Genomic Template Stability Variation in Zebrafish Exposed to Pharmacological Agents

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***Abstract*- Genotoxic agents, including drugs, compromise the integrity of genetic material and influence DNA expression, both directly and indirectly. Notwithstanding the fact that the large selection of pharmacological molecules has improved the health of individual humans and/or animals, they have also caused a notable damage to the environment due to their high reactivity. In the present study we evaluated genetic effects of nine pharmacological substances in 170 individuals of *Danio rerio* using RAPD-PCR analysis. The amplification products from the individuals exposed to the same concentrations of the environmental pollutants showed significant changes in their electrophoretic pattern with respect to the negative controls. The genotoxic activity of these drugs was also validated at the molecular level by means of mathematical and statistical approaches. The aim of this study was to confirm genotoxicity data previously obtained by the Comet test and Diffusion assay using the RAPD technique, comparing changes in RAPD profiles and evaluating the loss of genomic stability (GTS, %). These results show that all drugs have toxic effects on genomic template stability at the concentrations used.**

***Keywords*- Pharmacological Agents; Genotoxicity; RAPD-PCR; GTS; Zebrafish**

I. INTRODUCTION

The environmental pollution caused by pharmaceutical compounds has become a debated issue in scientific literature over the last few years [1-3].

It has been hypothesized that a wide variety of reproductive and developmental problems observed in some wildlife populations and in humans are caused by exposure to environmental contaminants that can interfere with endocrine signaling pathways (reviewed by [4]). This hypothesis is supported by a growing quantity of evidence from laboratory studies that show that pollutants present in the environment can disrupt the genetic functioning of wildlife species, thus causing permanent alterations in the DNA structure and function. DNA damage has both genotoxic and cytotoxic effects. There are, however, significant gaps in our knowledge of DNA damage in fish and humans, particularly as regards the causes and mechanisms of these phenomena. Although the interaction of pollutants with DNA continues to dominate the literature, there are many associations between exposure to environmental drugs and a variety of biological outcomes for which the mechanisms of action are poorly understood. Many drugs are extremely bioactive compounds and are unknowingly introduced into the environment as complex mixtures by many routes, especially in wastewaters (both treated and untreated). Certain pharmaceutically active compounds (such as caffeine, nicotine and aspirin) have been known for almost thirty years to be compounds discharged into the environment, especially in highly populated areas. A more complete picture has only recently emerged where it is evident that numerous drugs from a wide range of therapeutic categories can appear in the environment and even in drinking water (even if at very low concentrations), especially in natural waters that receive waste waters. For this reason the contamination of aquatic ecosystems and the consequent modifications of the balance of flora and fauna that live there, are recognized as one of the principal environmental emergencies [5, 6, 1]. Several studies have used the comet assay, micronucleus test or chromosome aberration assay to measure the genotoxic effects of environmental pollutants by *in vivo* and *in vitro* experiments [7-10].

Over the last few years there has been a notable increase in studies aimed at evaluating the genotoxic effects that certain substances, among which drugs, can induce on the organisms that directly or indirectly come into contact with them. In particular, the most used experimental model for the study of genomic damage caused by chemical and pharmacological substances is that of fish. These vertebrates, in fact, living in water, are particularly sensitive to pollutants and thus more susceptible to eventual damage that these substances can cause to DNA. They can produce the formation of additions and/or breaks of genetic material and/or punctiform mutations including insertions, deletions or chemical modifications of the purine and pyrimidine nitrogen bases. All these take place because fish are organisms that can metabolize, concentrate and bio-accumulate the contaminants present in water. Advantages of measuring effects of genotoxic chemicals directly on DNA are mainly related to the sensitivity and short response time. Advances in molecular biology have recently led to the development of a number of selective and sensitive assays for DNA analysis in the field of genotoxicology. Therefore, the genotoxicity assays based on molecular techniques have been exploited.

