MOLECULAR EFFECTS OF SOME HEAVY METALS ON *Tilapia niloticus* BLOOD DNA

BY

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The present study was conducted to determine the effects of three doses of cobalt, cadmium and zinc heavy metals as environmental pollutants for two and three weeks exposure periods on the blood DNA nucleotides sequence rearrangement of *Tilapia niloticus* compared to untreated ones as a negative control. Six different 10 mer random primers were used in randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) experiment for this purpose.

The obtained results showed that *Tilapia niloticus* blood DNA was affected significantly by all of the adopted treatments since great differences between the control and the treated fishes were documented. Generally, the lowest effect was recorded following cobalt treatments while cadmium and zinc treatments showed higher differences for the amplified bands number and intensity. The extension of exposure period from two to three weeks exhibited clear differences in number, size and intensities of the amplified fragments using the same primer as a result of blood DNA nucleotides sequence changes.

INTRODUCTION

Because of the ascending application of the agrochemicals and the removal of the industrial wastes into the irrigation canals and drains during the past few decades, the contamination with heavy metals increased up to the unsafe levels. These heavy metals are introduced to the human body either directly by fresh plants consumption or indirectly through the feeding on cattle and fish flesh. So many kinds of biohazards were induced and detected as a result of high concentration of heavy metals introduced to human bodies.

The PCR technique has been used frequently to discriminate the different fish species (**Taggart and Ferguson 1990, Carver *et al.* 1991, Franck *et al.* 1992, Hindar 1992, Bardakci and Skibinski 1994, Naish *et al.* 1995, Sultamann *et al.* 1995, Dinesh *et al.* 1996, Saad 1999 and Callejas and Ochando 2001**). Moreover the same techniqueis being used as an accurate and effective method to determine the genotoxicity of the environmental pollutants at the molecular level (**Theodorakis *et al.* 1994, Ferrero *et al.* 1998, Becerril *et al.* 1999, Muley *et al.* 2000, Lison *et al.* 2001, Audrey and David 2002**). Since the genetic material (DNA) composition is similar in all living organisms, so treated *T. niloticus* blood DNA was

used in the present study to determine the possible mutagenic effects of Co, Cd and Zn on the nucleotide sequence rearangment using RAPD-PCR technique.

MATERIALS AND METHODS

1. **Experimental fish:**

One well identified fish species was used in this study, namely Nile tilapia (*Tilapia niloticus*) with initial average weight of 30 gm.

2. **Heavy metals:**

Three heavy metals; i.e., cobalt, cadmium and zinc were applied in the form of nitrate compounds with four doses for two and three exposure periods. The tested doses were zero (as a control), 1/2 , 1 and 2 folds of the allowable concentrations (AL) according to the **Egyptian Environmental Law (1994)**. Table (1) shows the compounds and the concentrations of tested heavy metals in this study.

3. **Blood DNA extraction and purification** was carried out using Capture column kit according to the manfacturer's manual (Gentra).

4. **Polymerase Chain Reaction (PCR) reagents and protocol** was carried out according to **(Williams *et al.*, 1990)**. For PCR technique, Ready to Go PCR beads (Amersham Pharmacia Biotech No. 27. 9500-01) were used. Each bead contains all of the necessary reagents, except the primer and the template DNA for performing a 25µl PCR amplification reaction.

Primers. Six 10 mer random primers (Pharmacia biotech cat No. 27-9501-01) were used in this study. Their sequences are shown in Table (2).

Table (1): Compounds and concentrations of the tested heavy metals.

Compounds	Treatment	Concentration *	mg/L	No. of treated fish
Cobalt nitrate	Control	Zero	0	30
	Co_1	½ AL	1	30
	Co_2	AL	2	30
	Co_3	2 AL	4	30
Cadmium nitrate	Control	Zero	0	30
	Cd_1	½ AL	0.025	30
	Cd_2	AL	0.05	30
	Cd_3	2 AL	0.1	30
Zinc nitrate	Control	Zero	0.0	30
	Zn_1	½ AL	2.5	30
	Zn_2	AL	5.0	30
	Zn_3	2 AL	10	30

* AL: Allowable concentration

Table (2): Sequence of the six primers used in RAPD technique.

