lakes contaminated with radiocaesium (14).

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