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Flow Cytometric Evaluation of Apoptosis on The Effect of Mirazid on Gentamicin-Induced Renal Damage in Rats

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Abstract

The present study evaluates the effect of mirazid (mz) cd95 and cell cycle in rats of gentamicin (gm) induced renal damage for the detection of apoptosis. Albino male rats (*rattus norvegicus*), weighing 40–50 g were divided into 6 groups; normal saline, orally treated mirazid 10 mg/kg, gm i.p) for 10 days, mz at 2.5, 5, and 10 mg/kg, per oral for 10 days with the same concentration of gm, mz administered concurrently with gm for 10 days. Gm treatment caused nephrotoxicity as evidenced by marked elevation in serum creatinine and urea (152.3 ± 8.6 mg/dl, 1.6 ± 0.12 mg/dl resp) when compared to the saline treated group. Gm cause significant increase in both sub g1 (apoptosis) and cd95, marker of apoptosis, when compared to saline control whereas mz give significant decrease in cd95 (fas and fas ligand detection) and sub g1 (apoptosis).

INTRODUCTION

Aminoglycosides continue to represent highly effective antimicrobial agents since their introduction about more than 50 years ago [1, 2]. Despite the introduction of highly potent, wide-spectrum antibiotics, aminoglycosides are still considered to be very important against many life-threatening infections especially against gram-negative bacterial infections [3]. The most widely used drug in this category is gentamicin (GM) [4]. A major complication of GM treatment is nephrotoxicity, accounting for 10–20% of all cases of acute renal failure (ARF) according to experimental results [5, 6]. Nephrotoxicity induced by GM is a complex phenomenon characterized by increase in plasma creatinine and urea levels and severe proximal renal tubular necrosis, followed by deterioration and renal failure. [1, 7]. GM, though, becomes complicated by the dual direct effect (proliferation and apoptosis) exerted by this aminoglycoside on cultured mesangial cells [8]. A closer look indicates, notwithstanding, that in the experimental conditions and GM dose used in this study, proliferation was stronger than apoptosis, and thus a net increase in cell number was observed upon GM treatment. Yet, it might be speculated that growing doses of GM would induce further cytotoxicity (i.e. apoptosis), so that at a certain dose (the equilibrium dose) proliferation might equal apoptosis.

Mirazid (MZ), a drug introduced to the local Egyptian market over the last decade, is prepared from (Arabian or Somali) myrrh,

an oleo-gum resin obtained from the stem of Commiphora molmol Engier and some other thorny trees in the family Bursaceae [9]. MZ has been marketed in Egypt as an antischistosomal drug, following reports of its activity against schistosome infections in mice [10] and humans [11, 12].

Over the last few years, MZ has also been used, apparently successfully, in the treatment of both fascioliasis and Schistosomiasis in Egypt, in humans [13-15]. Treatment of the infected mice with *S. mansoni* with Citrus reticulate extract and MZ ameliorates the levels of the hepatic antioxidants to a great extent. Lipid peroxides were greatly reduced. GSH, levels of Vit C, E and CAT activity were increased [16]. In this work, we certify that the antioxidant effect of MZ as a treatment for the toxicity of GM.

MATERIALS AND METHODOLOGY

Animals. Albino rats (*Rattus norvegicus*), consisting of 60 adult males, weighing from 50-60gm, from Theodor Bilharzias Research Institute, Ministry of Scientific research, Giza were housed in groups of 10 rats in a cage and maintained at the animal house of the Genetic Engineering and Biotechnology Research Institute (GERI), Sadat City, Sadat City University . They were kept in animal house under constant conditions of 25°C, and 12h light / dark cycle for one week before the experimental work. All experiments were carried out in accordance with protocol approved by the local experimental animal ethics committee.