Primers No.	Sequences: 5' to 3'
1	5'-GCTGCGGGAA-3'
2	5'-GTTTCGCTCC-3'
3	5'-GTAGACCCGT-3'
4	5'-AAGAGCCCGT-3'
5	5'-AACCCCCAAT-3'
6	5'-CCCGTCAGCA-3'

DNA electrophoresis buffers were the same as in **Sumbrook *et al.* (1989)** and RAPD-PCR product analysis was carried out using Hoefer HE 99 x Max sub marine Electrophoresis unit. The different sizes of bands were determined against 100 Pb ladder marker (Boehringer Mannheim). The separated bands were stained with ethedium bromide and were visualized documented using UV transeliminator and photographed by Polaroid instant camera.

RESULTS AND DISCUSSION

In order to study the effect of the applied three heavy metals on DNA nucleotides rearrangement, anexperiment constisting of group of treated fish as well as untreated group (control) was conducted for two and three weeks. RAPD-PCR analysis was determined against six different 10 mer random primers. The results obtained following two weeks treatments were presented in Figure (1).

Generally, from Figure 1-a up to 1-f , it could be noticed clearly that a high degree of polymorphism was obtained either among the different treatments for each primer or following using the different primers for the same treatment. The obtained results following the application of primer No. 1 (Fig. 1-a), indecated that there were no differences between the control experiment and all of tested Co doses since all of them showed only two bands with about 500 and 550 bp size. On the contrary, the lowest doses of both Cd and Zn showed the highest number of the amplified polymorphic bands (lanes 4 and 7) since six bands were detected following Cd_1 and seven bands following Zn_1. The higher concentrations of Cd and Zn proved to be less efficient in inducing molecular abnormalities on *T. niloticus* blood DNA since one amplified band could be detected following Cd_2, with 900 bp size and two bands similar to those of control was detected following Cd_3. The application of Zn_3 showed almost the same results as in control experiment, while Zn_2 treatment showed an extra faint band such as those of Zn_1.

Fig. (1-b) showed the amplified polymorphic bands of *T. niloticus* blood DNA following application of primer No.2. It was clearly noticed that no amplified polymorphic bands could be detected neither in control experiment nor following Co_1 treatment. No differences between Co_2 and Co_3 treatments for two weeks were observed since both of them exhibited eight amplified bands with molecular size ranged from 350 up to 1100 bp having almost the same intensities. On the other hand, the lowest doses of Cd and Zn proved to be the highest effective ones on the rearrangement of DNA nucleotides sequences since both of them

