World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Cytotoxicity and apoptosis of glioblastoma multiforme cell line by arginine deiminase purified from a higher productive isolation *Enterococcus faecium M1*

Nada Z. Mahdi*[1], Shatha S. Al-Tahan[2] and Nahi Y. Yaseen[3]

[1]Department of Biology, College of Science, Al-Mustansiriya University, Baghdad, Iraq
[2]Department of Biology, College of Science, Baghdad University, Baghdad, Iraq
[3]Director General of Iraqi Cancer and Medical Genetic Researches, Al-Mustansiriya University, Baghdad, Iraq

Received: 17-06-2016 / Revised: 19-07-2016 / Accepted: 22-07-2016 / Published: 31-07-2016

ABSTRACT

Purified arginine deiminase from *Enterococcus faecium* M1 isolate is the strongest cancer treatment enzyme due to its activity and stability in different environmental conditions. The cytotoxicity of ADI to glioblastoma multiforme (ANG) cancer cell line and rat embryo fibroblast(REF) normal cell line for (24, 48 and 72h) were estimated, the inhibition rate(IR) increased with raising of ADI concentration and incubation period for ANG cell line but these results were opposite for REF cell line that IR decreased with increment of incubation period. Different concentrations (2-1000ng) of enzyme were used, the significant once were between (30-100ng) that they were safe for most cells of REF normal cell line but they inhibited the large numbers of glioblastoma cells, that the IC50 of ADI was 37ng/ml for this cancer cell line during 72h of incubation time and the IR reached to 75.4% after 48h incubation with 100ng/ml of enzyme. The ability of ADI to produce intrinsic mitochondrial apoptosis effect on ANG and REF cell lines was investigated, the results showed that the main reason of cell cytotoxicity was the induction of apoptosis process by ADI enzyme and they were compatible to the results of cytotoxicity test. We concluded that ANG cancer cell line could not produce arginine amino acid thus it was highly sensitive to arginine deprivation by the robust activity of arginine deiminase enzyme, but it was safe for REF normal cell line could produce arginine during the incubation time with enzyme.

Keywords: Cytotoxicity**,** Glioblastoma multiforme**,** Arginine deiminase, *E. faecium*

INTRODUCTION

Glyoblastoma multiforme (GBM) is the most common primary human malignant brain tumor and among the most lethal of all cancers [1]. Despite advances in surgical management, radiotherapy, and development of temozolomide, the median survival for patients is 12–16 months [2,3]. The discovery of anticancer drugs remains a highly challenging endeavor and cancer a hard-to-cure disease [4]. Arginine is required by all tissues in the human body for protein synthesis, and by some tissues for specialized needs. Tumour cells have a high requirement for arginine by enhances tumour growth [5]. Recently in Iraq there is a terrible number of unpublished cancer cases. The idea of overloading cancer cells with amino acids they don't want, and starving them of ones they do, has since proven to be a viable approach to cancer treatment [6]. This treatment attempts to inhibit the growth of, or necrotize the cancer cells by decomposing the nourishment required by the cancer cells and thus by shutting out the source of nourishment from the reach of the cancer cells. Arginine deprivation affects glyoblastoma cell adhesion and could inhibit the invasion process of highly malignant brain tumors [7]. Arginine deiminase regarded as arginine-depleting enzyme and being a potential anticancer agent [8]. Arginine deiminase isolated from a higher productive locally isolated strain *Enterococcus faecium* M1 is a very potent, stable and effective enzyme when used as a cancer theraputic agent [9]. The aim of this study was designed to investigate the cytotoxicity and apoptosis effect of arginine deiminase enzyme purified from the higher productive *Enterococcus faecium* M1 isolation on most lethal Glioblastoma multiforme cancer cell line and compare it with the effect of enzyme on Rat embrio fibroblast normal cell line in order to use this robust enzyme as a treatment for the highly malignant brain tumors in the future.

Corresponding Author Address:** Dr. Nada Z. Mahdi, Department of Biology, College of Science, Al-Mustansiriya University, Baghdad, Iraq; E-mail: **nada.zeki @ yahoo.co.uk

MATERIALS AND METHODS

Purified arginine deiminase enzyme*:* this enzyme was obtained from [9].

Cytotoxicity of glioblastoma and rat embrio fibroblast cell lines by ADI enzyme assay. The effect of purified arginine deiminase (ADI) enzyme on REF and ANG cell lines was determined. In the first step, five concentrations (200- 1000 ng/ml) of ADI were used and compared with controls. In the second and third step lower concentrations (10 to 100 ng/ml) and (2-8ng/ml) were used.

