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Studies on Clastogenic Effects of Alberk Swamp Water

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Abstract: The Clastogenic effects of water pollutants were studied using the micronucleus test in erythrocytes from peripheral blood of *Rana ridibunda* collected from different locations of Alberk (N 39,48-18^{0}C12 E 20,09,41^{0}C32) South west Saudi Arbia. Examination of blood smears showed that the formation of micronuclei was similar and not significant according to the control group. This unchanged in the ratio in the formation of micronucleus indicates that Alberk swamp water may has no clastogenic effects on peripheral erythrocytes of frogs but these observations does not mean that this type of water is safe for consumers till confirmed by another tests.

Key words: Genotoxicity, *Rana redibunda*, water pollution, Clastogenic effects, Micronucleus test.

INTRODUCTION

Gene mutations, occurrence of aneuploidi and chromosome structural aberrations are all involved in cancer development and inherited clinical disorders. This is -in general- due to industrial and agricultural contaminants and environmental contaminations (Cavallo *et al*., 2007). Because of that there is an increasing effort world-wide to determine the impact of genetic, life-style and environmental factors, on genomic stability in human. Some animal populations are used as good bio-indicators for screening the impact of environmental pollutions. Bio-indicators offer several types of information like early warning of potential harm to human health based on the response of wildlife to population. Biomarkers that have been validated for their predictive value may be used for the timely identification of increased cancer risk, and can be used in the prevention or control of disease. One of the most techniques that had been adopted by numerous laboratories is the measurement of micronuclei MN in different tissues (Fenech, 1999).

The micronucleus test, is an in vivo and in vitro short-time screening test (Heddle *et al*., 1978, Schmid, 1975) is widely used to detect genotoxic effects (Villarini *et al*., 1998). It is one of the simple, reliable, cheap and rapid screening system for both clastogenic effects (chromosome breakage, formation of a centric fragments) and an eugenic (chromosome lagging and effects on spindle) (Heddle *et al*., 1993). Clastogenic and an eugenic agents affect the spindle apparatus, which can be differentiated on basis of the relative induced micronucleus sizes or with the presence of kinetochores. Micronuclei formation can occur in any nucleated and dividing tissue of any species (Heddle *et al*., 1991).

In Anaphase, chromosome fragment or whole chromosomes which lack a centromere may not be integrated in the nucleus, because of the lack of an indispensable element for orientation in the spindle apparatus. After telophase, the fragments or whole chromosomes give rise to one or several secondary nuclei which are small than the main daughter nucleus and are therefore called micronuclei (Heddle, 1973, Schmid, 1975).

The usefulness of the micronucleus test for mutagenicity screening has been well established in several systems of several animals and human (Gonzalez-Yebra *et al*., 2009, Cavallo *et al*., 2009). plants, fish, birds and frogs were also investigated by micronucleus test to detect environmental pollution capable of producing genotoxic damage (Ma *et al*., 2004). Thus, MN provide a measure of both chromosome breakage and chromosome loss and it has been shown to be at least as sensitive an indicator of chromosome damage as classical metaphase chromosome analysis.

The key advantage of the MN assay is the relative ease of scoring and the statistical power obtained from scoring larger numbers of cells than are typically used for metaphase analysis .(Michael Fenech, 1999, Fenech *et al*., 1999).

Alberk (N 39,48-18^{0}C12 E 20,09,41^{0}C32) is a city located on the road to the west coast of South West Saudi Arabia, Asir region, and on the distance of one hundred and thirty kilometers south of Qunfudah. Because of it is moderate climate, the agriculture present to be one of the main activities. In their quest to improve and increase the agricultural productivity people had been using different types of pesticides and insecticides.

Using *Rana ridibunda* frogs as bio-indicator, we aimed to investigate the accumulation of clastogenic agents in the Swamp water of Alberk city.

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MATERIALS AND METHODS

Animals:

Healthy and actively living frogs *Rana ridibunda* were collected over one month from Alberk city (Saudi Arabia) considering five frogs for each collection. The animals were collected in February 2012. The weight of frogs was ranged from 25-35g. The same number of animals (5) was also collected from non polluted water to present the control group. Were positive control animals were kept maintained under standard conditions i.e. temperature (27±2) and clean water until they reached the target weight (25-35g) at this stage they were treated by Cyclophosphamide, a well-known genotoxic substance for its reliability for the MN test was used as a positive control for the MN test in amphibian tadpoles (Lajmanovich *et al*. 2005). The frogs were slaughtered to collect venous blood.

