ORIGINAL ARTICLE / ÖZGÜN MAKALE

UPREGULATION OF MIR-17 AND MIR-221 BY BENOMYL, CARBARYL, MALATHION AND DIAZINON PESTICIDES IN MICE BLOOD

FARE KANINDA MIR-17 VE MIR-221'İN BENOMİL, KARBARİL, MALATİYON VE DİAZİNON PESTİSİTLERİ İLE UPREGÜLASYONU

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ABSTRACT

Objective: *Increasing evidence demonstrate that the expression of miRNAs is affected by several known toxicants and environmental contaminants. To evaluate the toxicity effect of the pesticides including benomyl, carbaryl, malathion, diazinon on male Balb/c mice, expression profile of two oncogenic miRNAs were analysed by real-time PCR.*

Material and Method: *The 72 male mice were divided into 6 groups (n = 6 per group), including control (0 mg/kg), malathion (30 mg/kg), carbaryl (20 mg/kg), benomyl (30 mg/kg), diazinon (20 mg/kg) and mixture of all pesticides. Mice were intragastrically gavaged for 60 days, then sacrificed on the 30(th) and 60(th) day. The levels of oncogenic mir-17 and mir-221 in the serum were measured.*

Result and Discussion: *The results showed that compared with the normal controls, mir-17 and mir-221 were overexpressed in all treatment groups during 2 months. The expression level of miR-17 and mir-221 after 60 days were 9.2-17.7 fold and 1.9-4 fold higher than the first month respectively. The lowest increase was 1.9-fold, belongs to mir-221, which is still enough for easy diagnosis. These results provide new insights into the negative pesticide's carcinogenic probability via dysregulation of two oncogenic miRNAs. Our results suggest that due to positive association between mir-17 and mir-221 levels and the risk of toxicity, these miRNAs might be a useful biomarker in malignancy prediction and have a diagnostic value.*

Keywords: Dysregulation; miRNA; oncogene; pesticides; toxicity

ÖZ

Amaç: *Artan kanıtlar miRNA'ların ekspresyonunun bazı bilinen toksik maddeler ve çevresel kirleticiler tarafından etkilendiğini göstermektedir. Pestisitlerin toksisite etkisini değerlendirmek üzere erkek Balb/c farelerinde benomil, karbaril, malatiyon, diazinonun onkojenik miRNA ekspresyona etkisi gerçek zamanlı PCR ile analiz edildi.*

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Gereç ve Yöntem: *72 erkek fare 6 gruba ayrıldı: kontrol (0 mg/kg), malatiyon (30 mg/kg), karbaril (20 mg/kg), benomil (30 mg/kg) ve diazinon (20 mg/kg). Fareler 60 gün boyunca intragastrik yoldan sonda ile beslendi, daha sonra 30. ve 60. gününde öldürüldü. Serumda onkojenik mir-17 ve mir-221 düzeyleri ölçüldü.*

Sonuç ve Tartışma: *Sonuçlar normal kontrollerle karşılaştırıldığında, mir-17 ve mir-221 tüm tedavi gruplarında 2 ay boyunca aşırı eksprese edildiği görüldü. Mir-17 ve mir-221 ekspresyon düzeyi ilk aya göre 60 gün sonra sırasıyla 9,2-17,7 kat ve 1,9-4 kat daha yüksekti. En düşük artış 1,9 kat ile mir-221'e aittir ki, hala kolay teşhis için yeterlidir. Bu sonuçlar iki onkojenik miRNA'nın disregülasyonuyla pestisitlerin negatif karsinojenik olasılığına yeni bilgiler sağlamaktadır. Sonuçlarımız mir-17 ve mir-221 seviyeleri ve toksisite riski arasındaki pozitif ilişki nedeniyle, bu miRNA'ların malignite tahmininde yararlı bir biyobelirteç olabileceğini ve diyagnostik değeri olduğunu göstermektedir.*

Anahtar kelimeler: *Disregülasyon; miRNA; onkojen; pestisitler; toksisite*

INTRODUCTION

There are many different types of pesticides that are meant to control specific pests. The most important types are classified as four groups. Fungicides used to control fungi, herbicides remove unwanted weeds, trees or grasses [1], insecticides used to control insects and other arthropods and rodenticides that kills rodents like mice, rats, and gophers [2-4]. Another classification of pesticides includes organophosphate (OP), organochlorine (OC), and carbamate (CB) compounds. These families have special tense because of water pollution, soil contamination and persistent in the environment [5]. Pesticide exposure can happen in many ways such as eating, drinking, touching or breathing anything that bear pesticide residue [6].