Tissue culture cell line media (for cytotoxicity assay)*:* Rosswell Park Memorial Institute -1640 culture medium with or without Fetal calf serum.

Cell lines used in the study:

Ahmed Nahi Glioblastoma Multiforme (ANG) cell line: It was provided by Iraqi Center for Cancer and Medical Genetic Researches (ICCMGR) at passage 62-65 and the cells were growen on RPMI-1640 with 10% FCS. This human tumour culture was derived from a human cerebral glioblastoma multiform (GBM) obtained from a 72 year old Iraqi male who underwent surgery for intracranial tumour.

Rat Embryo Fibroblast (REF) cell line: It was kindly supplied by ICCMGR at Passage 63.This normal murine cell line were a mixture of fibroblastic and epithelial cells with normal chromosomal picture, tumorigenicity test of this cell line showed no tumor growth in injected rats during three months of monitoring.

In vitro cytotoxicity assay: Preparation and Maintenance of the cell lines has been done according to [10]**.**

Viable Cell Counting of control cell lines contained more than 95% cell viability of a confluent monolayer and It was performed according to [11].

Cytotoxicity assay: Cytotoxicity effect of various concentration of arginine deiminase enzyme on proliferation of the adherent cells in 96-well microtiter plate has been performed according to [11] method as follows: The purified arginine deiminase was diluted (when the cytotoxicity test was done) with serum free medium in a manner of concentrations. A set of five concentrations (1000, 800, 600, 400 and 200 nanogram/ml), then another set from (100 to10 ng/ml) and the last four concentrations (8, 6, 4 and 2 ng/ml) of ADI enzyme, were concerned, the exposure time assay were (24, 48 and 72 hours) for each concentration, the remaining steps of Cytotoxicity assay were completed according to [10]. The optical densities (O.D) at wave length 492 nm of 3 cell lines after 24, 48 and 72 hour exposure to all concentrations of (ADI) enzyme were compared to those of their controls (ADI-free treated groups). The change in O.D was referred as the percentages of Inhibitory Rate (IR) were calculated [11].

Intrinsic Mitochondrial apoptotic effect on cell-line. The principle of this assay depends on the disruption of the mitochondrial transmembrane potential, which is one of the earliest intracellular events that occur following the induction of apoptosis. The dye of Mitocapture reagent kit will be concentrated in the mitochondria of healthy cells, thereby creating red fluorescent region within the cell, while dispersed in apoptotic cells; these cells will not have red aggregates in the mitochondria, rather the entire cell will appear green. The assay can be carried out according to [13]. Dye reagent was aspirated and the chambers were removed from the slide, then the slide was examined under fluorescent microscope. The number of healthy cells (red fluorescent region), and apoptotic cells (green region) were counted in five fields and the mean of them were calculated then the percent of apoptosis was calculated from the following equation:- Apoptosis % = (No. of apoptotic cells/Total No. of cells) × 100. The final apoptosis % of treated groups = Apoptosis % of treated groups - Apoptosis % of their controls.

Statistical Analysis*:* The Statistical Analysis System [14] was used to determine the effect of different factors (Concentration and Time) on inhibition rate of different cell lines. LSD test was used to significant compare between the means of this study.

RESULTS AND DISCUSSION

Cytotoxic Effect of ADI concentrations on cell lines: The results showed a variable effect of treatments on the cell lines proliferation among the three periods of incubation. When high concentrations of enzyme(200-1000ng) used as showed in (table 1), all treated groups of glioblastoma(ANG) cell line with ADI, exhibited a significant toxic effect started from the concentration 200ng/ml till the concentration of 1µgm/ml. Statistical analysis of differences (Mann-Whitney test) between each concentration and its control group showed that all concentrations of ADI revealed significant differences at $P \leq 0.05$ in comparison with control and LSD value between concentrations and times.

The results explains that IR% values were between (74-85%) at (200- 1000 ng/ml respectively) of

enzyme after 24h of incubation, these results indicates that the sensitivity level of ANG cell line to ADI may be due to a higher requirement of arginine to produce important metabolites for growth and proliferation of ANG cell line depending on control group which had very low inhibition rate values. The effect of ADI on ANG cell line after 48hr and 72 hr. of exposure was more toxic than 24 h exposure with high level of inhibition rate for all concentrations, the IC90 of ADI was 200ng after 72hr of incubation.