One such assay is the random amplified polymorphic DNA (RAPD) assay, based on PCR amplification of random DNA fragments of genomic DNA with short primers of arbitrary nucleotide sequences under low-temperature annealing conditions [11]. This technique is already extensively used for species classification, genetic mapping and phylogeny [12, 13]. In addition, their use in surveying genomic DNA for evidence of various types of DNA damage and mutation shows that RAPD-PCR may

potentially constitute a biomarker assay for the detection of DNA damage and mutational events in cells of bacteria, plants, invertebrate and vertebrate animals [14]. Detection of genotoxic effects using RAPD-PCR involves the comparison of profiles generated from control (unexposed) and treated (exposed) DNA. As described in the most recent literature, the damage caused to genomic DNA would induce the modification of the binding sites of the aspecific primers used, determining the presence of various electrophoretic PCR patterns (polymorphisms) [15-18, 10]. This evidence supports the use of the method to investigate eventual genotoxicity of polluting agents [19, 14] or of some chemical substances [20]. The RAPD technique is a reliable and sensitive method that can identify a wide range of damaged DNA and genetic mutations [16] and therefore can be applied to genotoxicity and carcinogenesis studies. In this study, we assessed the potential of the RAPD assay to be used as a tool for detecting the genotoxicity of nine pharmacological agents (Table 1) using DNA damage as a marker in the teleost *Danio rerio* (Osteichthyes, Ciprinidae). The pharmacological agents were chosen because previous studies reported their presence in high concentrations in Italian treated wastewater [21]. As an experimental model, we used zebrafish (*Danio rerio*, Hamilton, 1822), not only because this fish is a component of the throphic chain of the waters of the ecosystem under study, but also because this species has already been used as a bio-indicator for genetic toxicology studies [22-24]. The aim of this study was to confirm genotoxicity data previously obtained by the Comet test and Diffusion assay using the RAPD technique, comparing changes in RAPD profiles and evaluating the loss of genomic stability (GTS, %) [25].

TABLE I PHARMACEUTICAL COMPOUND AND RELATIVE CONCENTRATION USED FOR EXPERIMENTAL DESIGN

Pharmaceutical compound	Category	Exposure concentration (ng/L)
Diclofenac	non-steroid anti-inflammatory agent	810
Carbamazepine	antiepiletic	310
Atorvastatine	regulator of hematic level of lipids	13
Gemfibrozil	regulator of hematic level of lipids	380
Bezafibrate	lipid regulator	33.86
Ibuprofene	painkiller	
Furosemide	diuretic	611.08
Ranitidine	antiulcer	245.55
Sildenafil citrate	vasodilatator	26.25

II. MATERIALS AND METHODS

A. Samples

Following the recommendations of OECD international guidelines for *in vivo* genotoxicity tests [26], the tests were carried out on 170 individuals of zebrafish obtained from a local source; these were placed in a tank containing 80 L of water with a filter system. The physical parameters such as temperature and pH were constantly monitored; in particular, the water temperature was maintained at 28°C, while the pH was maintained at 7.6. The concentration of ammonia was also monitored (values less than 0.01 mg/L); the fish were maintained with a photoperiod of 14 h light and 10 h dark. Fish were fed every 2 days with commercial fish-food (Tetramin). The fish were maintained under these conditions for 2 weeks before the experiments. After these 2 weeks of adaptation, groups of zebrafish were transferred to smaller tanks each containing 5 L of water, maintained at the same conditions as mentioned above, in which the pharmacological agents were dissolved at concentrations found in treatment waters. These tanks, unlike those of the aquarium, were without filters in order to maintain the concentrations of the pharmacological agents for the time of exposition; however, the water in the tanks was replaced totally every 7 days and the same drug concentration was added. The drugs and their relative concentrations used are reported in Table 1. The experimental design was completely randomized with these 9 treatments. Thus, the samples of *Danio rerio* were divided into groups as shown in Table 2.

TABLE II EXPERIMENTAL CONDITIONS OF TREATMENT

Drug	Days of Treatment	Number of specimens examined
Diclofenac	5-7-14	20
Carbamazepine	5-7-14	20
Atorvastatine	5-7-14	20
Gemfibrozil	5-7-14	20
Bezafibrate	5-7-14-21-28	20
Ibuprofene	5-7-14-21-28	20
Furosemide	5-7-14-21-28	20
Ranitidine	5-7-14-21	20
Sildenafil citrate	5-7-14-21-28-35	20

B. Genomic DNA Isolation and RAPD-PCR Procedures

The genomic DNA was extracted by using the High Pure PCR Template Preparation kit (ROCHE) from about 100 µL of whole blood from each specimen. The concentration of DNA was estimated using the NanoDrop 1000 Spectrophotometer (Gene Company Limited). The DNA was stored at −20°C.