Most pesticides are intrinsically toxic and cause potential hazard to human health. Cancer, endocrine disruption, reproductive and sexual dysfunction [7] and dermatitis are among the health effects [8]. Carbaryl is a carbamate insecticide which can inhibit acetylcholinesterase, and associate with lower birth weight in rats and mice [9].

Early accurate diagnosis of diseases like cancer increases the chances for successful treatment. The improvement of genomic technologies and the ability to evaluate the toxicant risks are valuable in therapeutic targets. Several preclinical and clinical trials have been approached for miRNA-based therapeutics [10]. Also microRNAs (miRNAs) are a class of endogenous noncoding RNAs with 18 to 25 nucleotides in length that play an important regulatory role in developmental and physiological mechanisms in human body [11]. Dysregulation of miRNAs is correlated with toxicogenomics, disease aetiology and the effect of toxicants. Circulating miRNAs are useful in diagnostics as biomarkers in the evaluation of toxicant risks [12, 13].

MiR-222/221 cluster is a typical up-regulated miRNA in human cancer[14]. Another example of miRNA overexpression with oncogenic effect is miR-17–92 cluster that is highly overexpressed in different types of cancers, suggesting a mechanism of involvement in human tumorigenesis [15, 16].

In this study we aimed at investigating the expression levels of two oncogenic miRNAs (mir-17 and mir-221) in blood samples of mice treated with four pesticides including benomyl, carbaryl, diazinon and malathion.

MATERIAL AND METHOD

Chemicals and treatment of mice

All pesticides (benomyl, carbaryl, diazinon and malathion) were dissolved in corn oil and administered intragastrically to female mice daily for 60 days. All of the mice had the average weight of 35 gr. For this study 72 BALB/c mice were classified into 6 groups, including control which received normal saline (9%), the second group of mice received 30 mg/kg malathion, the third group received 20 mg/kg carbaryl, the forth group received 30 mg/kg benomyl, the fifth group received 20 mg/kg diazinon and the last group received mixture of all pesticides. (*Formulated* product by Iranian companies were 57% emulsifiable concentration for malathion, 85% wettable powder for carbaryl, 85% wettable powder for benomyl and 60% emulsifiable concentration for diazinon).

At week 4 post gavage, 6 mice from each group (36 mice altogether) as described above, were sacrificed and their sera were separated for RNA extraction and cDNA synthesis. cDNA kept at -20° C. At week 8 post gavage, again 6 mice from each group (the rest of 36 mice) were sacrificed. Their blood was separated for RNA extraction. The sera were collected by centrifugation at 5500 r/min for 10 min.

RNA extraction and cDNA synthesis

Total RNA was isolated from serum samples using RNXTM reagent (Cinnagen, Iran) following the manufacturer's instructions. Two steps including chloroform for removing proteins and isopropanol for RNA precipitation were performed respectively. RNA purity was determined with a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). The miRNAs assessed in the present study included mir-17 (Ensembl:ENSMUSG00000065508; miRBase:MI0000687) and mir-221 (Ensembl:ENSMUSG00000065422; miRBase:MI0000709).

