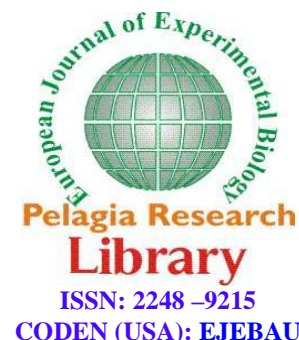




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European Journal of Experimental Biology, 2014, 4(5):1-6



Apoptotic activity of Paracetamol on normal lymphocytes by DNA fragmentation measurement

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ABSTRACT

DNA is the central molecule in cells. The correct function of cells depends on the structure of DNA, and any mutation led to drives a wave of cellular multiplication associated with gradual increases in cancer occurring. The effect of paracetamol on DNA was studied using proliferative lymphocyte in vitro. DNA fragmentation was measured by quantitative method using (DPA). This study showed, that the Paracetamol drug at all concentrations (500µg/ml; 250µg/ml; and 125µg/ml) showed potent induction of DNA fragmentation in DNA after different exposure periods (24hrs; 48hrs; and 72hrs) respectively. Current results showed, that the paracetamol at 500µg/ml had highly apoptotic activity against normal lymphocytes, the percentage of fragmented DNA was 47.13 %, 69.32%, and 72.81% at 24hrs, 48hrs, and 72hrs respectively. The results showed significant effect ($p < 0.05$) of paracetamol on treated cells at 250µg/ml after 24hrs of exposure time, the percentage of DNA fragmentation was 51.94%, 60.00% and 57.89%, after 24hrs, 48hrs, and 72hrs of exposure time, respectively. However, the ability of paracetamol at low concentrations (125 µg/ml) were achieved a significant fragmentation ($p \leq 0.05$) on lymphocytes DNA; percentages of DNA fragmentation was 44.76%, and 61.66%, and 70.17% respectively after 24hrs, 48hrs, and 72hrs of incubation period in. Indeed, the inhibition rate was increased with increasing time of incubation. On the other hand, present results showed low percentage of DNA fragmentation (21.14%, 21.78%, and 23.42%) in untreated cells after different exposure periods (24, 48, and 72hrs) respectively.

Key words: Paracetamol, Apoptosis, lymphocytes, DNA Fragmentation.

INTRODUCTION

Paracetamol is among the most widely used drugs in the world (1) and the most popular over the counter analgesic and antipyretic agent (2, 3). There are around 40 registered products of paracetamol (4) and it is the most popular analgesic and antipyretic. It is estimated that over about 60 million daily defined doses of paracetamol (adult and pediatric dosages) were dispensed in the public sector alone in 2005.

Paracetamol associated problems were not heard until the first case of paracetamol poisoning was reported in 1966 (5, 6). Several reports have indicated genotoxic effects of Paracetamol. It was reported that Paracetamol also causes chromosomal aberrations in Chinese hamster fibroblast cells in vitro (7). Paracetamol has been shown to induce liver-cell tumours in mice after long- term feeding (8), and to induce DNA damage in mouse-liver cells in culture (9). In mammalian cell lines The genotoxicity of paracetamol, including covalent binding to DNA, induction of DNA single-strand breaks (SSBs), and inhibition of replicative and repair synthesis of DNA, where (10) found that

paracetamol covalent binding to liver DNA, when administered intraperitoneally (i.p.). The maximal binding was 8.4 +/- 3.1 pmol/mg of DNA after 2 hrs in male ICR mice. However, published data giving clear evidence that paracetamol causes chromosomal damage in vitro in mammalian cells at high concentrations and indicating that similar effects occur in vivo at high dosages. Available data point to three possible mechanisms of paracetamol-induced genotoxicity: (1) inhibition of ribonucleotide reductase; (2) increase in cytosolic and intranuclear Ca²⁺ levels; (3) DNA damage caused by NAPQI after glutathione depletion. All mechanisms involve dose thresholds (11, 12, 13). For all that present study was carried out to investigate the DNA fragmentation in human lymphocytes after paracetamol exposure.

MATERIALS AND METHODS

Culture media RPMI-1640 (Rosewall Park Memorial Institute) medium (14):

It prepared by mixing RPMI-1640 (5.2g), NaHCO₃ (7.5 ml), Benzyl penicillin (0.25 ml), Gentamicin (0.125 ml), and Nystatin (0.125 ml), and adjusted pH to 7.2, thereafter FCS (5-10 %) was added and the volume brought to 500 ml of deionized distill water (DDW), medium was sterilized by Nalgene filter unit (0.2µm), distributed into vials and incubated at 37°C for 3 days as check sterility and stored at 4°C until used.

Preparation of different concentrations of paracetamol

Serial concentrations of paracetamol (0, 500, 250 and 125, µg/ml) with serum free media (Its RPMI-1640 media but without FCS, sterilized by Nalgin filter.

Collection of Blood samples

The study included 20 subjects, venous blood samples were obtained from healthy individuals, 15 were treated with paracetamol and 5 of them did n't exposed to paracetamol were considered as negative controls. Peripheral blood (5 ml) was aspirated from each subject. Blood samples were immediately transferred to sterile heparinized tubes for lymphocyte separation.