The conditions of DNA amplification were optimized following the procedure of [15] with some modifications. PCR amplification was carried out in a total volume of 25 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP and 12.5 µL of puREtaq Ready-to-go-PCR (Roche). Optimization of amplification conditions were carried out by ranging the template DNA from 10 to 50 ng, and the primers from 5 pmol/µl to 10 pmol/µl. It was found that 40 ng of DNA template and 5 pmol/ µl for primer were the optimal conditions for a satisfactory PCR amplification. The sequence of the primer utilized is 5'-CCCGTCAGCA-3'. For amplification, the reaction mixtures were denatured at 94°C for 5 min, followed by 45 cycles consisting of denaturation for 1 min at

95℃, primer annealing for 1 min at 36℃, and extension for 2 min at 72℃. The amplification was concluded with a final extension period (72℃). Reaction mixtures were stored at 4℃ prior to use.

C. Agarose Gel Electrophoresis and Analysis of DNA Profiles

Electrophoresis of RAPD-PCR reaction products was performed in 2% w/v agarose, using a Tris-borate-EDTA buffer (TBE) system (1X TBE= 90 mM Tris-base, 90 mM boric acid, and 2 mM EDTA). Amplified DNA was mixed with one-fifth volume of gel loading buffer (analytical grade water containing 25% ficoll, 0.25% bromophenol blue, and 0.25% xylene cyanol), and 15 ml of this solution was loaded onto the agarose gel. DNA molecular size marker (100 bp marker; ROCHE) was used for each agarose gel. The DNA samples were subjected to electrophoresis at 100 volts for 2 h, after which the gels were stained in a 1X TBE solution containing ethidium bromide (0.5 $\mu g/ml^{-1}$) for a period not less than 40 min. Gels were photographed under ultraviolet illumination using a Digital camera (Coolpix 950, Nikon).

D. Estimation of genomic template stability

Each separate DNA effect observed in the RAPD-PCR profiles (disappearance of bands, appearance of new bands and variation in band intensities in comparison with control profiles) was considered in order to assess any DNA damage and the template genomic stability (percent) was calculated for each experimental group of fish with the chosen primer.

Genomic template stability (GTS, %) was calculated as follows:

$$GTS\ (\%) = (1 - a/n) \times 100$$

where "a" is the number of RAPD polymorphic profiles detected in each sample treated and "n" is the number of total bands in the control [25]. Polymorphisms observed in the RAPD profile include disappearance of a normal band and appearance of a new band in comparison with control profile [11]. The average was then calculated for each experimental group exposed to different pollutants. To compare the sensitivity of each parameter (genomic template stability), changes in these values were calculated as a percentage of their control (set to 100%). The statistical analyses were carried out using the software package SPSS 9.05 for Windows.

III. RESULTS

A. RAPD Based DNA Fingerprinting

The amplification products obtained by RAPD-PCR showed various bands of lengths between 300 and 2000 bases. In particular, the bands at about 400, 500, 600, 800, and 1100 base pairs were present in all the controls. The RAPD primer used in our study yielded specific and stable results and they indicate that the primer used has a discrete efficiency to amplify the genomic DNA of zebrafish (Fig. 1).

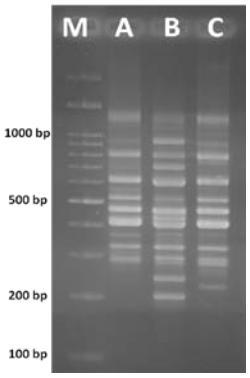

Fig. 1 RAPD-PCR fingerprint generated by selected primer from three different zebrafish genomic extracts (A: zebrafish size small, B: zebrafish size large and C: zebrafish size extralarge)

M= 100 bp molecular weight marker

B. GTS % Evaluation and Transformation of the Data

Changes in the RAPD patterns are expressed as decreases in GTS, a qualitative measure reflecting the change in the number of RAPD profiles generated by the nine drugs, in relation to profiles obtained from the control specimens. GTS values are reported in Fig. 2.