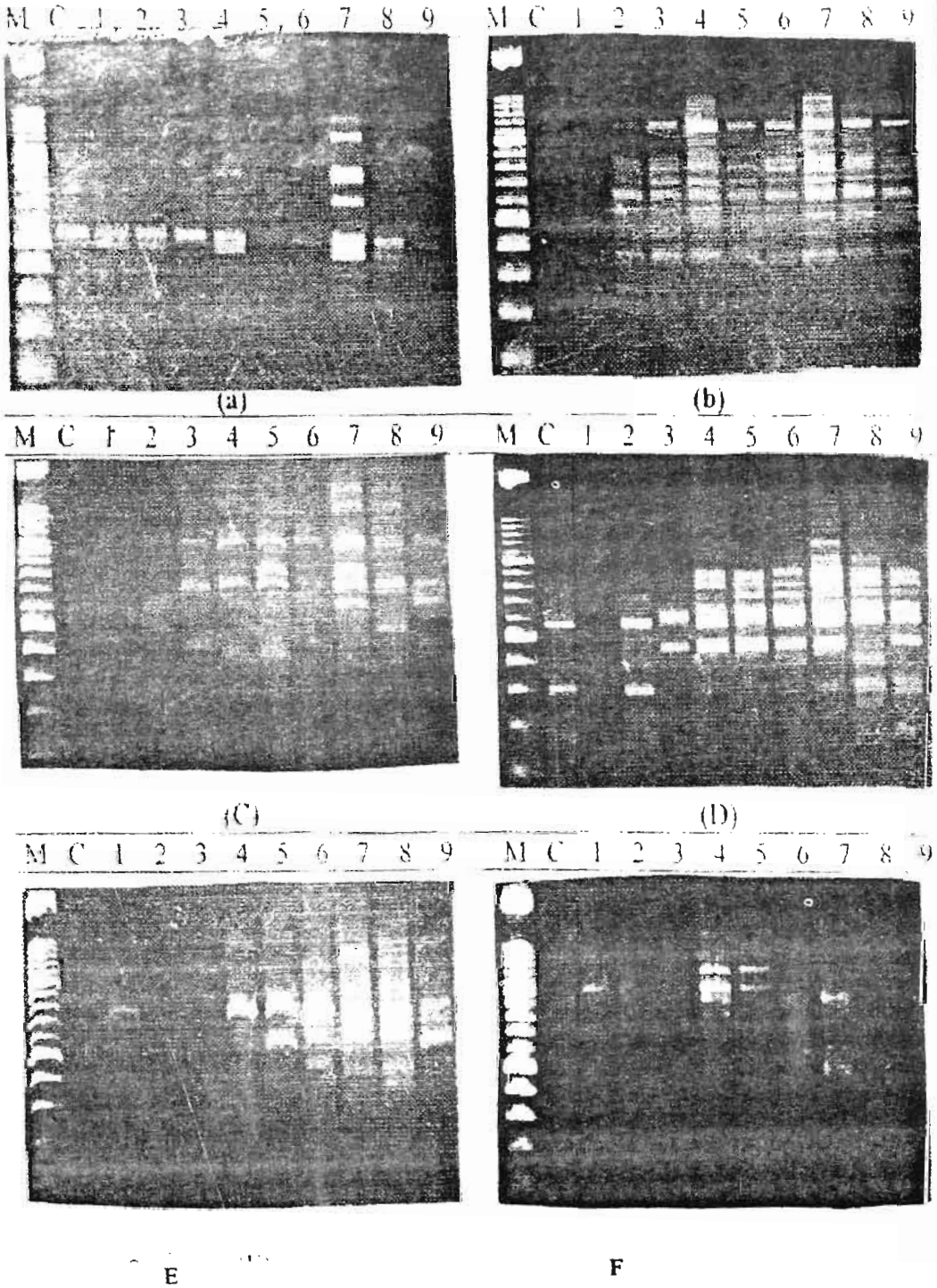

Fig. (1): RAPD banding patterns of *T. niloticus* blood using primers1-6 (Fig. a- f).

M : 100 base pair ladder marker
C : Control (untreated fish).
1-3 : Co_1, Co_2 and Co_3 treatments for two weeks.
4-6 : Cd_1, Cd_2 and Cd_3 treatment s for two weeks
7-9 : Zn_1, Zn_2 and Zn_3 treatments for two weeks.

with the primer No. 2 amplified the highest number of DNA segments (lanes 4 and 7). Moreover, Zn_1 was considered as the highest effective dose than all of Cd and Zn doses as shown in Figure (1-b lanes 4-9). In spite of all other doses of Cd and Zn (lanes 5, 6, 8 and 9) which exhibited high polymorphic differences than the control experiment, however, their effects were less than that of the lowest doses of Cd and Zn.

Regarding primer No. 3, (Figure 1-c), it was clearly shown that no complementary nucleotide sequence to this primer was found neither in the untreated DNA nor Co_1 treatments lanes. However, following Co_2 and Co_3 treatments, some nucleotide rearrangement were induced since three major bands were detected with very faint to brightness in lane 2 and more brightness in lane 3. The highest effects on DNA nucleotides sequences were detected as a result of Cd_1, Cd_2, Zn_1 and Zn_2 treatments, since seven amplified bands were detected following Cd treatments and nine amplified bands occurred following Zn treatments as a result of two weeks treatments.

Fig. (1-d) showed the amplified polymorphic bands of blood DNA following heavy metals treatments for two weeks using primer No. 4. It was clearly noticed that no differences existed between the control experiment and Co_2 since both of them showed two amplified bands with the size of 300, 600 bp, respectively. The highest dose (lane 3) of Co showed two amplified bands with size of 400 and 600 bp, respectively. On the contrary, it was clearly noticed that no amplified bands could be detected in Co_1 treatment. All doses of Cd and Zn showed high number of amplified bands. The increase of Cd concentration showed an increase in number of amplified bands, since five bands were detected following Cd_1, six bands following Cd_2 and seven bands following Cd_3 treatments with sizes ranged from 450 up to 800 bp. On other hand, the lowest dose of Zn proved to be the highest effective one on the rearrangement of DNA nucleotide sequences, since it amplified the highest number of DNA segments with the primer No. 4 (eight major and eight minor segments). The other two doses of Zn (lanes 8, 9) exhibited high number of amplified bands, since nine bands were determined following Zn_2 and Zn_3 with sizes lower than those obtained following Zn_1 treatment.

