

Atrazine Genotoxicity on Human Placental Cells

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Abstract: Atrazine is one of the most commonly used herbicides. After exposure to Atrazine during pregnancy, it can be found in placental tissue. We investigated the toxic effects of different Atrazine concentrations on human cytotrophoblasts. These cells were isolated from fresh placenta and were exposed to Atrazine for 24 hours. Our results showed that very low doses of Atrazine induced apoptotic placental cell death. It decreased the relative survival of human cytotrophoblasts up to 50% of control. It significantly decreased P53 and TNF- α genes expression while Bcl2 gene expression was increased insignificantly. Our findings suggested that exposure of placental cells to Atrazine even in low doses causes harmful effects, leading to adverse pregnancy outcomes such as preeclampsia, intrauterine growth restriction, prematurity and pregnancy loss.

Keywords: Atrazine, Placenta cytotrophoblasts, mRNA, Bcl2, P53, TNF- α

1. Introduction

Atrazine, a herbicide used to control weeds in cultivation (Oh et al¹), is classified as moderately toxic herbicide by US environmental protection agency (USEPA²). In Egypt; its utilization was increased since 2000 (Mona et al.,³).

After oral ingestion of atrazine, it passes through the lining of intestine then enters the bloodstream and is distributed to many parts of body especially placenta (Rohr and Crumrine³). Apoptosis plays a critical role in different stages of placental development, that is essential for fetal growth as it delivers nutrients and oxygen to the fetus. Placental apoptosis occurs through immune-mediated extracellular ligands and receptors such as TNF- α and its receptor type1. As well as endogenous death signal molecules such as the P53 and Bcl-2 family genes (Allaire et al⁴). TNF- α is produced in the villous placenta throughout pregnancy and is implicated in regulating placental apoptosis (Levy and Nelson⁵). The p53 protein is a tumor suppressor gene product, whose wild-type is able to negatively regulate cell proliferation by arresting the growth in G1 and maintain genetic integrity (Cantemir et al⁶). Enhanced cell apoptosis is associated with increasing the expression of the TNF- α gene and pro-apoptotic proteins p53 along with decreasing the expression of the anti-apoptotic protein Bcl-2 (Kharfi et al⁷). Disruption of the reproductive system by Atrazine begins

when disruption of cell signaling occurs. However, the exact mechanism by which atrazine exerts its toxic effects in human placenta aren't clear (Oh et al¹; Pathak & Dikshit⁸).

2. Aim of the Work

Enhancing apoptosis in human trophoblasts that exposed to different low doses of Atrazine were evaluated by detecting the expression levels of P53, Bcl2, TNF- α genes and measuring human cytotrophoblast relative survival.

3. Materials and Methods

Dulbecco's modified Eagle medium (DMEM), fetal calf serum, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) were purchased from GIBCO BRL (Grand Island, NY, USA). Trypsin 2.5%, penicillin, streptomycin, atrazin and all other chemicals employed in this study were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, USA).

3.1. Cell Culture and Cell Proliferation Assay

Atrazine was dissolved in complete DMEM, the pH value was adjusted to 7.2 and sterilized through a 0.2 μ m filter to the desired working solutions. Human placental cell line was cultured in DMEM medium supplemented with 5% fetal

bovine serum (FBS), 100 mg/mL streptomycin and 100 units/mL penicillin at 37 °C in a humidified incubator in an atmosphere of 5% CO₂. Cells were seeded in 96-well plates (10³- 10⁴ cells/well) for 24 hours incubation, cell viability was evaluated using MTT assay as described by Cui et al⁹. In brief, cells were treated with atrazine at a various concentration 0, 1, 10, 30 µM/ml for 24 hours and untreated cells served as a control. Prior to determination, 10 µL MTT (2.5 g/L) was added to each well. After 4 hours incubation, the culture media were discarded followed by addition of 100 µL of detergent reagent to each well and vibration for 10 min. The absorbance (A) in the experimental wells was measured at 570 nm with a microplate reader (ELISA reader). The absorbance in the experimental wells to that of the control wells. The cytotoxicity percentage of viable cells was calculated as follows: (A of experimental group/A of control group) × 100%.