Briefly, the input RNA was polyadenylated using 10 µl of RNA in a final volume of 20 µl including 2 µl of 10x poly(A) polymerase buffer, 0.2 µl of 5 U/ µl Poly A polymerase, 1µl of 10 mM rATP and 6/8 µl DEPC water. The mixture was incubated at 37°C for 30 min follow by enzyme inactivation at 65°C for 20 minutes.

cDNA synthesis was performed using BONmiR miRNA 1st-Strand cDNA synthesis kit following the manufacturer's protocol. To brief a report, 10 µl of polyadenylated RNA was reverse-transcribed to cDNA using RT enzyme (BONmiR, Iran) and a BON-RT universal primer (BONmiR, Iran). The

following reaction conditions were used: 55 °C for 5 min, 25 °C for 15 min, 42 °C for 30 min and 95 °C for 5 min.

Quantitative real-time PCR for miR-17 and miR-221 expression in the BALB/c blood

SYBR green gene expression assay was carried out for mir-17 and mir-221 to evaluate their different expressions in control and treated mice. Real-time PCR analysis was performed in the Bioneer thermocycler with 20 μL volume reaction containing 1 μL cDNA, 0.5 μL miRNA-specific forward primer (BonMir), 0.5 μl universal reverse primer, 6.5 μl 2× miRNA QPCR master mix and nuclease-free, PCR-grade H2O up to 13 μl. The reactions were incubated in 96-well plates at 95°C for 20 secs, following by 40 cycles (95°C for 5 secs, 60°C for 30 sec). miR-93 (reference gene) was measured by the same method and used for normalization. The relative levels of each miRNA in mice blood, normalized to miR-93 and relative to the expression in control, was calculated using $RQ = 2^{-\Delta\Delta^{CT}}$ equation, in which $\Delta\Delta C_T = (C_{T\,miRNA} - C_{T\,mir\text{-}93})_{test} - (C_{T\,miRNA} - C_{T\,mir\text{-}93})_{control}$ and C_T is the threshold cycle to detect fluorescence.

Statistical analysis

Data analysis was performed using SPSS software and Graphpad Prism (Prism 7.0 Graphpad Software Inc., La Jolla, USA). Comparisons between groups were done using parametric unpaired t-test to measure the statistical difference of expression levels between 5 groups that were not normally distributed followed by one-way Anova analysis of variance. The data were expressed as the means ± SEM. P-values <0.05 were considered statistically significant.

RESULT AND DISCUSSION

Epigenetic alterations such as DNA methylation reprogramming, altered histone modification, maternal effects and X chromosome inactivation are the consequences of applying a number of toxicant pesticides [17, 18]. For instance, rat-liver epithelial cell lines treated with arsenic showed decrease in S-adenosyl-methionine (SAM) levels and DNA methyltrasferase activity. Toxic materials such as pesticides can modify gene expression in organisms expose to them. One of the most commonly genetic hallmark is miRNA. Dysregulation in miRNA expression can affect the protein expression in cells, leading negative biological effects. Due to such power of miRNA in changing the expression of proteins, measuring RNA profiling data could be a suitable tool to prognosticate the effective capacity of pesticide's toxicity [19].

For the purpose of keeping homeostasis, miRNAs play a key role as a mediator between cellular response and extracellular signals [20].

For example, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is an environmental pollutant that could increase tumor suppressing miRNAs and decreased oncogenic miRNAs in the liver and brain of mouse [21]. Also exposure to metal-rich particulate matter (PM) and b*isphenol A* (*BPA*) unregulated miR-222 and miR-638 [22, 23].

In the present study, we evaluated the effects exerted by four different pesticides and a mixture of them on the expression of two oncogenic miRNAs in the blood of BALA/c mice. We chose four pesticides (benomyl, malathion, diazinon and carbaryl) that have high consumption in agriculture and were also detected as probably non-carcinogen compounds [24-26]. The precise dose of lowest observed effect level (LOEL) of our used pesticides for BALA/c mice is not yet being determined. So based on the overall results of articles (lower than LOEL) and LD_{50} similarity that was almost being among benomyl-malathion together and diazinon- carbaryl together, we choose the gavage amount of 30 mg for benomyl and malathion and 20mg for diazinon and carbaryl.

Different expression of mir-17 in mice treated with pesticides corresponding to healthy mice

The expression of oncogenic mir-17 was analyzed using quantitative real-time PCR. The results showed that compared with the normal controls, mir-17 was overexpressed in all treatments during 2 months (Figure 1).