The results mean that ANG cell line was very sensitive to ADI enzyme at these concentrations because this cancer cell line is not able to synthesize arginine that lead to deprivation of this amino acid in the presence of ADI enzyme in growth medium and inhibit the production of essential proteins and metabolic products for growth and proliferation. The cytotoxicity effect of ADI high concentrations for cancer cell line may be partly due to pH effectiveness of NH_3 produced as a product of enzyme activity during the time and due to accumulation of cell line metabolic products with the time which increased the pH of culture media, because the color converted from neutral orange to pink alkaline not suitable value (in the presence of phenol red indicator) for the activity of enzymes and proteins, while this effect was diminished as concentration dropped.

These results describes that arginine decreases with the time leading to induce more dead cell ratios and this probability raised with time, that explain this enzyme was very toxic to ANG cell with these efficient concentrations. Syed *et al*., (2013) found that 1µg amount of ADI enzyme inhibited glioblastoma cultures by 78% IR after 24h of incubation and they mentioned that ADI causes arginine auxotrophy, either by nutritional starvation or induces adaptive transcriptional upregulation of *ASS1*(arginino succinate synthetase gene) and *ASL*(arginino succinate lyase gene) in glioblastoma multiforme cultures. Many studies reported that arginine deiminase has cytotoxic effect at low concentrations toward many cell lines because ADI induces G0/G1-phase arrest then sub-G1 accumulation [16, 17, 18].

All treated groups IR% of REF cell line began with low level of cytotoxicity then gradually decreased whereas vice versa about control groups which were began with lower IR% level then increasing occurred gradually according to time, these results indicated that arginine was decreased in culture medium during the first time of incubation in treated normal REF cell line by arginine deiminase enzyme which lead to starvation of some cells to this amino acid then to cell cycle arrest and inhibited the proliferation of them, but during the time the cells could synthesize the needed amount of arginine by induction the production of two enzymes argininosuccinate synthetase and argininosuccinate lyase because their expression, localization and regulation differs significantly depending on the tissue specific needs for arginine, thus the arrested cells were retained their ability in proliferation and decreasing the I.R. during the time, the best and significant con. of ADI was 200ng/ml after 72h incubation which revealed a slight cytotoxicity effect on the viability of (REF) normal cell line but it was very toxic for ANG cancer cell line. About control groups the I.R. enhanced with time due to accumulation of metabolites and deprivation of nutrients in cell line culture medium. In the other hand this normal cell line can tolerate the low difference of pH value the optimum pH for cell growth varies among different cell strains [15]. These data described the lowest IR to REF cell line produced by ADI after 72h of incubation which confirmed the ability to produce arginine increased with the time of incubation lead to gradually diminish of the cytotoxicity effect to this normal cell line and exit the cells from stationary stage then proliferation of them. [19] found that arginine deprived normal cells will have become quiescent but soon recover on restitution of the missing nutrient, whereas tumor cells in cycle can be hit by low doses of cycle-dependent cytotoxic drugs.

Effect of low ADI concentrations on ANG and REF cell lines: The high toxic effect of the previous concentrations lead to use lower ADI concentrations (10-100 ng/ml) to determine the lower significant con. of enzyme which will be toxic for ANG but safe for REF cell line. 1. The cytotoxic effect of ADI against ANG cell line after 24h incubation with ten concentrations (10-100ng) is described in table (2), the IC50 of ADI was 60ng/ml after 48h of incubation and IR significantly expanded with increasing the concentrations and reached to 75.4% at 100ng ADI. That prove this cell line was very sensitive to deprivation of enzyme. When the incubation time increased to 72h, the cytotoxic effect of the ten concentrations significantly raised to higher cytotoxic effects, these data indicate that the IC50 of enzyme is 37ng/ml and the IR reached to 80.6% when 100ng of enzyme used after 72h. of incubation with significant differences at $P \leq 0.05$ in comparison with control and LSD value between concentrations and times. The results identified the robust cytotoxic ability of ADI enzyme to inhibit the proliferation of ANG cell line especially after 72h of incubation, which mean ANG couldn't express (ASS) enzyme the rate-limiting enzyme for the biosynthesis of arginine from citrulline. ASS-

negative cancer cells require arginine from extracellular sources for growth and survival, thus the absence of arginine in the presence of ADI in culture media components lead to suffering the cells from starvation then to death. Some authors [20] found that melanoma and hepatocellular carcinoma (HCC) are auxotrophic for arginine, because they do not express (ASS) enzyme thus they die, because of arginine starvation; where as normal cells which express ASS were able to survive.