3.2. Total RNA Isolation

Cells were detached by trypsin (2.5%) and total RNA was isolated with RNA assay Mini Kit (Qiagen) and further analyzed for quantity and quality with Beckman dual spectrophotometer (USA). The RNA integrity and the GAPDH-RNA (housekeeping gene) ratio were used as the quality control.

3.3. Real Time PCR (qRT-PCR) for Quantitative Expression of P53, Bcl2 and TNF-Alpha

The mRNA expression level was quantified by qRT-PCR (Real time PCR). 1000 ng of the total RNA from each sample were used for cDNA synthesis by reverse transcription using High capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48-

well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 minutes at 95°C for enzyme activation followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 55°C and 30 second at 72°C for the amplification step. Changes in the expression of each target gene were measured relative to the mean critical threshold (CT) values of GAPDH housekeeping gene by the Ct method. We used 1µM of both primers specific for each target gene. Primers sequence specific for each gene demonstrated in (table 1).

3.4. Statistical Methods

Data were statistically described in terms of mean ± standard deviation. Comparison of quantitative variables was done using Mann-Whitney test. Correlation was done to test for linear relations between quantitative variables by Spearman correlation coefficient. A probability value (P value) less than 0.05 was considered statistically significant. All statistical calculations were done using SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 21(Chan¹⁰).

Table (1). Primers sequence specific for P53, Bcl2 and GAPDH genes.

Target gene	Primer sequence: 5' - 3'
P53	Forward: AGAGTCTATAGGCCACCC
	Reverse: GCTCGACGCTAGGATCTGAC
Bcl2	Forward: TTACGAGAAGCTTGGAGAG
	Reverse: TGTGCAGATATCAGAGTGG
GAPDH	Forward: CCTCTACTGGCGTGCCAAGGCT
	Reverse: GTCCACCACTGACACGTTGG

4. Results

Our results showed that small doses of Atrazine (1, 10 and 30µM/ml) were cytotoxic for human cytotrophoblasts, as they induced apoptotic and necrotic cell death (Table 2).

Table (2). Apoptotic effects of different Atrazine's concentrations in human cytotrophoblasts after 24 hours.

Cell line treated with Atrazin Apoptotic parameters	0µM/ml	1µM/ml	10µM/ml	30µM/ml
Extrinsic pathway parameter				
TNF-alpha	1.3±.89	.69±.14	.25±.11	.15±.06
Intrinsic pathway parameters				
p53	1.42±.31	.16±.04	.13±.14	.04±.02
Bcl2	1.35±.21	1.65±.65	.78±.26	.44±.22
Cell viability				
MTT assay	.58±.10	.28±.08	.15±.05	.14±.15
Relative survival (%)	100	49.9±17.5	25.6±6.9	23.8±26.9

Cytotoxicity of Atrazine was determined through relative survival and MTT assay of human cytotrophoblasts. The Atrazine concentrations 1, 10 and 30 µM/ml induced nearly 50, 75 and 77% growth inhibition in relation to the control and significantly altered the growth of the cells (table 2 and figure1). Moreover, Relative survival of human cytotrophoblasts showed significant negative correlation with Atrazine's concentrations (figure 2).

We demonstrated that Atrazine was able to affect intrinsic apoptotic pathway through affecting p53 and Bcl2 genes

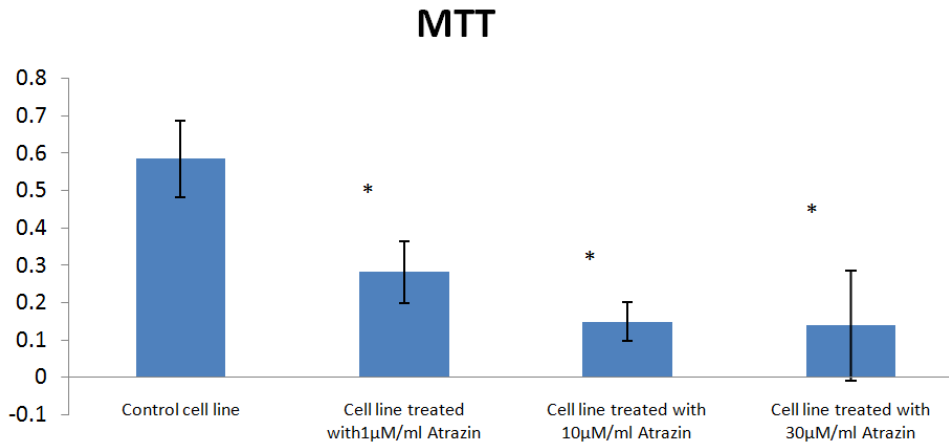
expression. P53gene expression decreased significantly (0.16±.04, 0.13±.14 and 0.04±.02) with increasing Atrazine dose (table 2 and figure 3). Moreover, it showed a significant positive correlation with Bcl2 genes (figure 4).