SlidePpreparation and Staining:

For each frog, two microscopic slides were prepared. Fresh blood samples taken from the venous of each frog within the experimental and control groups collected were smeared onto clean slides. The slides were air dried for 30 min. and then fixed in cold Carnoy fixative for 10 min. After fixation, the slides were stained in aqueous Giemsa (5%) (Sigma) for 10 min (Saleh and Zeytinoglu 2001).

Examination of Slides:

Five frogs were used for each of the control, positive control and polluted habitats for each frog, 4.000 cells/frog were analyzed, totaling 20.000 erythrocytes/collection. The frequencies of micronuclei in erythrocytes were detected under a binocular microscope (OLYMPUS) using a 1000× oil-immersion lens. Only cells with intact cellular and nuclear membranes were scored. The following criteria was used as described by the previous studies: (i) micronuclei should be one-tenth and one-third the diameter of the main nucleus, (ii) they should be on the same plane of focus, (iii) they should have the same color, texture and refraction as the main nucleus (iv) they should be clearly separated from the main nucleus (Fenech, 2000).

Micronuclei formation indicating variations in shapes and numbers per cell were designated M1, M2, M3 and M4 to indicate one, two, three and four micronucleus per cell respectively. Moreover, this study was investigated the apoptosis and necrosis rate to achieve the parameters of NDCI (nuclear division cytotoxicity index) and NDI (nuclear division index), following the equations suggested by (Fenech, 2000)

Statistical Analysis:

The present data was statistically analyzed using one-way ANOVA.

Results:

This study of the clastogenic effect of Alberk Swamp water using micronucleus test revealed that there was no significant induction of micronucleus in frog *Rana ridibunda* erythrocytes. Comparing results showed that control group and the experimental group have almost same value of MNE (f=5.317, df=1). Whereas the positive control showed more significant comparing with experimental and negative groups (f=5.98, df=1). The microscopic investigation of micronuclei also not showed a variation in their shapes and number per cell as shown in (Table1). The micronucleus type (M1) was found in all groups (Fig. 1A, 1B), while, (M2) and (M3) (M4) types were not present in all groups including negative control were it was very slightly in positive control (Fig. 1C).

NDI is often calculated according to the method of (Eastmond and Tucker, 1989). Five hundred viable cells are scored to determine the frequency of cells with 1, 2, 3 or 4 nuclei and calculate the NDI using the formula: NDI = (M1) + 2(M2) + 3(M3) + 4(M4)/N, where M1–M4 represent the number of cells with one to four nuclei and N is the total number of viable cells scored (Michael Fenech, 1999, Fenech, 2000).

NDCI = nuclear division cytotoxicity index, NDCI = (Ap + Nec + M1 + 2(M2) +3(M3) + 4(M4))/iV), Ap = number of apoptotic cells, Nec = number of necrotic cells, M1-M4 = number of viable cells with 1–4 nuclei and N = total number of cells scored (Fenech, 2000). There were no observations recorded regarding apoptosis and necrosis in all groups (Table 1). In this study we evaluated our results in the direction of the formulas given above.

Discussion:

Early warning of environmental damage is one of the most important bioindicators. (Saotome and Hayashi, 2003) used aquatic organisms to assess the genotoxicity of water in the field and in the laboratory. (Villela *et al*., 2007), confirmed that the micronuclei frequencies may vary according to many factors like; season, the kind of pollution and the organism species. Amphibian also used before to evaluate the impact of radiation (Siboulet *et al*., 1984) and environmental pollutions (Ma *et al*., 2004). So amphibian is a suitable group to investigate the

clastogenic effectors in different water resources. In the light of these studies we are convinced that *Rana ridibunda* considered to be good indicator for Albark pollutions.