Drugs. Mirazid (Commiphora extract) capsules were purchased from Pharco, Pharmaceuticals Company, Alexandria, Egypt. Each capsule containing 300 mg of purified *Commiphora* extract as soft gelatin forms. Mirazid was used in a freshly prepared 2% suspension with saline. Gentamicin (Gentamicin Sulphate) vials were purchased from Sigma company, U.S. A. Each vial contains 80 mg per ml of gentamicin sulphate.

Experiment Protocol. The experimental animals were divided randomly into six groups, ten rats in each as the following: group 1, given only standard pellet diet and tap water (Control); group 2, Mirazid (MZ) treated rats (10 mg/kg body weight (B.W)); group 3, gentamicin (GM) (100 mg/kg B.W) treated rats; group 4, rats treated with GM plus MZ (10 mg/kg body weight); group 5, rats treated with GM plus MZ (5 mg/kg B.W); group 6, rats treated with GM plus MZ (2.5 mg/kg B.W). MZ suspension dissolved in 1ml saline was given orally at concentrations (10, 5, 2.5, mg/kg B.w) to groups 2, 4, 5 and 6 respectively [12] through an intragastric feeding tube over a period of ten consecutive days. GM was given interiteneally (100 mg/kg B.W) over a period of ten consecutive days [17]. Rats were sacrificed 24 hours post the last injection.

Sample Collection and Biochemical Assays. After 24 hr from the last injection, rats were sacrificed, blood samples collected by cardiac puncture. Serum samples used for determination of serum urea, creatinine according to urease and Jaffé methods, respectively, these methods done by Beckman auto analyzer in Shebin El-kom teaching hospital. Kidney tissue was used for detection of cell cycle and also CD95 (apoptosis) by Flowcytometric method.

Detection of apoptosis with fas ligand (CD95) by flowcytometry [18], using FITC (Fluorescein Isothiocyanate) anti-human Fas (CD95), Clone DX2 (the solution is free of unconjugated FITC), Cata. No./ Size(305606/100 test) (Phosphate buffered solution (PBS), pH 7.2, containing 0.09% sodium azide and 0.2% (w/v) Bovine serum albumin (BSA) (origin USA) Isotype: Mouse IgG1, kappa and Fluorochrome Antibody Code No F 3056. Data was analyzed by flow cytometry (FACS caliber flow cytometer (Becton Dickinson, Sunnyvale, CA, USA) with a compact air cooled low power 15 mwatt Argon ion laser beam (488 nm) at the Mansoura Children Hospital. The average number of evaluated nuclei /specimen 20.000 and the number of nuclei scanned were 120/second [18, 19] using propidium iodide.

Cell cycle analysis by flowcytometric technique. The ability of flow cytometry to estimate cellular DNA content is based on the measurement of fluorescence from dyes which bind in a stoichiometric manner to DNA. Cellular DNA content is usually expressed as DNA ploidy representing the DNA content of cells under investigation as a ratio to that of normal control (diploid). As the DNA content is duplicated prior to cell division, mathematical models have been derived which can estimate the percentage of cells in different phases of the cell cycle (G0/1, S-phase, G2/M), for diploid and aneuploidy cycle, coefficient of variation (CV), Apoptosis %, DNA index (DI), diploid % and aneuploidy % [20], using ethanol (96-100%), propidium iodide (0.05µl/ml). Data analysis was conducted using DNA analysis program MODFIT (verity software house, Inc. Po Box 247,

Topsham, ME 04086 USA, version: 2.0 powers Mac with 131072 KB Registration No: 42000960827-16193213 Date made: 16-Sep., 1996). This was done by the same flowcytometer at Mansoura Children Hospital.

RESULT

Data represented in Table (1) show the results of serum urea and creatinine of control and experimental groups of rats treated with GM and MZ. Marked significant elevation ($p < 0.05$) in both urea and creatinine were observed in the group 3, GM intoxicated rats when compared with group 1, control rats. Activities of urea and creatinine in serum were significantly ($p < 0.05$) maintained at near normal levels in the group 4 rats that were pre treated with GM plus MZ (10mg/kg B.W) compared to group 3. Non significant ($p > 0.05$) in group 5 and 6, rats pre-treated with GM and MZ (5 and 2.5mg/kg B.W) respectively compared to group 3. Group 2, rats administered with MZ alone did not show any changes when compared to group 1, control group.