Although, Bcl2 was not significantly enhanced (1.65±0.65) at Atrazine concentration 1µM/ml, it significantly decreased (0.78±.26 and 0.44±.22) with Atrazine concentrations ranged between 10 and 30µM/ml (table 2 and figure 5).

Atrazine was also able to affect the extrinsic apoptotic pathway through modulation of TNF-α gene expression.

Indeed, we revealed that mRNA levels of TNF- α reduced significantly ($0.69\pm.14$, $0.25\pm.11$ and $0.15\pm.06$) by Atrazine doses (1, 10 and 30 $\mu\text{M}/\text{ml}$ respectively) (table2 and figure 6).

TNF- alpha gene showed a significant positive correlation with p53 and Bcl2 genes expression (figure 7).



* significant difference at $P < 0.05$.

Figure (1). MTT- assay in human cytotrophoblasts exposed to different Atrazine's concentrations. Statistical analysis.

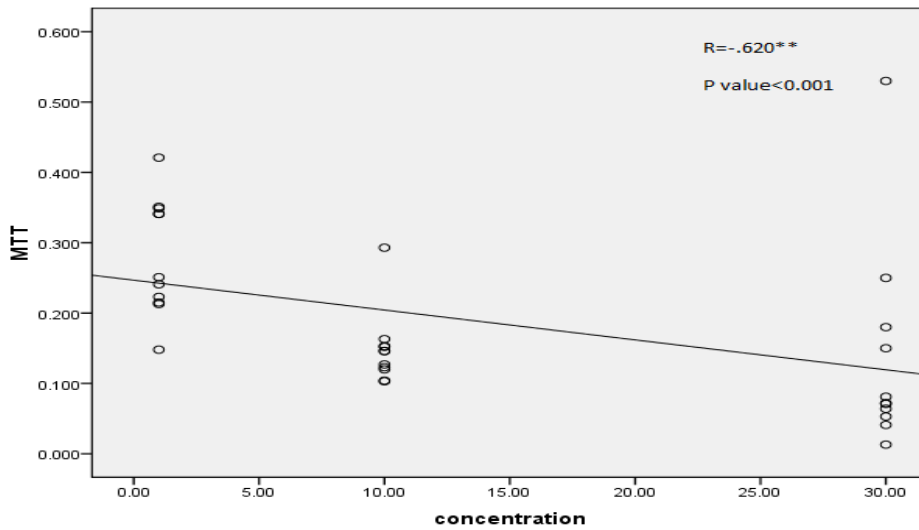
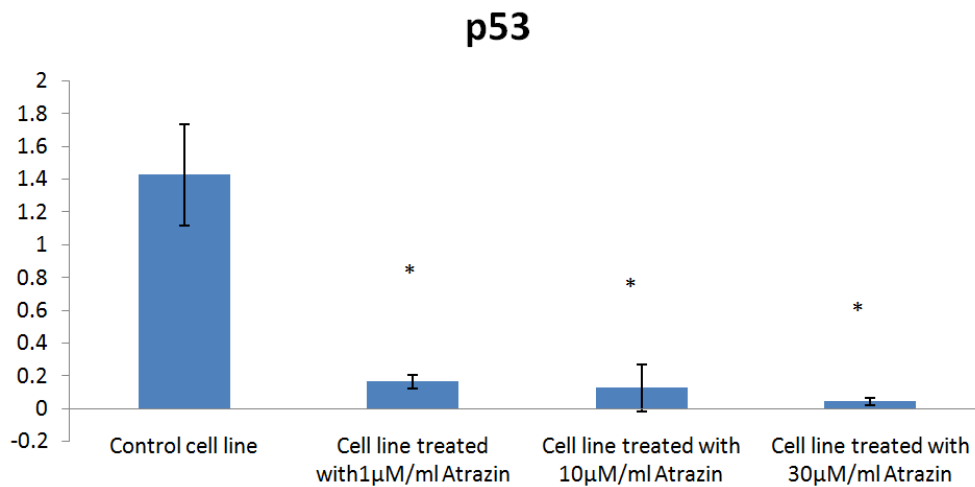


Figure (2). correlations between MTT assay and different Atrazine's concentrations in human cytotrophoblasts.