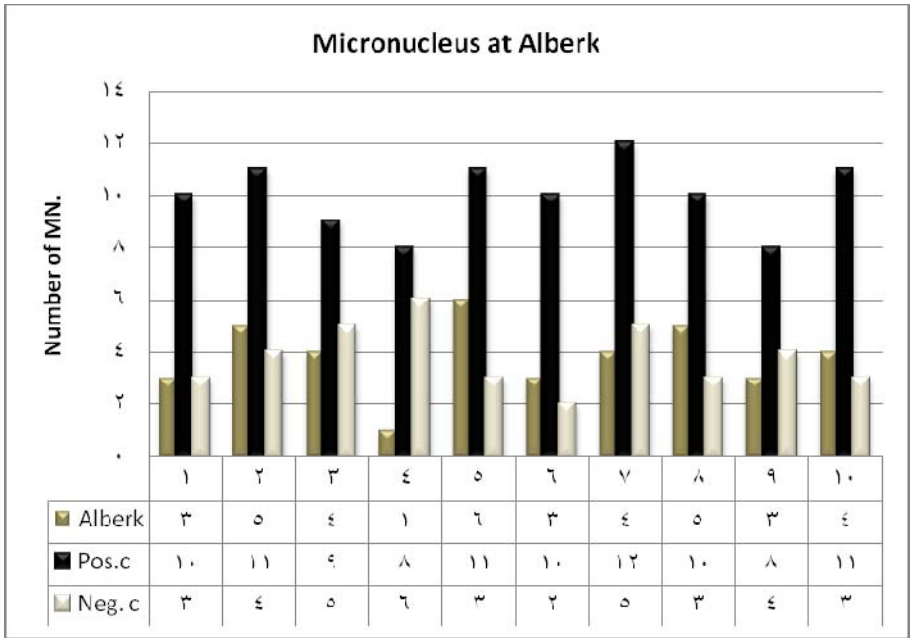

Fig. 1: Micronucleus frequencies within red blood cells. results with standard deviation.

Table 1. NDCI and NDI for all frog groups.

Frog No.	Slide No.	Eryth .Cell No.	M1	M2	M3	M4	Ap.	Nec	NDI	NDCI
1	1	2000	3	0	0	0	0	0	0.00050	0.00050
	2	2000	5	0	0	0	0	0	0.00117	0.00117
2	1	2000	4	0	0	0	0	0	0.00067	0.00067
	2	2000	1	0	0	0	0	0	0.00017	0.00017
3	1	2000	6	0	0	0	0	0	0.00100	0.00100
	2	2000	3	0	0	0	0	0	0.00050	0.00050
4	1	2000	4	0	0	0	0	0	0.00067	0.00067
	2	2000	5	0	0	0	0	0	0.00083	0.00083
5	1	2000	3	0	0	0	0	0	0.00050	0.00050
	2	2000	4	0	0	0	0	0	0.00067	0.00067
Pos.c		40000							0.00216	0.00216
Neg.c		40000							0.0054	0.0054

Abbreviations:
M1: Erythrocyte with one micronucleus.
M2: Erythrocyte with two micronuclei.
M3: Erythrocyte with three micronuclei.
M4: Erythrocyte with four micronuclei.
Ap. : Apoptotic cell.
Nec.: Necrotic cell.
NDI: Nuclear division index.
NDCI: Nuclear division cytotoxicity index.
Pos.c: Positive control.
Neg.c: Negative control.

Micronucleus is a very sensitive test, but many references reported that MNE can be affected by different factors like age, sickness, species, feeding chemical and physical agents and environmental conditions (Ma *et al*., 2004). So healthy, young and active individual frogs had been chosen.

NDI and NCDI are very good and useful parameters for comparing the clastogenic effects of agents. The present study showed no significant variation of MN frequencies, this indicate that Alberk water pollutants were may not contain clastogenic agents. To assess potency of different pollutants impact obtained by necrotic and apoptotic cells which indicate the total number of cells becoming non-viable. In the light of our results observations, the Swamp water of Alberk is not contaminated by clastogenic agents.

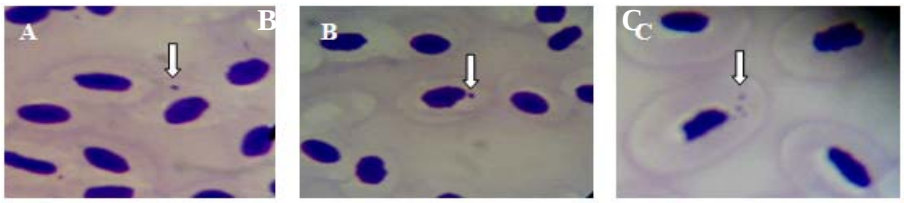

Fig. 2: Arrows in A and B indicates one micronucleus, while C more than one Micronucleus per erythrocyte. Cells were stained with Giemsa Stain for 10min.

Cyclophosphamide, which is an indirect alkylating agent well-known for its genotoxic properties, was used as a positive control for the MN test in amphibian tadpoles(Lajmanovich *et al*., 2005) and also in our experiment. Our observation is agreed with the previous studies.

In conclusion, our study did not observed accumulation of clastogenic agent in Alberk Swamp water, but the water throughout the speech may contains different pollutants that can't screened by micronucleus test.

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