The level of mir-17 in mice was significantly upregulated by $\geq$ 25.13-fold (day 30th) and 233.33-fold (day 40th) respectively after treatment with all pesticides in comparison with the control group. Also mir-17 was upregulated by $\geq$ 7.21-fold and 122.72-fold respectively after 30 and 60 days' treatment with diazinon. At the time of using carbaryl, mir-17 was overexpressed by $\geq$ 10.94-fold and 152.2-fold after 30 and 60 days respectively. In return to malathion, mir-17 was increased about $\geq$ 4.48-fold (after 30 days) and $\geq$79.53-fold (after 60 days). In return to benomyl, mir-17 was increased about $\geq$ 1.12-fold (after 30 days) and $\geq$17.06-fold (after 60 days). All the results were significant at *P*< 0.05 (Figure 1.a). Next, we comparison the expression level of miR-17 on the 60(th)/30(th) day for each treatment group. The results have shown in figure 3.1.B indicated that mir-17 was extremely higher (at least 9.2-fold to the most 17.7-fold) after 60 days.

Different expression of mir-221 in treated mice corresponding to healthy mice

The change in the expression of oncogenic mir-221 was confirmed by real-time PCR. The results showed that mir-221 was overexpressed in all treatments compared to healthy mice during 2 months (Figure 2). Compared to the non-treatment group, oncogenic mir-221 expression in mix pesticide's treatment group was upregulated by $\geq$ 476.88-fold and 4715-fold after 30 and 60 days respectively. Also compared to the non-treatment group, the diazinon- treated group showed increased
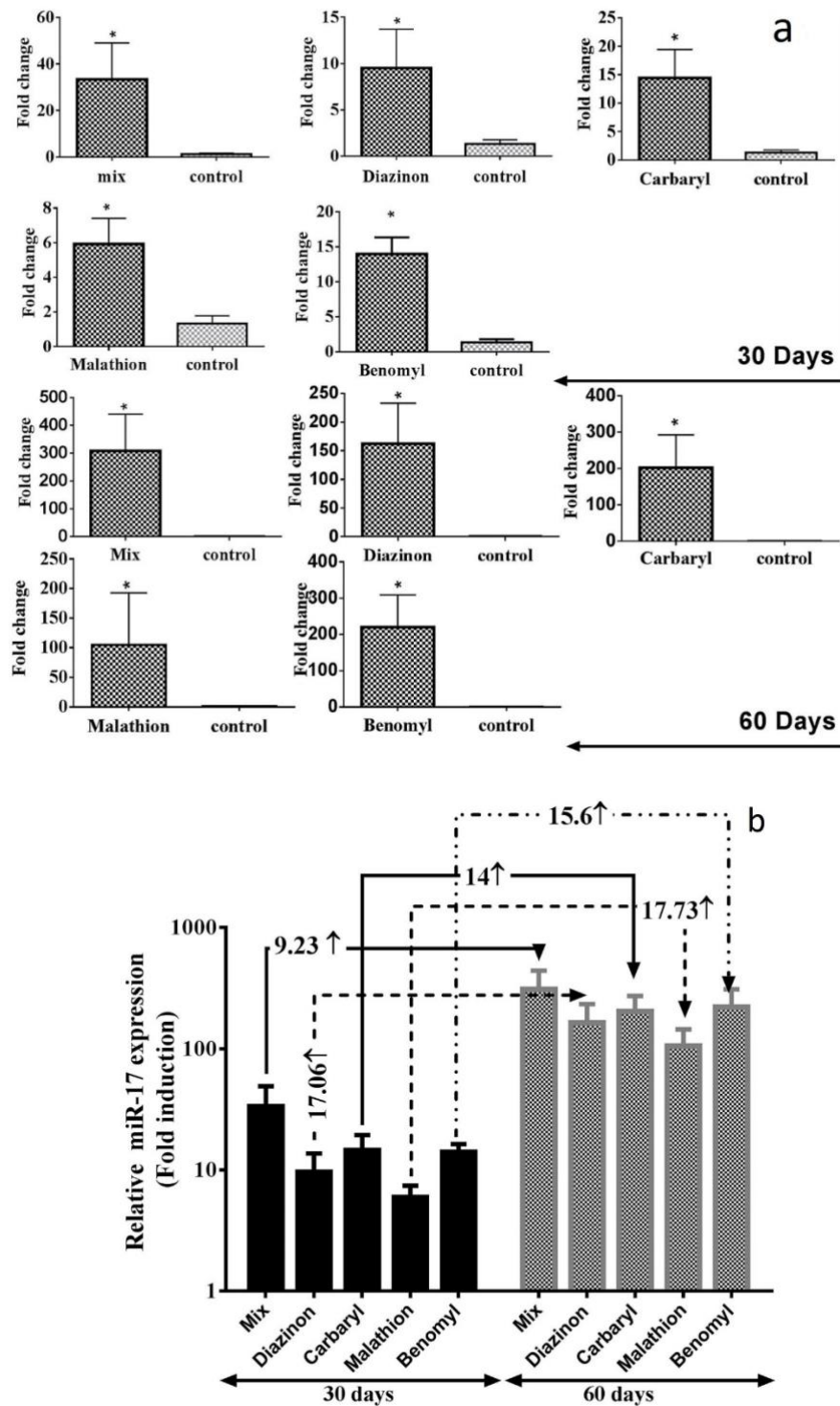
Figure 1. The expression level of mir-17.