* significant difference at $P < 0.05$

Figure (3). p53 gene expression in human cytotrophoblasts exposed to different Atrazine's concentrations. Statistical analysis.

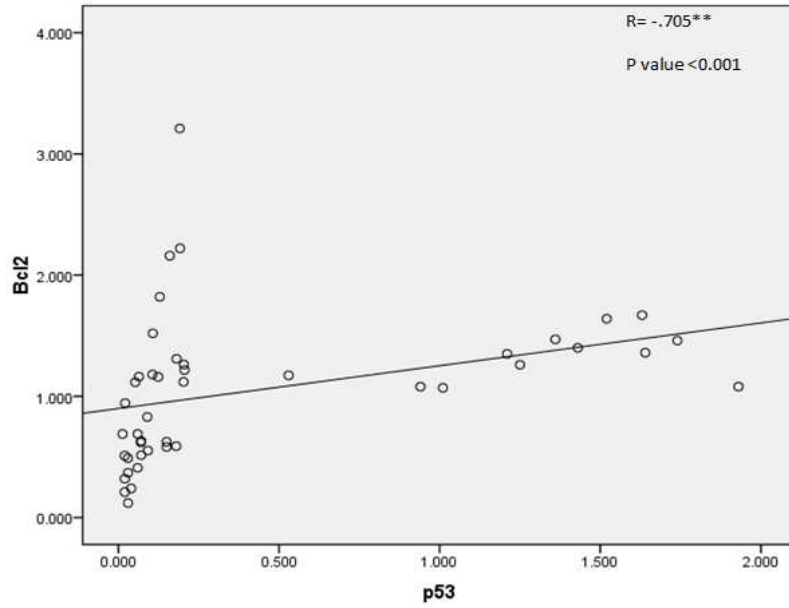
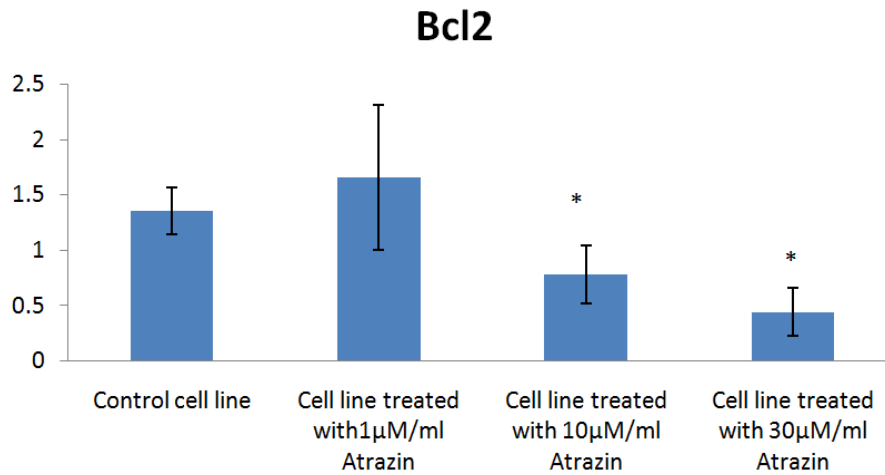
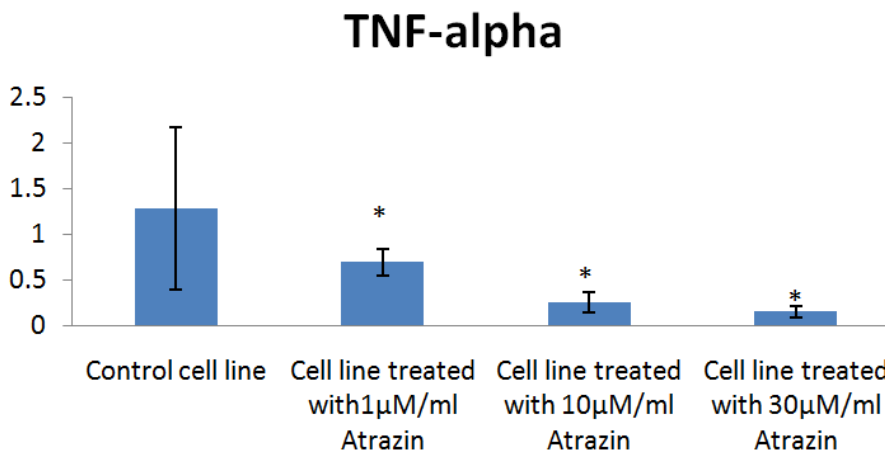