Regarding primer No. 5, Figure (1-e) clearly showed that no complementary nucleotide sequence to this primer was found neither in the untreated DNA nor following all Co treatments. However, following all Cd treatments, some nucleotide rearrangement were induced since one major band was detected following Cd_1 and Cd_3 treatments (lanes 4, 6) with size of about 700 bp and two bands were detected following the Cd_2 with size of 700 and 500 bp, respectively. The highest effects on DNA nucleotides sequences were detected as a result of the three doses of Zn treatments since high number of amplified bands occurred after Zn_1, Zn_2, Zn_3 treatments in descending order.

The results following the application of primer No. 6. (Figure 1-f) showed that no amplified bands could be detected neither in control experiment nor following Co_2 and Zn_3 treatments, only one faint band could be detected following Co_1 treatment and four faint bands following Co_3. The highest effects on DNA nucleotides sequences were detected as a result of Cd treatments since 7, 5 and 5 amplified bands occurred as a result of Cd_1, Cd_2 and Cd_3 treatments, respectively. The highest concentration of Zn proved to be not efficient to induce

molecular abnormalities on blood DNA since no amplified bands could be detected following Zn_3 treatment. However the two other treatments of Zn (Zn_1 and Zn_2) exhibited three major amplified bands.

The results following three weeks treatments are presented in Figure (2). Generally, from Figures 2-a up to 2-f it could be noticed clearly that a high degree of polymorphism was obtained either among the different treatments for each primer or following using the different primers for each treatment. The obtained results following the application of primer No. 1, (Fig. 2a) indecated that four amplified bands were clearly detected in control experiment with size rangig from 300 up to 1000 bp. Only one amplified band was detected following Co_1 treatment with size about 600 bp, while three amplified bands with size ranging from 150 bp to 700 bp were detected following Co_2 treatment. Two bands with size about 150 and 300 bp were detected following Co_3 treatment. The highest effects on DNA nucleotides sequences were detected as a result of Cd_1, Cd_2, Zn_1 and Zn_2 treatments, since 6, 7, 5 and 6 amplified bands were occurred respectively after three weeks of treatments. On the other hand, it was clearly noticed that no amplified bands could be detected following Zn_3 treatments.

Regarding the application of primer No. 2, Figure (2-b) clearly showed that only four very faint amplified bands were detected in the control experiment. No complementary nucleotide sequence was detected following Co_1 treatment and three different bands occurred following Co_2 and Co_3 treatment. The highest effects on DNA rearmament occurred as a result to the two low doses of Cd since 3 amplified bands were detected following Cd_1 and Cd_2 treatments. On the contrary the highest dose of Cd (lane 6) and all doses of Zn showed that no complementary nucleotides sequence to this primer were found.

Figure (2-c) showed the amplified polymorphic bands as a result of using primer No. 3 against the same samples of *T. niloticus* blood DNA following heavy metals treatments for three weeks. The obtained results indecated that five amplified polymorphic bands we detected in control experiment. On the other hand it was clearly noticed that no amplified polymorphic bands were detected neither in Co_1 nor following Co_2 treatments. The highest doses of Co exhibited three amplified polymorphic bands with size ranging from 800 to 1200 bp. The lowest two concentrations of Cd exhibited the highest number of amplified bands since seven and six bands occurred as a result of Cd_1 and Cd_2 treatments, respectively. It was clearly noticed that no amplified bands could be detected neither in Cd_3 nor following Zn_1 and Zn_3 concentration treatments. Meanwhile, Zn_2 treatment exhibited one major band with size of 200 bp.

Primer No. 4 (Figure 2-d) showed that all examined heavy metals except Zn_3 treatment showed an amplified band with length of about 900 bp as the largest amplified band with this primer. The number of amplified bands following all Co doses were 3, 2 and 4, respectively. Three, four and three amplified polymorphic bands occurred as a result of treatment with Cd_1, Cd_2 and Cd_3 treatments. The two low concentrations of Zn exhibited one and four amplified bands due to the treatment with Zn_1 and Zn_2. On the other hand, no amplified bands were detected following Zn_3 treatment using Primer No. 4 for three weeks.