Table 2 showed all treated groups IR% of REF cell line, it revealed a slight effect on the viability of normal (REF) cell line. The IR% of the first three concentrations (10- 30 ng/ml) were 0% and other seven concentrations(40-100ng) had very low cytotoxic effect which will be diminished during the time, whereas vice versa about control groups which were began with low IR% level then increasing occurred gradually according to time. These results indicated that the enzyme had a very little cytotoxic effect on REF normal cell line with those ten concentrations (which were less than the effect of first five higher concentrations (200-1000ng/ml). The results confirmed the ability of REF normal cell line to synthesize arginine by ASS and ASL enzymes induced during the time. Statistical analysis showed no significant differences between each concentration and its control group showed non-significant difference at P>0.05 in comparison with control but they were significant (LSD) value between different ADI concentrations and times.

Effect of very low concentrations (2-8 ng/ml) of purified arginine deiminase on REF and RD cell lines: In order to know the cytotoxicity of ADI at lower concentrations on the proliferation of two studied cell lines, four amounts were used (2- 8 ng/ml). In (table 3) results indicate that the cytotoxicity of ANG cell line was gradually deprived with decreasing of ADI con, and during the time, which mean that the enzyme had a concentration and time depending effect, Non-significant differences at P≤ 0.05 between inhibition rates in comparison with control but they were significant LSD values between concentrations and times. Inhibition rate reached to 22.1% when 8ng/ml of ADI used, but it was not sufficient to inhibit ANG cell line like higher concentration (100ng/ml) which was the best amount of enzyme to inhibit about 80.6% of ANG cell line. Table 3 showed all treated groups IR% of REF cell line, the IR values were 0% with all enzyme concentrations. From the results of three groups of concentrations used it can be concluded that all concentrations of arginine deiminase possess a cytotoxic effect toward the cancer cell lines but the severity of cytotoxicity was varied between enzyme quantities and two cell lines.

Intrinsic Mitochondrial apoptotic effect of arginine deiminase on cell-lines: The significant concentrations of ADI caused inhibitory effect on cell proliferation, particularly in ANG tumor cell line and in normal REF cell line were chosen to investigate their ability to cause apoptotic effect (table 4).The apoptotic cells (green regions) and healthy cells (red regions) of both ADI-treated and untreated (control) cells have been illustrated and computed under the fluorescent microscope using cationic fluorescence dye (mitocapture reagent). The percentage of apoptosis was calculated as a mean of five fields for every test which was clearly showed apoptosis induction in treated groups in comparison to their controls. Arginine deiminase treatment caused significant induction of apoptosis in ANG cell lines. IC50 and other important enzyme quantities that produced high percentage of inhibition rate for cancer cell lines were chosen here. The results of apoptosis test were compared with the results of cytotoxic test. The results in this study showed the ability of ADI enzyme to induce (Intrinsic mitochondrial) apoptosis in ANG tumor cell line, the number of apoptotic cells were ADI dose and time dependent which are directly proportional to each other. The apoptosis ratio in ANG cell line (table 4) and figures (3, 5, 7 and 8) showed that the main reason of cell cytotoxicity was inducing the mitochondrial apoptosis process by different concentrations of ADI enzyme with high significant differences at $P \leq 0.05$ in comparison to control. Therefore, apoptotic effect of ADI to cancer cell lines may be due to its ability to cause deprivation of arginine, growth factors and other stimulatory survival signals leading to cause the production of anti-apoptotic members of the Bcl-2 family and survival facter deprivation then activate the Intrinsic apoptotic pathway [21].