Figure (4). correlation between Bcl2 and p53 gene in Atrazine exposed human cytotrophoblasts.



* significant difference at P<0.05.

Figure (5). Bcl2 gene expression in human cytotrophoblasts exposed to different Atrazine's concentrations. Statistical analysis.



* significant difference at P<0.05

Figure (6). TNF-alpha gene expression in human cytotrophoblasts exposed to different Atrazine's concentrations. Statistical analysis.

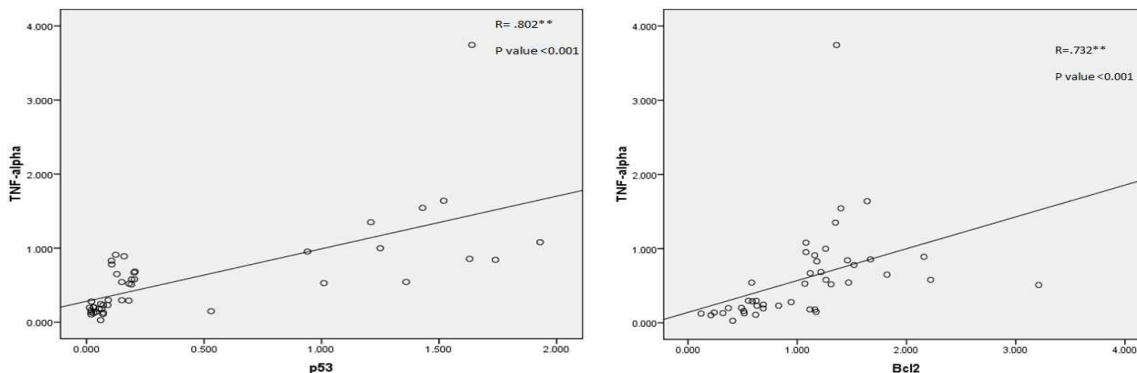


Figure (7). correlation between p53, Bcl2 and TNF- alpha gene in Atrazine exposed human cytotrophoblasts.

5. Discussion

In this study, we showed a dose-dependent enhanced apoptotic cell death of human cytotrophoblasts after exposure to atrazine in different doses.

Regarding atrazine's effects on cytotrophoblast relative survival, we were not in the same line with Jung Kim and Chun Ryu¹¹ who investigated the effect of different doses of atrazine on Rat2lacI transgenic fibroblasts, found 20% cell growth inhibition was resulted from using 261.7µg/mL of atrazine that was higher than ours. This may be due different sample type.

Our results were similar to those of Nwani *et al*¹² who evaluated the genotoxic and mutagenic effects of the Rasayanzine herbicide, whose active ingredient is atrazine. They stated that all tested concentrations up to 8.48 mg/L showed significant genotoxic and mutagenic effects and arranged a dose response curve for their results.

Atrazine induced apoptosis through affecting the intrinsic pathway more than extrinsic one. Our finding coincided with the study results carried by Zhang *et al*¹³ in which mice administered atrazine exhibited a significant apoptotic activity in a dose dependent manner through increasing the expression of Fas, FasL and caspase-3.

Concerning TNF- α , Benachour *et al*¹⁴ stated that TNF is produced in the villous human placenta throughout pregnancy and induced placental apoptosis. If TNF- α is modulated by low doses of Atrazine, the placenta will be harmfully affected.

Regarding p53, Deng *et al*¹⁵ discovered that the direct apoptogenic role of p53 is induced through induction of p53-Bcl2 binding that causes DNA damage.

Elevated apoptotic factors, as shown in our study especially p53, cause failure or deficiency in trophoblast proliferation or differentiation, this will compromise placental development in pregnancies and complicated by IUGR and fetal death (Benachour¹⁴).

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