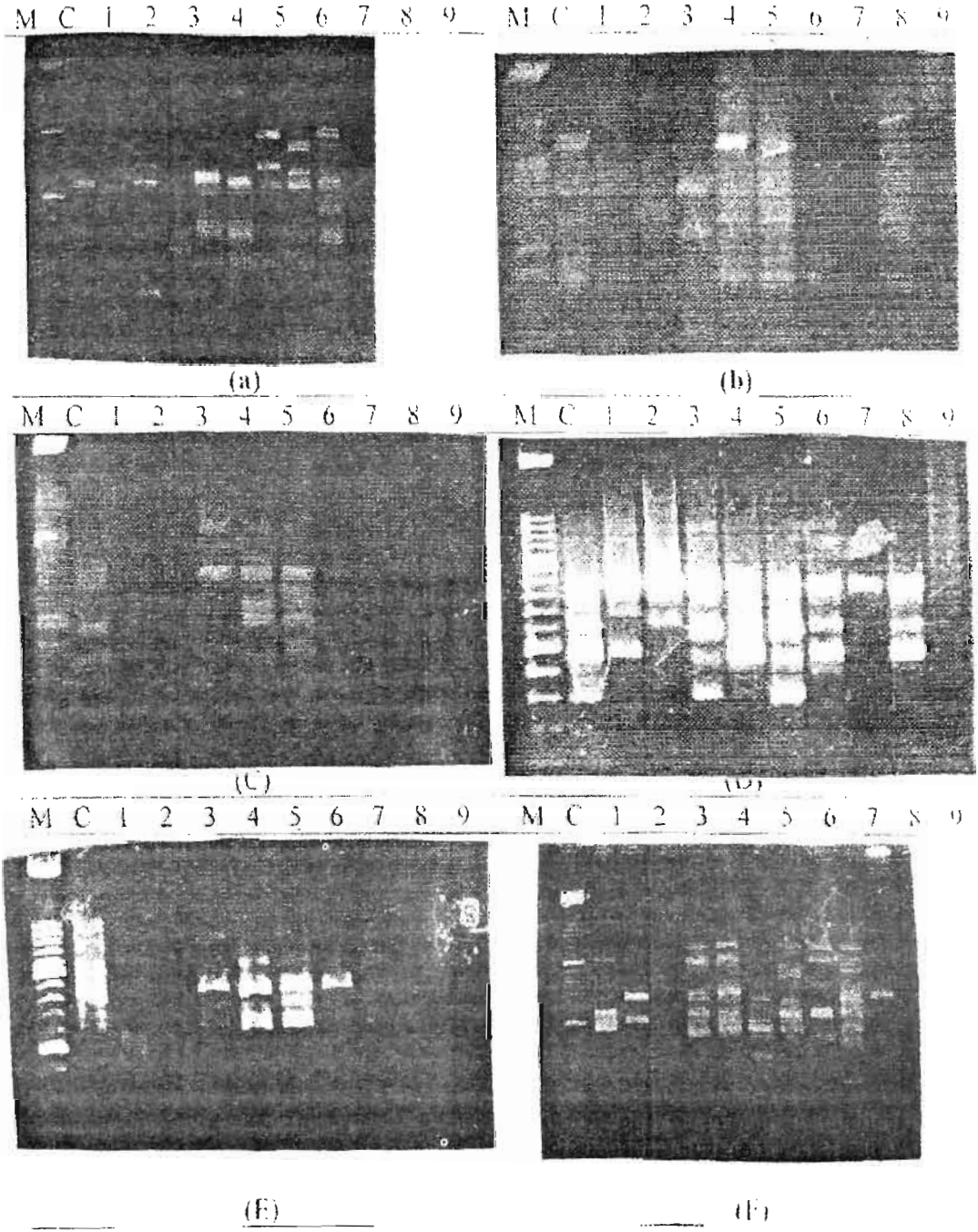

Fig. (2): RAPD banding patterns of *T. niloticus* blood using primersl-6 (Fig. a - f).

M : 100 base pair ladder marker
C : Control (untreated fish).
1-3 : Co_1, Co_2 and Co_3 treatments for three weeks.
4-6 : Cd_1, Cd_2 and Cd_3 treatment s for three weeks
7-9 : Zn_1, Zn_2 and Zn_3 treatments for three weeks.

Primer No. 5 (Figure 2-e) clearly showed that there were differences in the number of amplified bands following the treatment with all Co and Cd concentrations, since 3, 3, 2, 4, 4, 3 and 1 amplified bands occurred following control experiment, Co_1, Co_2, Co_3, Cd_1, Cd_2 and Cd_3, treatments, respectively. The heighest rearrangement in DNA nucleotide sequence were induced following the treatment with Zn since no complementary nucleotide sequence occurred using primer No. 5.