The apoptotic effect increased with increasing incubation time and ADI concentration, this indicates that the enzyme is potent with low amounts in death program of ANG cell line, but it was safe with REF cell line when the same concentrations were used as described in (table 4) and in figures (4 and 6) with non-significant differences at $P \leq 0.05$ in comparison to control. Many researchers[22] found that the killing of cancer cell lines by arginine deprivation is also selective because deprived normal cells will have become quiescent but soon recover on restitution of the missing nutrient, whereas tumor cells in cycle can be hit by low doses of cycle-dependent cytotoxic drugs. The results described in (table 4) showed that the ADI enzyme had very low cytotoxicity for REF cell line which increased with

increasing /ml of ADI used, the apoptosis ratio of the amount of enzyme. When 400 REF cell line was 3% during 72h of incubation, this may be due to inability of these cells to recover 100% of cells in the presence of high amount of ADI. Many researchers were described the tumor cells (in the presence of ADI enzyme)in many cases as: 1- Lost the ability to make arginine from citrulline[23,24]. 2- Stay in cycle instead of moving out of it into G1 or G0 [25]. Die within 3-4 days in many cases, probably as a result of trying to cycle when insufficiently resourced [22, 25]. Because they stay in cycle, they continue to be suitable targets for cell cycle-dependent cytotoxic agents [26], where as normal cells become quiescent and relatively resistant. 5- As long as arginine is reduced to the micromolar level, many cancer cells will die, while normal cells recover from quiescence when enzyme is removed [22].

Table1: Cytotoxic effect(Inhibition Rate%) of ADI high concentrations on REF and ANG cell lines after different times of incubation

Cytotoxic effect (Inhibition Rate%)						
REF				**ANG**		
Inc. time	**24 hrs**	**48hrs**	**72hrs**	**24hrs**	**48hrs**	**72hrs**
Control range	0.3-0.9	1.2-2.4	2.5-3.3	1-1.2	1.7-2.1	3-3.2
Test ADI con. ng/ml						
200	18.2	9.0	6	74	85	90
400	17.7	9.6	6.7	77	88.6	91.1
600	19.7	10.2	6.2	81	90	90.4
800	20.3	11	7.8	84	92	91.5
1000	22.3	11.6	8	85	92.7	92.8

Table 2:Cytotoxic effect(Inhibition Rate%) of ADI lower concentrations on REF and ANG cell lines after different times of incubation

Cytotoxic effect (Inhibition Rate %)						
REF				**ANG**		
Inc. time	**24 hrs**	**48hrs**	**72hrs**	**24hrs**	**48hrs**	**72hrs**
Control range	0-0.6	1.1-2.4	2.9-3.5	0.4-0.9	2.6-3	3.2-3.8
Test ADI con. ng/ml						
10	0	0	0	5.2	13	26.4
20	0	0	0	9.7	22	34
30	0	1.8	0	7.3	36	42
40	2.1	1.6	0	12	30	53.3
50	4.4	4.1	0	18.4	44	55.8
60	3.2	3.7	1.1	22.1	50.8	61.9
70	5	4.9	3.4	28.5	62	70.4
80	5.7	5	2.5	27.6	60.9	68.3
90	6.1	5.3	4.8	33	72.1	77.3
100	6.8	5.5	5	41	75.4	80.6

Table3: Cytotoxic effect(Inhibition Rate%) of very low concentrations of ADI on REF and ANG cell lines after different times of incubation

	(Cytotoxic effect) Inhibition Rate%					
Cell line	**REF**			**ANG**		
Inc. time	**24 hrs**	**48hrs**	**72hrs**	**24hrs**	**48hrs**	**72hrs**
Control range	**0-0.1**	**1.1-2**	**2.3-3.1**	**0-0.3**	**1.3-2.2**	**2.4-2.9**
ADI con. ng/ml						
2	**0**	**0**	**0**	**0**	**5.6**	**7.4**
4	**0**	**0**	**0**	**3.6**	**7.8**	**11.5**
6	**0**	**0**	**0**	**3**	**9.1**	**17.8**
8	**0**	**0**	**0**	**4.5**	**13.4**	**22.1**

Table (4): Mitochondrial intrinsic apoptosis ratio of cell lines induced by significant concentrations of ADI enzyme during incubation times.

ADI concentration (Nanogram/ml)	Apoptosis percent and incubation time used at 37C°	
	ANG	REF
37	50% during 72h	0% during 72h
100	75.4% during 48h	5.5% during 48h
200	93% during 72h	6% during 72h
400	100% during 72h	6.7% during 72h