Kidney tissues data for CD95 are shown in Table (2) and Figure (1). Significant change on CD95 percent for all groups during experiment. The analysis showed high significant increase (P value < 0.05) in the amount of CD95 in group 3, rats treated with GM intoxication in CD95 compared to normal control. Also significant decrease ($P < 0.05$) in the amount of CD95 in group 4, rats that were pre-treated with GM and MZ (10 mg/kg B.W) compared to group 3 and non significant ($P > 0.05$) to group 5 and group 6 rats that were pre-treated with GM and MZ (5 and 2.5 mg/kg B.W) respectively compared to group 3. Group 2, rats administered with MZ alone show a significant decrease ($P < 0.05$) in the amount of CD95 when compared to group 1, control rats. Also it is evident from Table (2) and Figure (2) that a significant increased in sub G1 (P value < 0.05), significant decreased G2/M (P value < 0.05) and non significant change in S phase and G0/1 in group 3, rats treated with GM intoxication compared to normal control group. Significant decreased (P value < 0.05) in sub G1, G0/1, S, G2 /M in group 4, rats pre-treated with GM plus MZ (10mg /kg.B.W) compared to group 3. While, it was obvious that significant decreased (P value < 0.05) in G2/M and non significant change (P value > 0.05) in sub G1, G0/1 and S phases in group 5 rats pre-treated with GM plus MZ (5mg /kg.B.W) compared to group 3. Also, it was clear that there is significant increased (P value < 0.05) in G0/1 and S phases in group 6, rats pre-treated with GM plus MZ (2.5 mg /kg.B.W) and non significant change (P value > 0.05) in G2/M and sub G1 phases compared with group 3. Group 2, rats administered with MZ alone show significant decrease in the amount in sub G1, G0/1, S, G2/M with a (P value < 0.05) compared to group 1, control rats.

DISCUSSION:

Results of the present study showed that i.p administration of 100 mg/kg body weight of GM daily for 10 days led to a significant increase in serum urea and creatinine levels as compared to those of the control group. The elevation in blood urea and creatinine levels in GM treated rats is considered as significant markers of renal dysfunction (glomerular damage marker). These findings are in agreement with reports of the authors [21-25]. However our results are in contrast with that of Guidet and Shah 1989, who did not observe any change in these

parameters following treatment with gentamicin (100 mg/ kg/day) for 5 consecutive days [26].

Table.1: Effect of GM different concentrations of MZ on serum urea and creatinine among different studied groups.

Analytical parameter	Group I (n=10)		Group II (n=10)		Group III (n=10)		Group IV (n=10)		Group V (n=10)		Group VI (n=10)		P-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Urea mg/dl	57.3#	2.5	57.3	3.1	152.3*#	8.6	59*	1	65*	4.6	100.3*	2.5	<0.05
Creatinine mg/dl	0.3#	0.03	0.4	0.01	1.6*#	0.2	0.5*	0.02	0.5*	0.05	0.9*	0.05	

Table 2: Effect of GM different concentrations of MZ on kidney Cell cycle stages among different studied groups

Analytical parameters	Group I (n=10)		Group II (n=10)		Group III (n=10)		Group IV (n=10)		Group V (n=10)		Group VI (n=10)		P-Value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
SubG1(Apoptosis)%	32.8#	0.8	13.2	0.2	41.2*#	0.2	14.3*	0.3	36.1*	0.1	36.6*	0.6	<0.05
G0/1 %	22.7#	0.7	6.2	0.2	24.7*#	0.7	3.2*	0.2	23.2*	0.2	35.5*	0.5	
S%	12.4#	0.4	2.6	0.5	11.6*#	0.6	1.02*	0.02	12.2*	0.2	20.7*	0.7	
G2/M%	12.3#	0.6	0.8	0.06	8.6*#	0.6	0.2*	0.05	4.6*	0.6	8.3*	0.6	
CD95	40.5#	9.1	22.5	15.95	54.6*#	14.1	32.6*	8.2	44.6*	21.7	46.8*	11.6	