Separation of lymphocyte from blood samples (15)

Two milliliters of heparinized blood were diluted at ratio 1:1 ratio with phosphate buffer saline PBS, then two milliliters of mixture were carefully layered on the top of equal volume of ficoll which was dispersed in ten milliliters glass centrifuge tube. Mixture was centrifuged in cooling centrifuge at 2100rpm, for 25 min at 20° C. After centrifugation lymphocyte forms a white buffy coat at the interface of the blood plasma and separating medium. The white buffy coat of lymphocyte was aspirated (not disturbed) by Pasteur pipette and transferred into a ten ml tube. The aspirated lymphocyte was washed by PBS, centrifuged at 2500 rpm until a pellet was formed. The supernatant was discarded; this procedure was repeated for three times. Finally, the lymphocyte pellet was resuspended in 0.5 ml of PBS.

Exposure stage of lymphocytes to paracetamol

Serial concentrations of paracetamol were added to 4.5ml of RPMI.1640 (growth media). The final volume of the mixture must be taken into account. About 1x10⁶ (0.5ml) of cell suspension (lymphocytes) was added to each tube, the contents were mixed gradually by inversion and incubated at 37° C for 1, 24, 48, and 72hrs, in diagonally way, tubes mixed each 24hrs. 0.3 ml of PHA was added to each tube. One tube cultured without extraction as a negative control.

Detection of paracetamol effect on the viability of lymphocytes isolated from healthy individuals (16):

After incubation periods a known volume of lymphocyte suspension (100µl) was mixed with an equal volume of trypan blue dye and examined immediately under light microscope using Hemocytometer counting chamber to calculated of viable cell depending on viability formula: % viability: viable cell count / total cell count×100.

Detection of paracetamol fragmentation effect on lymphocytes DNA using DPA reagent.

Culture tubes were divided to three groups depending on method of (17,18): First group: prepared as in exposure stage of lymphocytes to paracetamol (incubated for 72hrs); Second group: also prepared as in exposure stage but cells incubated with paracetamol for 48hrs; while third group of exposure lymphocytes were incubated with paracetamol for 42hrs

Harvesting stage:

After completed of incubation periods of exposure cells Colcimied (0.1ml), was added and incubated at 37°C for another 30 min. Cultured cells were centrifuged at (1500 rpm for 10 min), the cell pellet was re- suspended by adding 5 ml of pre-warmed KCl solution, and then incubated at 37°C for 30 min. the cells Supernatant was dividing to 3 group A, B and C and centrifuged. A volume of 1.0ml TTE solution was added to the pellet in tubes. A volume of 1.0ml of 25% TCA was added to tubes C, A, and B and vortexes vigorously, then heated for 15 min at 90°C in water bath. To each tube, 320µl of freshly prepared DPA solution was added, then vortex, allowing color to develop for about 4hrs at 37 C or overnight at 25 C.

The optical densities of the tubes were measured with spectrophotometer on wave length of 600 nm. The percentage for DNA fragmentation which indicated the programmed cell death of cancer cells was calculated, according to the following equation:

$$\% F = \frac{B+C}{A+B+C} \times 100$$

Since; %F: The percentage of fragmentation. **A, B, and C:** Reading of optical density of three tubes.

RESULTS

Current results showed, that the paracetamol at 500µg/ml had apoptotic activity against normal lymphocytes after 24hrs of exposure time; the percentage of fragmented DNA was 47.13%, and there was significant differences ($p \geq 0.05$) when compared with untreated cells, where the percentage of fragmented DNA was 21.14% (table 1). On the other hand, the percentage of fragmented DNA increased with increasing of exposure period at same concentration, the percentages reach to 69.32%, 72.81% at 48hrs and 72hrs respectively. There was significant differences ($p \geq 0.05$) when compared with untreated cells.

Notable, 250µg/ml of paracetamol had a potent anti-proliferative activity; the percentage of fragmented DNA was 60.00% and 73.89%, after 24hrs, 48hrs, and 72hrs of exposure time, respectively in comparison with untreated cells (21.14%). However, the ability of paracetamol at low concentrations (125 µg/ml) were achieved a significant fragmentation ($p \leq 0.05$) on lymphocytes DNA; percentages of DNA fragmentation was 44.76%, and 61.66%, and 70.17% respectively after 24hrs, 48hrs, and 72hrs of incubation period in. Indeed, the inhibition rate was increased with increasing time of incubation.

On the other hand, present results showed low percentage of DNA fragmentation (21.14%, 21.78%, and 23.42%) in untreated cells after different exposure periods (24, 48, and 72hrs) respectively (table 1).