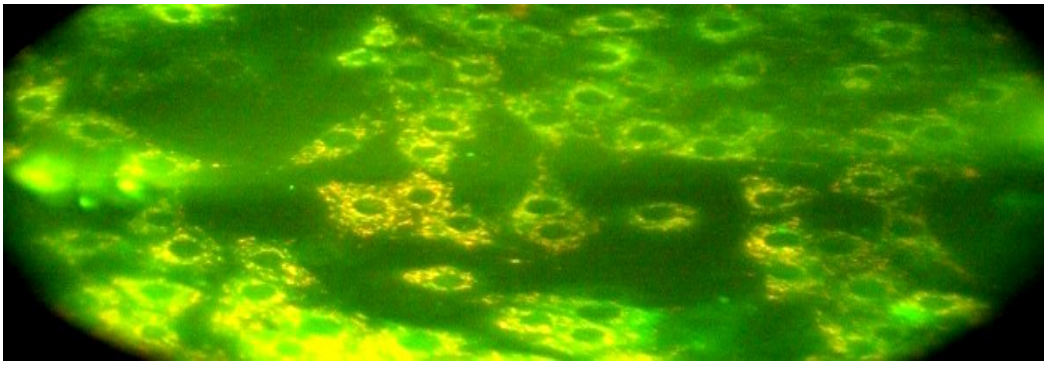

Figure 1: Non treated(ANG cell line) after 72h of incubation, presented healthy red cytoplasmic cells. M.p. power:400X using cationic fluorescence dye(for all the following figures).

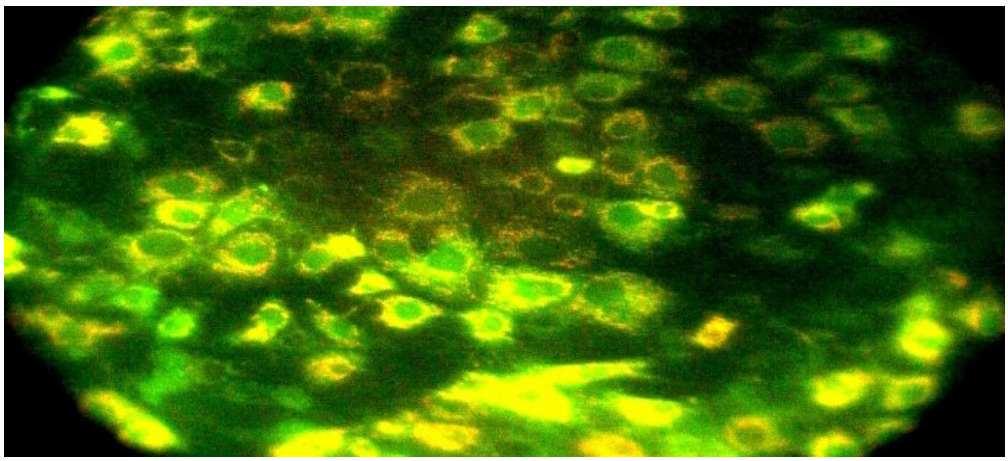

Figure 2: Untreated REF cell line Shows 0% apoptosis after 72h ofincubation presented healthy cells. Magnification power: 400X, using cationic fluorescence dye.

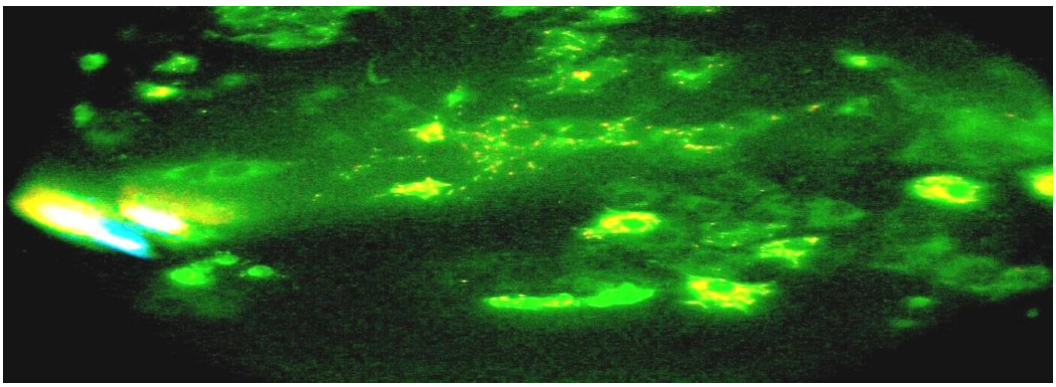

Figure 3: Apoptosis (50%) of ANG by 37 ng/ml of ADI incubated for 72 h displayed dispersed cells, some are healthy (fluorescent cytoplasmic reigns) and the others are (green shrinking) dead cells