Group I: Control, Group II: Rat treated with MZ (10mg/kg B.W), Group III: Rat treated with GM (100mg/kg B.W), Group IV: Rat treated with GM + MZ (100mg/kg B.W and 10mg/kg B.W respectively) Group V: Rat treated with GM + MZ (100mg/kg B.W and 5mg/kg B.W respectively) Group VI: Rat treated with GM + MZ (100mg/kg B.W and 2.5mg/kg B.W respect)

G0: gape 0 phase , g1: gape one phase, g2: gape two phase, s: synthesis phase, m: mitosis phase, #: comparisons are made between group i & group iii, (*): comparisons are made between group iii & group iv, v, vi and p: is considered significant when < 0.05.

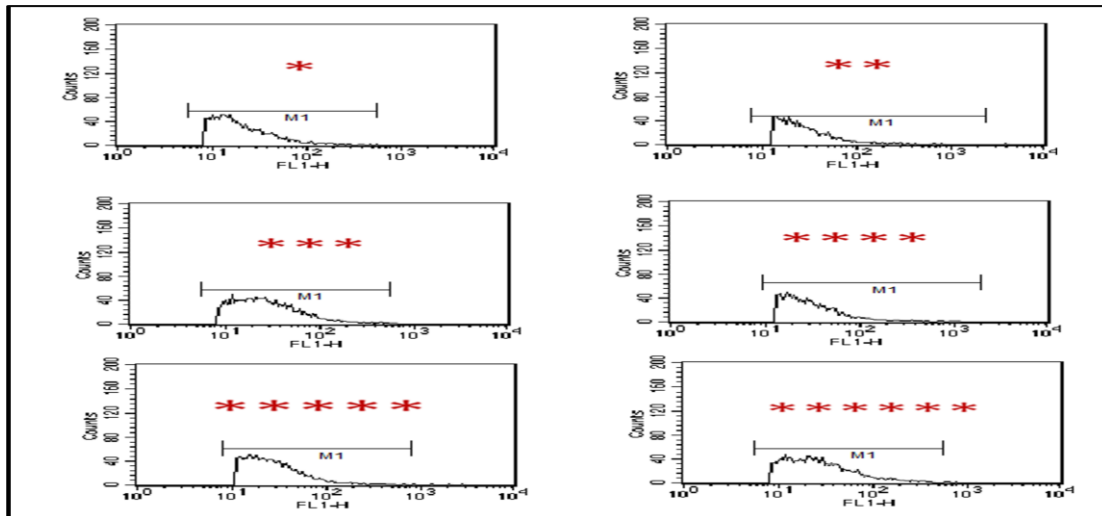


Figure (1): effect of gm and mz efficiency on cd95; (*) group 1,(**) group 2, (***) group 3, (****) group 4, (*****) group 5 , (*****) group 6.