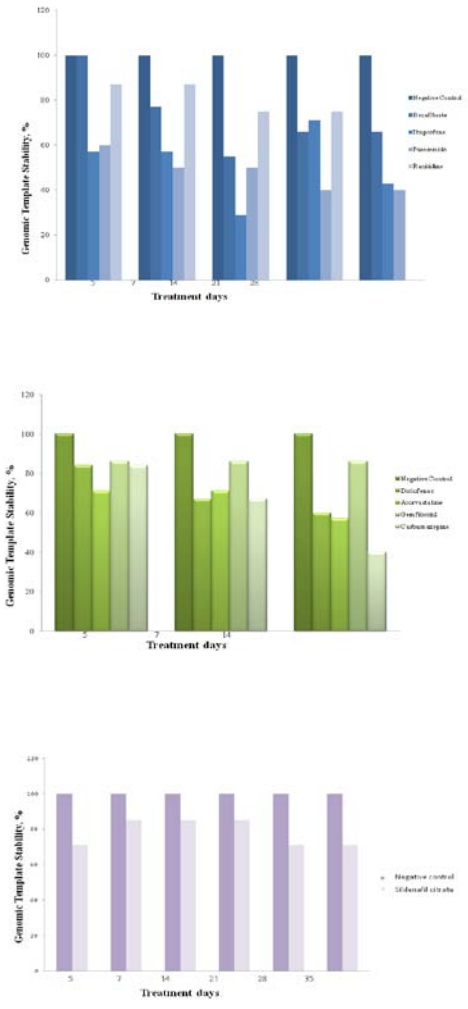

Fig. 2 Changes of GTS in Danio rerio erythrocytes exposed to nine drugs as evidenced by RAPD-PCR

This statistical qualitative analysis allowed the correlation of genomic stability variations with exposure time to the chosen drugs. The resulting GTS graph shows that the genome stability was slightly reduced with each day of exposure to sidenafil citrate, gemfibrozil and ranitidine. On the other hand, the genomic stability varied more with the other six drugs, even if in different ways for the various exposure times. However, in this case, the graph shows that stability tended to decrease over the exposure time.

IV. DISCUSSION

It is well known that numerous pharmacological substances used for human health are discharged into the waters of treatment plants, while those for veterinary use end up directly in surface waters or the soil. The studies carried out on the waste waters of both Italian and European treatment plants have shown that among the chemical compounds most commonly found in the waste waters of these treatment plants, and thus responsible for contamination, there are many active pharmacological agents. These are biologically active compounds that can interfere with specific biological systems, such as DNA, causing more or less severe genetic damage. From these investigations, it was seen that the pharmacological classes most present in environmental waters are the following: lipid regulators, antibiotics, anti-inflammatory drugs, anti-hypertensives, diuretics and broncodilators, in concentrations in the order of mg-ng/L [27]. Results of chemical analyses showed that concentrations of drugs in the environment were rather elevated (from 10 to 749 ng/L) [28, 29]. The present study shows that nine drugs found in the waste waters of some Italian water treatment plants: gemfibrozil and atorvastatin (two regulators of the hematic level of lipids), sildenafil citrate (a vasodilator), carbamazepine (a known anti-epileptic), diclofenac (a non-steroid anti-inflammatory agent), furosemide (a diuretic), ranitidine (an anti-ulcer drug), bezafibrate (a lipid regulator) and ibuprofen (a painkiller) are able to damage the DNA of the erythrocytes of zebrafish.

In [15] demonstrated, using the RAPD-PCR technique and the experimental model of zebrafish, that some aquatic contaminants such as phosphamide and dimethoate are able to induce genotoxicity, confirming the accuracy and the sensitivity of this technique for the analysis of genotoxic damage induced by environmental contaminants. The technique was used with success by our group to evaluate the genotoxicity of several drugs found downstream of some Italian wastewater treatment plants. In ecotoxicology, the specific evaluation and environmental monitoring of potentially genotoxic agents would be improved by the development of sensitive and selective methods to detect toxicant-induced alterations in the genomes of a wide range of biota. Aquatic pollution due to the discharge of chemical substances, among which are active pharmacological agents, into aquatic ecosystems is becoming a threat for the environment. The active pharmacological agents discharged into superficial waters can, in fact, interact with the DNA of the organisms that make up the trophic chain of these environments and induce significative genetic damage.