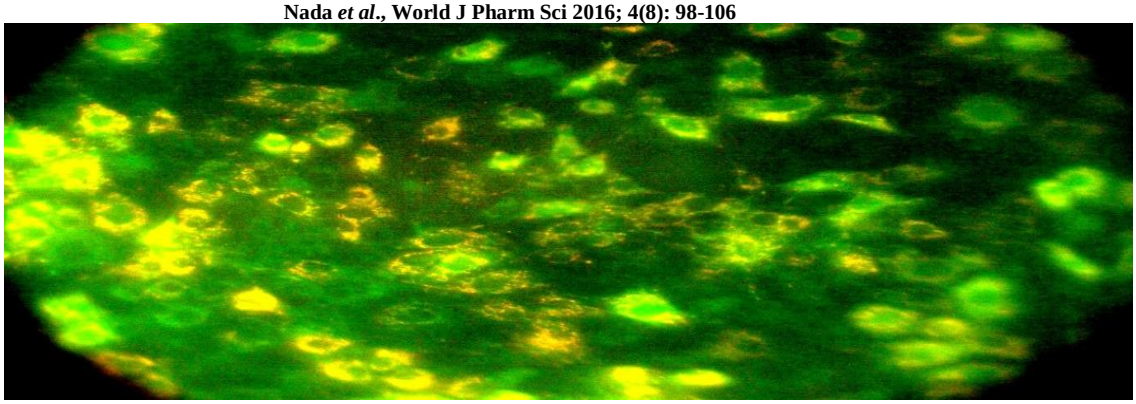

Figure 4: REF cell line Shows 5.5% apoptosis when treated with 100ng of ADI after72h ofincubation presented healthy cells for most of them.

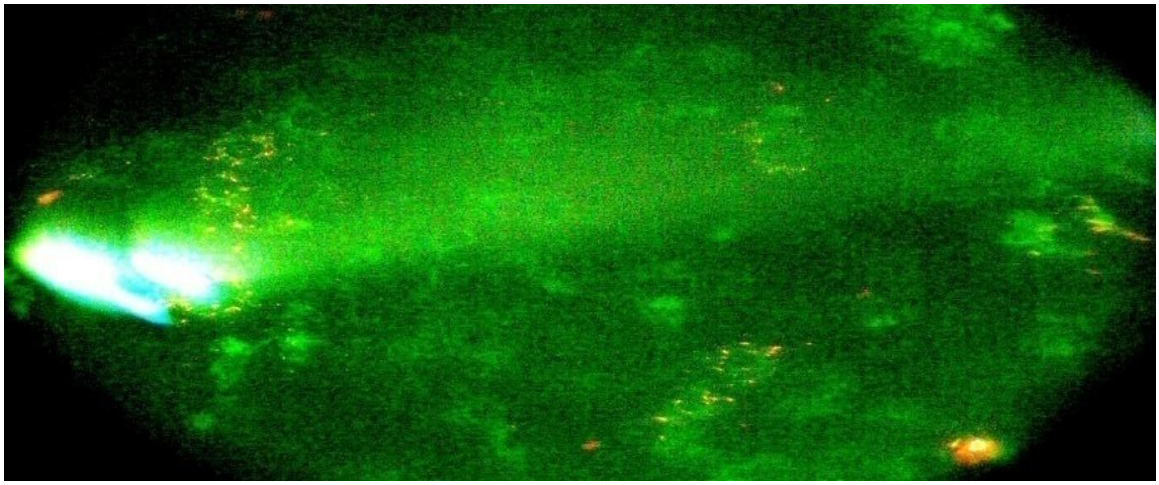

Figure 5: ANG cell line Shows 75.4% apoptosis when treated with 100ng of ADI after72h of incubation with ADI enzyme, presented green dead dispersed cells for most of them