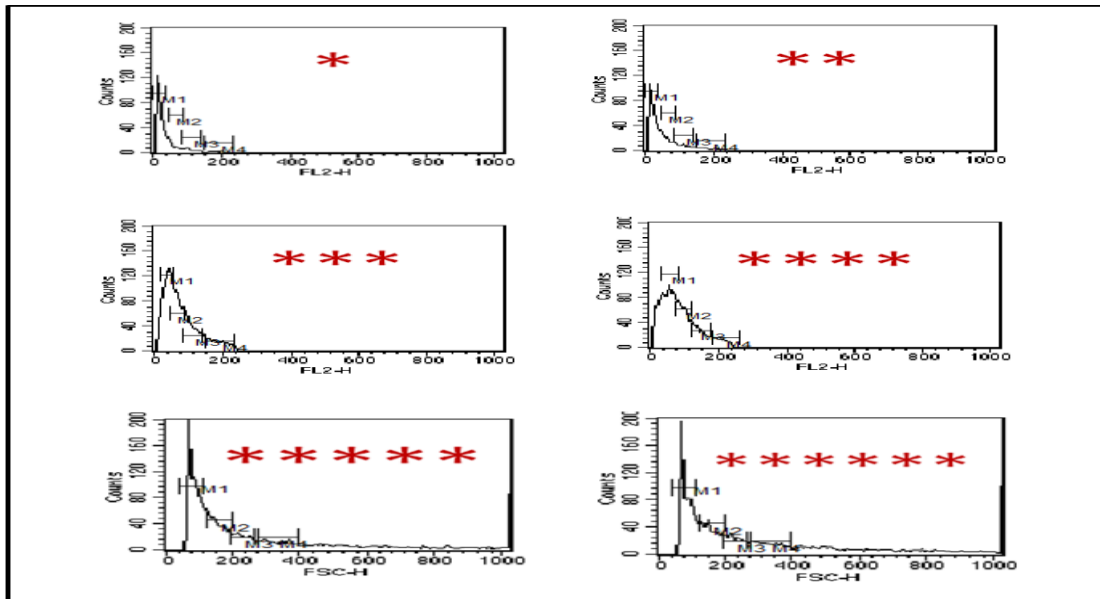


Figure (2): Effect of gm and mz efficiency on cell cycle; (*) group 1, (**) group 2, (***) group 3, (****) group 4, (***** group 5, (*****) group 6.

In our study, a dose of 100 mg/kg/day gentamicin for 10 days was administered to rats as opposed to 5-day treatment in the study of Guidet and Shah, 1989 [26]. Our results are in line with the observation that a decline in glomerular filtration rate and increase in serum creatinine and urea are not usually apparent until 10 days of treatment with GM ([27]. Our results showed that there is a significant decrease in serum urea and creatinine level in rats that are orally treated with MZ (10 mg/kg body weight) daily for 10 days as compared to those of GM-treated group, and shows no significant changes in urea and creatinine when compared to control group.

Also we showed that there are different changes in the level of urea and creatinine in groups treated with MZ and GM, as there are significant decrease in urea and creatinine level at high concentration of MZ (10 mg/kg .B.W) better than at concentration 5 mg/kg body wt, and non significant decrease at concentration 2.5 mg/kg B.W. One of the main side effects of GM is that it increased apoptosis in mesangial cells in vivo and also in vitro. This apoptosis seems to be mediated by an elevation in ROS production (at least, O_2^-). Simultaneously, GM induces mesangial cell proliferation. In addition, GM treatment in vivo also induced glomerular cell proliferation and apoptosis (mainly in the mesangium). GM-induced proliferation and apoptosis either in vitro or in vivo are associated with an early increase in the proapoptotic protein Bax and a delayed increase in the survival-promoting protein Bcl-2. We propose that the simultaneous occurrence of cellular proliferation and apoptosis may be a mechanism regulating glomerular cell number after acute treatment with GM [28]. In this study apoptosis detected by detection of Fas and Fas ligand, which is detected by CD95. CD95 (APO-1/Fas) is a member of the death receptor family, a subfamily of the TNF-R superfamily [29]. Cross linking of CD95 with its natural ligand CD95L (CD178) [30] or with agonistic antibodies such as anti-APO-1 induces apoptosis in sensitive cells [31]. In addition, triggering of CD95 induces a number of non-apoptotic activities [32, 33]. The death-inducing signaling