a: The expression level of mir-17was remained significantly higher in comparison with the control group *versus* control ($^{*}P < 0.05$). b: The expression level of mir-17 was extremely higher after 60 days in comparison with day 30th. The folds are written on the arrows connected the months of every pesticide together.

mir-221 expression by $\geq$ 150.48-fold and 2056-fold after 30 and 60 days respectively. The mir-221 expression was also found increased in carbaryl-teatment group about $\geq$ 215.54-fold (after 30 days) and $\geq$3577-fold (after 60 days). At the time of using malathion, mir-221 was overexpressed by $\geq$ 114.32-fold and 1610-fold after 30 and 60 days respectively. In return to benomyl, mir-221 was increased about $\geq$ 184.68-fold (after 30 days) and $\geq$1877-fold (after 60 days). All the results were significant at $P <$ 0.05. Next, we determined the expression level of miR-221 on the 60(th)/30(th) day for each treatment group. The results have shown in figure 3.2.B indicated that mir-221 was at least 1.92-fold higher after 60 days in comparison with 30 days.

The current study shows up-regulation of mi-17 and mir-221 levels in treatment mice compared to non-treatment controls. As mir-17 and mir-221 are both considered as an oncogene [27, 28] increased expression of these two miRNAs represents their properties of dysregulation in facing with pesticide's toxicity. Although both miRNAs had significant dysregulation but most applied changes were made on mir-17. The lowest increase was 1.9-fold, belongs to mir-221, which is still enough for easy diagnosis. It is true that the rise of mir-17 and mir-221 reflects the toxicity of these materials, but may also be suspected of being carcinogen compounds.