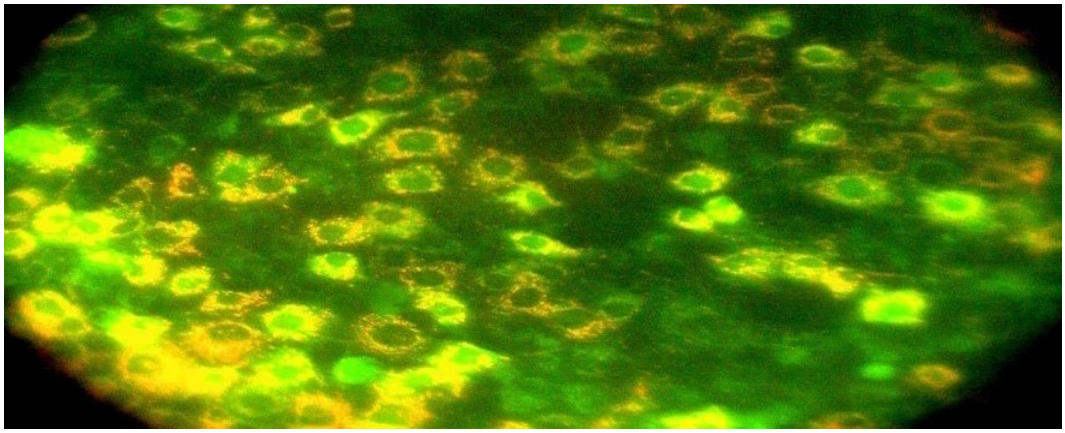

Figure 6: REF cell line Shows 6.7% apoptosis when treated with 400ng of ADI after72h ofincubation presented healthy cells for most of them.

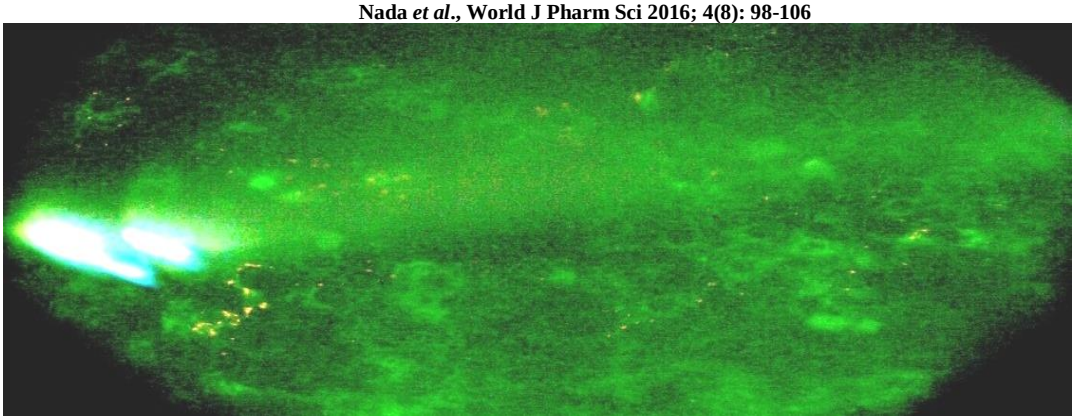

Figure 7: presented 93% apoptosis of ANG cell line after adding 200 ng ADI for 72h of incubation presented dispersed dead shrinking cells with their exudes and a few number of healthy red cytoplasmic cells.

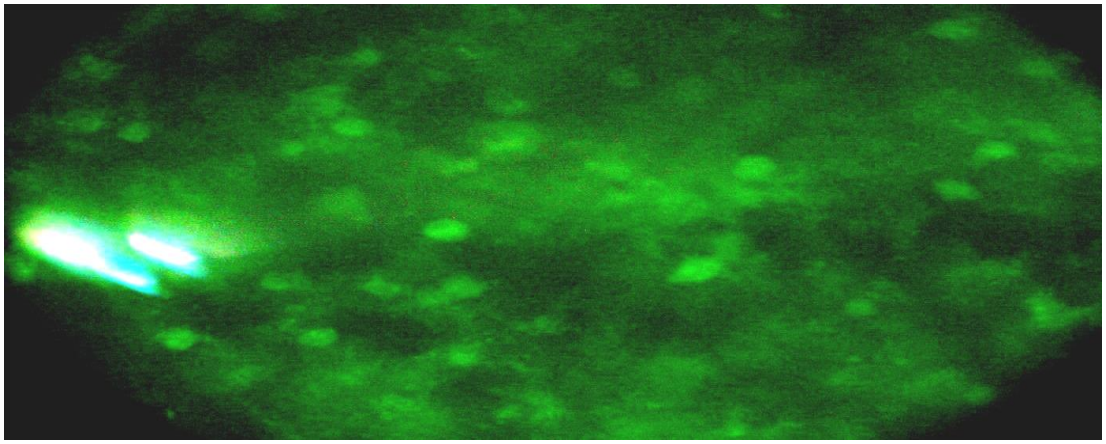

Figure 8: ANG cell line Shows 100% apoptosis when treated with 400ng of ADI after72h of incubation with ADI enzyme, presented debris of lytic cells and their exudes

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