complex (DISC) is formed within seconds after CD95 stimulation [34]. The DISC consists of oligomerized CD95, the adaptor molecule FADD, two isoforms of procaspase-8 (procaspase-8/a and procaspase-8/b), procaspase-10. Our Kidney tissue results show significant change on CD95 percent for all groups during experimental. The analysis showed high significant increase in rats treated with GM intoxication in CD95 ($P < 0.05$) compared to normal control. Also significant decrease in the amount of CD95 ($P < 0.05$) rats treated with GM and MZ (10 mg/kg B.W) and non significant ($P > 0.05$) to rats treated with GM and MZ (5 and 2.5 mg/kg B.W) respectively compared to GM-treated rats group. Rats administered MZ alone show significant decrease in the amount of CD95 ($P < 0.05$) when compared to normal control, this is in agreement with Watanabe-Fukunaga et al., 1992, when studied the interaction between Fas receptor and FasL, that plays an essential role in the maintenance of immunological tolerance which leads to immune dysfunction in association with particular genetic backgrounds includes glomerulonephritis ([35]. These results showed increase in apoptosis due to GM intake, this is may be due to increase production of ROS, which decreased by treatment of GM by MZ, as MZ lowers the production of ROS. Also our results indicated that best concentration of MZ is 10 mg/kg body weight, which ameliorates the increase in apoptosis due to GM intake.

The results that obtained in the present study indicate that GM treatment resulted in increased level of renal DNA damage as shown in our results as GM treatment affects the cell cycle. This finding is confirmed by the result of Alkahtani *et al.*, 2009, who reported that GM induced apoptosis and DNA fragmentation in renal DNA [36]. Also, they illustrated that DNA damage by GM is due to production of ROS, also Servais et al., 2005, reported that GM accumulates in lysosomes and induced apoptosis and DNA damage in kidney proximal tubules and renal cell cycle [37] as results showed that significant increase in sub G1 (apoptosis) ($P < 0.05$), non significant change in S phase and

G0/1 and significant decreased G2/M ($P < 0.05$) in rats treated with GM intoxication compared to normal control group, this indicates that GM treatment induce apoptosis and stop the synthesis phase especially DNA synthesis, also it decreases the amount of mitosis compared to normal cells, this is in agreement with Alkahtani *et al.*, 2009, who reported that GM induced apoptosis and DNA fragmentation in renal DNA, so it affected the synthesis phase and stop normal progression of the cell [36]. Also, they illustrated that DNA damage by GM is due to production of ROS, as GM is accumulated in renal tubules and then promotes cellular damage, by multiple mechanisms including oxidative stress, DNA damage and apoptosis. Also, Servais *et al.*, 2005, reported that GM accumulates in lysosomes induces apoptosis and DNA damage in kidney proximal tubules and renal cell cycle (37). In GM +MZ 100mg/10mg group it was significant decreased in sub G1, G0/1, S, G2 /M ($P < 0.05$) as compared to GM treated rats, this indicates that MZ improves the amount of apoptosis better than GM group, and also give significant decrease in synthesis of DNA and mitosis of the cell compared to GM-treated group. In group that administered MZ alone show significant decrease in the amount in sub G1, G0/1, S, G2/M ($P < 0.05$) compared to control group, this indicate that MZ decrease the amount of apoptosis and this concentration of MZ (10 mg /kg B.W) is better than 5mg and 2.5 mg/kg B.W in improvement of GM toxicity towards the cell and DNA, as it was obvious that significant decrease in G2/M ($P < 0.05$) increased and non significant change in sub G1, G0/1 and S phases in rats treated with GM plus MZ 5mg /kg B.W. GM ($P < 0.05$) compared to group 3. Also, it was clear that there was significant increased ($P < 0.05$) in G0/1 and S phases in group 6, rats treated with GM plus MZ 2.5 mg /kg B.W. Non significant change ($P > 0.05$) in G2/M and sub G1 phases compared with GM- treated group.

CONCLUSION

This study provides scientific evidence of the nephroprotective effects of orally administered mirazid in a albino rat model employing gentamicin as a toxicant that directly induces renal damage. It further proposes that observed protective effects of mirazid in gentamicin nephrotoxicity could be attributed to its well-known antioxidant potential, and the best renoprotective concentration of mirazid is 10 mg/kg body weight and not less than this dose for best free radical scavenger effect of mirazid.

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