The aim of this research was to evaluate, by visualization of DNA band profiles and GTS % changes, the potential genotoxic effects of nine pharmacological agents present in the waste waters of some Italian treatment plants. Genetic damage was evaluated by RAPD-PCR profiles to detect increases after some days of exposure to the pharmacological agent. Using RAPD-PCR, bands were characterized in both quality and quantity at the genomic level of the zebrafish exposed to atorvastatin, sildenafil citrate, gemfibrozil, carbamazepine, diclofenac, furosemide, ranitidine, bezafibrate and ibuprofen and at different times of exposure. The disappearance or the loss of PCR amplification products can reveal a change in the DNA sequence due to mutations, showing new annealing events and/or large deletions, bringing two pre-existing sites nearer or separating them farther. Furthermore, the amplification pattern of the DNA of the individuals exposed showed the acquisition or the loss of bands and/or the change of intensity of the same, caused by a variation in the number of recognition sites of the sequence of the primer and thus of mutations. The variations of frequency of the bands could be the result of structural changes induced by the genotoxic events. In fact, previous studies [15, 16] showed that the changes in electrophoretic patterns reflect the alterations of the DNA due to a single change in the bases (point mutations) or a more complex chromosomal reorganization. Analogously, in this study, the DNA damage induced by atorvastatin, sildenafil citrate, gemfibrozil, carbamazepine, diclofenac, furosemide, ranitidine, bezafibrate and ibuprofen is shown by means of the changes in the variations of intensity of the bands, in the loss and/or acquisition of new bands and in the assessment of genomic template instability. Furthermore, RAPD-PCR confirmed, agreeing well with our previous Comet and Diffusion results, that the damage induced by these pharmacological agents at the concentrations used was already visualized after 3 days for carbamazepine and diclofenac, 5 days of exposure for atorvastatin and gemfibrozil, 14 days for furosemide and ranitidine, 21 days for bezafibrate and 28 days of exposure for ibuprofen, and 35 days for sildenafil citrate.

In general, our results indicate a variation in RAPD-PCR polymorphisms with increasing concentration and time of exposure of these drugs. In fact, we have demonstrated a dose- and time-dependent relationship between the DNA changes in *Danio rerio* and genotoxicity in the RAPD analyses. On the other hand, the genomic template stability in the zebrafish decreased with increasing concentrations or exposure time, which indicated that genomic template stability was significantly affected by the pollutant stressors. Thus, in these experiments, changes in GTS % exhibited a relationship with drug concentration, indicating its potential use as a biomarker.

The techniques we used in this work, together with the results performed, should be framed within a broader vision of the most appropriate procedures to optimize the standard genetic toxicology battery for prediction of potential human risks, and to provide guidance on interpretation of results, with the ultimate goal of improving risk characterization for

mutagenic and/or carcinogenic effects that have their basis in changes in the genetic material [30].

The experimental procedure adopted, and the successive analysis of the results, allowed us to evaluate with extreme precision the damage induced by these classes of pharmaceutical agents on the genome of zebrafish. The simultaneous use of qualitative and quantitative analyses of electrophoretic patterns makes the observations more reliable as regards the various genotoxic effects that these substances can be induced. RAPD profile analysis in conjunction with the evaluation of GTS % would prove a powerful eco-toxicological tool. Thus, DNA polymorphisms detected using RAPD analysis could be used as an investigation tool for environmental toxicology and as a useful biomarker assay that can be used as an early warning system.

Finally, this present study confirmed the DNA damage induced by drugs compared to those from our previous studies and also demonstrated the potential of RAPD-PCR analyses (also as a quantitative indicator) to monitor the genotoxicity on the teleost *Danio rerio*.

V. CONCLUSIONS

We can summarize our findings as follows:

a. In all cases, the RAPD patterns generated by the pollutant-exposed zebrafish are clearly different from the control groups, and exhibit a distinct change with increasing pharmacological agent concentration and exposure time.

b. The differences in RAPD patterns refer to band intensity, loss of normal bands and appearance of new bands as compared with the control.

c. In addition, we have also shown that the reproducibility of the RAPD patterns was optimal when PCR was performed twice with the chosen primer, confirming that the variation of bands was stable.

d. The number of modified RAPD bands correlated positively not only with the concentration of the pollutants used but also with the exposure time.

e. Changes in GTS% exhibited a relationship with drug concentration, indicating its potential use as a biomarker.

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