Value of LSD	concentration				Time (hrs)
	125	250	500	0	
7.883 *	44.76± 1.81	51.94± 2.27	47.13± 1.75	21.14 ± 1.45	24
7.025 *	61.66± 2.42	60.00± 3.51	69.32± 2.86	21.78± 1.62	48
8.319 *	70.17± 2.58	73.89± 2.89	72.81± 3.19	23.42± 1.77	72
----	6.973 *	5.217 *	6.983 *	NS	LSD

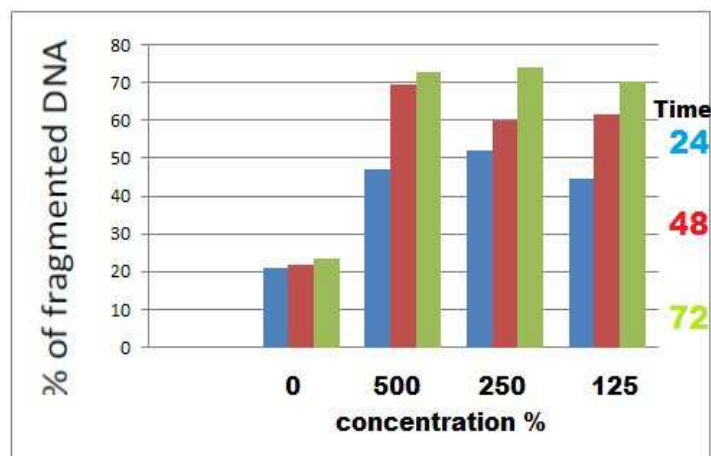


Figure (1) The percentage of DNA Fragmentation of normal lymphocytes by Paracetamol

DISCUSSION

From the results above, it can be noticed that the toxic effect of paracetamol is concentration and time-dependent. In addition, the results showed significant difference between DNA fragmentation percentages of treated and untreated cells at various concentrations of paracetamol during different periods of incubation. However, current results concluded that paracetamol drug had highly toxic effect on the blood cells (lymphocytes) and that may reflected on all the body activity in the future because these cells represent important defense line of the body. However, this is the first study shed the light on toxic activity of paracetamol on lymphocyte isolated from healthy patients. Paracetamol has powerful cytotoxic effects against lymphocyte ($p < 0.001$), after exposure to different concentrations (500, 250, and 100 $\mu\text{g/ml}$), when it compared with untreated cells.

Current results showed potent ability of paracetamol to induce DNA fragmentation in lymphocytes, after different exposure time. The genotoxicity of paracetamol, including covalent binding to DNA, induction of DNA single-strand breaks (SSBs), and inhibition of replicative and repair synthesis of DNA, As well as 34, found that the Paracetamol uses lead to blocks DNA replication by inhibiting deoxyribonucleotide (dNTP) synthesis and may therefore also interfere with DNA repair, by using Alkaline elution to assay DNA single-strand breaks plus alkali-labile sites (= SSBs) in Resting human mononuclear blood cells (MNC) when 0.3 mM paracetamol.

The potent toxic activity of paracetamol contributed to early mitochondria depolarization is an initial injury which is followed by ATP depletion and cell death (19). One possible mechanism of cell death is that covalent binding to critical cellular proteins results in subsequent loss of activity or function and eventual cell death and lysis. Primary cellular targets have been postulated to be mitochondrial proteins, with resulting loss of energy production, as well as proteins involved in cellular ion control (20). Alteration in mitochondrial function was monitored (21) that lead to cell damage. This result agree with previous study (22), also reported that paracetamol was able to induce significant DNA strand break formation in cells after treatment with paracetamol, and gradually increased in both dose- and time-dependent manners. Direct cytotoxic effects of paracetamol have previously been reported using V79 Chinese hamster cells (23).

Furthermore (24) found that paracetamol leading to DNA single strand breaks in hepatoma cells. As well as, it consistent with previous suggestion (25) who mentioned that the generation of apoptotic cells after treatment with paracetamol closely follows the rapid induction of DNA-strand breaks and inhibition of nucleotide excision repair. Present results came in harmony with the previous study by (26) how studied DNA damaging by paracetamol in vitro and in animal experiments, where showed it's covalently bond to DNA, and inhibit its replication as well as DNA repair synthesis, and causes chromosomal aberrations in somatic cells. Also, (27) revealed that paracetamol causes DNA fragmentation in a dose-dependent concentration manner. Further experiment done by (28;29) revealed that DNA strand breakage products increased gradually depending to comet assay results, in mouse liver; as well as, Binding of paracetamol to liver and kidney in mice leading to DNA and protein damages (30).

Previous study done by(31) showed oral administration of the paracetamol for laboratory rats showed that this drug has severe toxicological effects on most of the vital organs in the body like kidney, liver and heart. The present data indicate that paracetamol interferes with nucleotide excision repair in several mammalian cell types. This constitutes a mechanism by which paracetamol may contribute to genotoxicity in humans. Overall, the data indicate higher risk of cancer after exposure to paracetamol depending on concentration and exposure time.

CONCLUSION

The present study has reached the truth that paracetamol at all concentrations (500, 250, and 125mg/ml) had highly apoptotic effect on the human proliferative lymphocytes. As well as, it's induced highly DNA fragmentation in lymphocyte depending on exposure time and its can considered highly mutagenic material, where had highly side effect more than its beneficial role.

Acknowledgements

This work was supported by AL-Yarmouk University Collage/ Department Of Medical Laboratory technique. A grateful support by Miss, SalaNajem and Malak Abdulbast, Ms. Mawj Wasfi and Fatima Alzahraa Mudher.

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