Regarding Primer No. 6, (Fig. 2-f) showed six amplified polymorphic bands with size ranging from 450 to 1250 bp which were detected in control experiment. Two amplified polymorphic bands with length 500 and 700 bp, respectively were detected following Co_1 treatment. No complementary nucleotide sequence could be found following the treatment with Co_2. All other treatments (with the exception of Zn_3) exhibited higher number of DNA fragments, since 10, 9, 9, 7, 6 and 11 amplified polymorphic bands occurred as a result of the treatments with Co_3, Cd_1, Cd_2, Cd_3, Zn_1 and Zn_2, respectively. On the contrary, the highest dose of Zn exhibited the lowest number of amplified polymorphic bands, i.e., one band with a size of 700 bp.

Table (3) showed that the application of the different heavy metals doses for two and three weeks caused high level of molecular abnormalities on blood DNA nucleotides sequences. The extension of exposure time to three weeks instead of two weeks exhibited more variations and rearrangement of nucleotides in about 50 percent of cases.

Table (3): Effect of different concentration of Co, Cd and Zn on number of fragments generated by each primer.

Treatment	Primer 1		Prime 2		Primer 3		Prime 4		Primer 5		Prime 6	
	2W	3W	2W	3W	2W	3W	2W	3W	2W	3W	2W	3W
Cont.	2	4	0	4	0	5	2	4	0	3	0	6
Co_1	2	1	0	0	0	0	0	3	0	3	1	2
Co_2	2	3	8	3	3	0	2	2	0	2	0	0
Co_3	2	2	8	3	3	3	2	4	0	4	4	10
Cd_1	6	6	9	3	7	7	5	3	1	4	7	9
Cd_2	1	7	5	3	5	6	6	4	2	3	5	9
Cd_3	2	3	6	0	5	0	7	3	1	1	5	7
Zn_1	7	5	12	0	9	0	16	1	8	0	3	6
Zn_2	3	6	8	0	11	1	9	4	8	0	3	11
Zn_3	1	0	6	0	6	0	9	0	5	0	0	1

It could be concluded that there were a molecular rearrangements of nucleotide sequences as a result of the treatments with different concentrations of the tested heavy metals as well as the difference in exposure time. This changes in DNA may be due to DNA breakage **(Theodrakis *et al.*, 1994 and Lison *et al.*, 2001)** or inhibition of DNA repair by Co compounds **(Lison *et al.*, 2001)** and Cd ions **(Audrey and David, 2002)** which displaced Zn ions from active sites on proteins involved in the repair process. Moreover, some heavy metals, i.e. Cd, Pb induced a decrease in DNA content in all tissues of *Cyprinus carpio* **(Muley *et al.*, 2000)**. Furthermore , it could be postulated that *T. niloticus* could be considered as an accurate

and sensitive organism to monitor the toxicity of the environmental pollutants at the molecular level. Even one half of the allowable concentrations of Co, Cd and Zn proved to be highly injurious of DNA constitution.

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الملخص العربى

التأثيرات الجزيئية لبعض المعادن الثقيلة على الـ DNA فى دم سمك البلطى النيلى

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أجرى هذا البحث لدراسة التأثيرات الجزيئية للمعاملات بثلاث تركيزات مختلفة (٢/١ الجرعة الآمنه ؛ الجرعة الآمنه ؛ وضعف الجرعة الآمنة) من الكوبالت والكادميوم والزنك كملوثات بيئية لمدة أسبوعين وثلاثة أسابيع على ترتيب النيكليوتيدات فى الـ DNA المستخلص من دم سمك البلطى النيلى مقارنة بتلك غير المعامل فى تجربة للمقارنة. أستخدم لذلك ستة بوادئ عشوائية (عشرة نيكليوتيدات لكل منها) فى تجربة RAPD-PCR.

أوضحت النتائج أن DNA المستخلص من دم سمك البلطى النيلى قد تأثر معنويا بجميع المعاملات تحت الدراسة حيث تم تسجيل اختلافات واسعة بين الأسماك فى تجربة المقارنة وبين تلك المعاملة. وبصفة عامة أدت معاملات الكوبالت الى أقل تأثير بينما أدت معاملات الكادميوم والزنك الى اختلافات أكثر من حيث عدد وكثافة حزم الـ DNA.

أدت إطالة فترة التعريض من أسبوعين الى ثلاثة أسابيع الى ظهور إختلافات أوضح فى عدد وحجم وكثافة الحزم سواءً باستخدام نفس البادئ أو بين البوادئ المختلفة.