Among four pesticides, benomyl and carbaryl was attracted our attention. Although their LD_{50} was lower than diazinon, but mir-17 and mir-221 of the serum had the greatest change in expression against them. This can be due to benomyl and carbaryl ability on changing the expression of oncogenic miRNAs. This property can also increase the pesticide's carcinogenic probability. It may also be expected that potential targets of mir-17 and mir-221 could contain genes encoding oncogenic proteins that increase their carcinogenic impact. Taken together, these findings indicate that there is a positive association between mir-17 and mir-221 levels and the risk of malignancy, contributing towards improved predictive human toxicity. This result suggests that both miRNAs might be a useful biomarker and have a diagnostic value. However, the detailed mechanism will require further investigations.
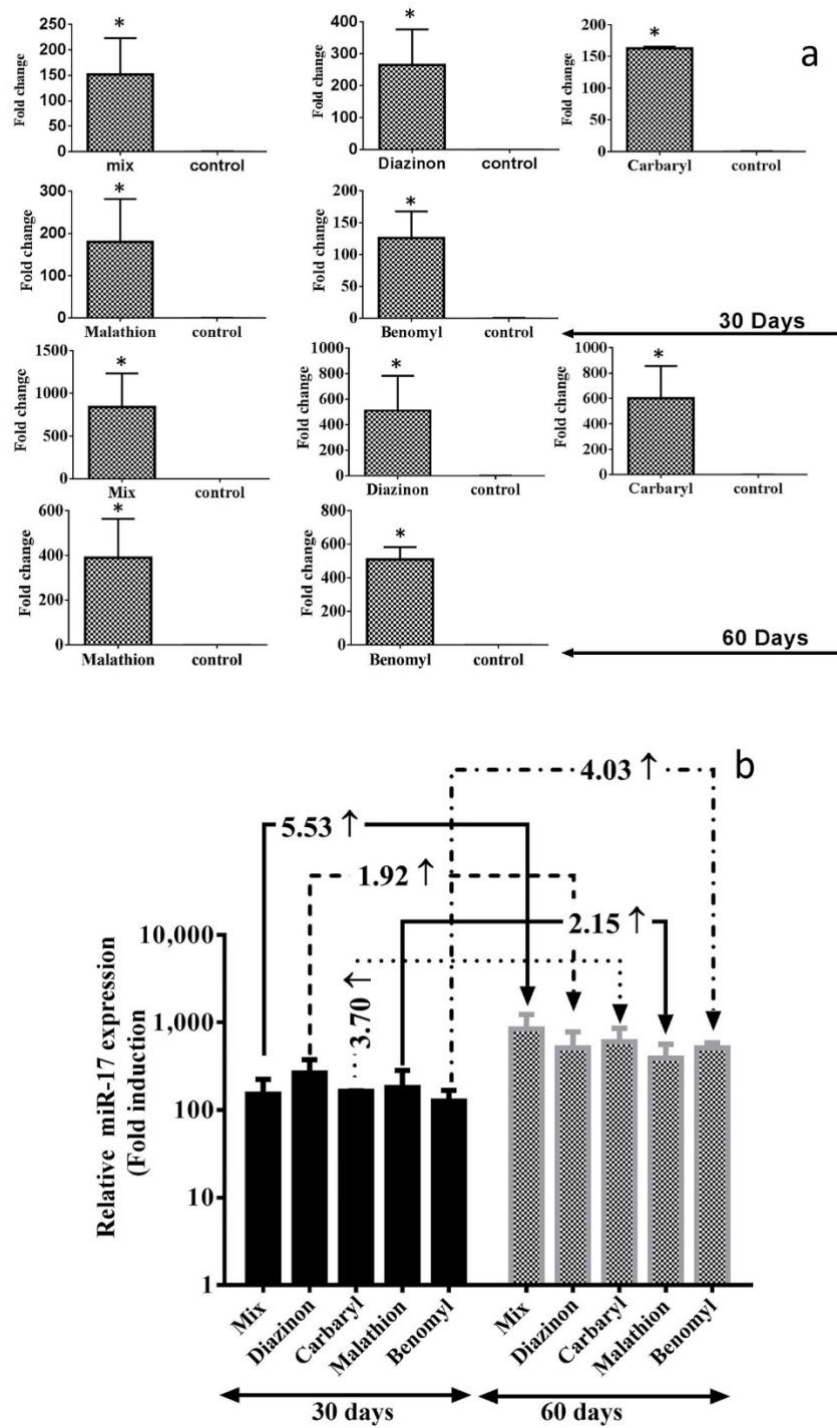
Figure 2. The expression level of mir-221.

a: Compared to the non-treatment group, the expression level of mir-221 was remained significantly higher after 2 months *versus* control ($^*P < 0.05$). b: The expression of mir-221 varied significantly among the treatment groups ($^*P < 0.05$). mir-221 levels were significantly increased in second month. The folds are written on the arrows connected the months of every pesticide together

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