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Assessment of Autophagy as Possible Mechanism of the Antitumor Effects of Arsenic Trioxide and/or Cisplatin on Ehrlich Ascites Carcinoma Model

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ABSTRACT

Arsenic trioxide (As_2O_3) (ATO) and Cisplatin (CIS) have potent antineoplastic effects in several types of cancer. Autophagy is important for normal cell function and survival, it is also used by tumor cells so, and we studied its role as possible mechanism of ATO and/or CIS antitumor effect on mice bearing Ehrlich Ascites carcinoma (EAC) and checked whether ATO can enhance the antitumor potential of CIS. The study was carried out on eight groups of female mice; GP1 (negative control), GP2 (Erlich tumor only), GP3 (Normal +ATO), GP4 (Normal + CIS), GP5 (Normal + ATO+CIS), GP6 (EAC+ATO), GP7 (EAC+CIS) and GP8 (EAC +ATO+ CIS). In this study, viable cells were counted, where percentage of viability (%) was calculated. Flow cytometry of autophagosome was appointed. Glutathione *S*-transferase (GST) and catalase (CAT) enzymes activities, total thiol and malondialdehyde (MDA) concentrations in liver tissues were determined to evaluate the effects of these drugs. Treatment with ATO (GP6) induced a significant decrease in tumor volume and percentage of viability with best result in combination of ATO with low dose of CIS (GP8) against EAC. Our results showed that a reduction in fluorescence intensity of autophagosome marker that determined by flow cytometry especially in combination treated group (GP8). CAT, GST enzymes activities and total thiol level were decreased, while MDA level was increased in ATO treated group (GP6) and CIS treated group (GP7) as compared to EAC group. On the other hand, combining both ATO and CIS in EAC treated group (GP8) decreased MDA level and augmented the level of total thiol and activities of CAT and GST enzymes. Therefore, our result revealed that combination of ATO and CIS have anti-tumor effects against EAC better than each one alone. So, we recommended the combination of ATO with CIS treatment due to their synergetic effect on cancer

1. INTRODUCTION

Arsenic trioxide (ATO), have a noticeable therapeutic effect on solid tumor cell, lines, such as cervical cancer cells, human sarcoma cells, hepatoblastoma cells and HCC cells. ATO treatment activated both p38 MAPK and JNK pathway in Hela cells. Activation of MAPKs, such as p38 MAPK and JNK, were important for cancer prevention by drug therapy against cancer (Xia et al., 2018).

Autophagy is a lysosome-dependent cellular degradation process that has functions in nutrient recycling and energy generation. Damaged proteins and organelles were clearance by this process.

Autophagosomes are emerging double membrane vesicles where the cytoplasmic materials targeted for autophagic destruction are sequestered, and delivered for lysosomal degradation. Autophagy contributes to survival during starvation and other forms of cellular stress; it also functions in differentiation and development, anti-aging, innate and adaptive immunity, and tumor suppression (Sunitha *et al.*, 2018).

Under normal physiological conditions, cells control reactive oxygen species levels by balancing the generation of reactive oxygen species with their elimination by scavenging system (glutathione *S*-transferase (GST) and catalase (CAT), but under oxidative stress conditions, excessive reactive oxygen species can damage cellular proteins, lipids and DNA, leading to fatal lesions in cells that contribute to carcinogenesis. Cancer cells exhibit greater reactive oxygen species stress than normal cells do, partly due to oncogenic stimulation, increased metabolic activity and mitochondrial malfunction, this was confirmed by Abdel-Hamid *et al.*, 2017 who found that disturbed hepatocellular anti-oxidant mechanisms suppress apoptosis, reflecting failure in combating chemical hepatic-carcinogenesis. Oxidative stress is the one of most important mechanisms involved in CIS and ATO toxicity (Dasari and Tchounwou, 2014).

We hypothesized that ATO may be used to improve CIS efficacy while reducing its adverse effects. Therefore, the aim of the present study was to investigate the antitumor effect of ATO and to assess whether it can enhance the anticancer potential of CIS against mice bearing EAC. We also tried to elucidate the possible underlying mechanisms associated with the ATO-induced inhibition of cancer cells with special focus on autophagy.

2. MATERIAL AND METHODS

2.1. Chemicals and Reagents

Arsenic trioxide and Cisplatin were purchased from Sigma-Aldrich USA (St.Lous, MO, USA). The drugs were dissolved in phosphate buffer saline (PBS) with the appropriate doses. Autophagy assay kit for fluorometric tests were purchased from Sigma-Aldrich USA (St.Lous, MO, USA).Ferric tripyridyltriazine (Fe^{III}-TPTZ) and other chemicals were purchased from Sigma Aldrich (St. louisMo., USA).

2.2. Animals

Female BALB/c, weighting 20-25g purchased from National Cancer Institute (Cairo University, Egypt), were housed at the Medical Biochemistry Department, Faculty of Medicine, Tanta University, Egypt. Mice were maintained on laboratory standard experimental conditions (temperature $23^0C \pm 2^0C$, relative humidity 55% ±5 %, balanced diet and free access to water) and were left one week before experimentation to adapt laboratory conditions. Animal's procedures were consistent with the guidelines of Ethics by public Health Guide for Care and Use of Laboratory Animals approved by Animal Ethical committee of Tanta University, Egypt.

2.3. Experimental Design

Mice were weighted in the beginning and the end of the experiment then divided into eight groups (10 mice each) and received intraperitoneal injection. The eight groups were as follows: GP 1: Naïve (0.2 mL of saline.), GP 2: EAC only (0.5×10^6 cells.), GP 3:ATO only (ATO (5 mg/kg) (Jing et al., 2001), GP 4:CIS only (CIS (40 µg/mouse) (Puisset et al., 2014**)**, GP 5: ATO & CIS (ATO (5 mg/kg) + CIS (10 µg/mouse).), GP 6:EAC-treated with ATO (EAC mice as GP2 + ATO (5 mg/kg.), GP 7: EAC-treated with CIS (EAC mice as GP2 + CIS (40 µg/mouse).): and GP 8: EAC-treated with ATO & CIS(EAC mice as GP2 + ATO (5 mg/kg) + CIS (10 µg/mouse).). we used low dose of CIS in combination with ATO in GP5 and GP8 because when we combined between ATO and CIS (40mg/kg) all mice were died. All groups were received all treatments at day zero from inoculation of EAC cells.

2.4. Sampling

Two weeks after tumor inoculation, EAC cells were isolated from the peritoneal cavity of mice from each group, suspended in a sterile isotonic saline, mixed with a 0.4% trypan blue staining and counted in a hemocytometer. Percentage of viability (%) = (number of viable cells x 100) / total number of cells. Also, the ascetic volume was measured.

2.5. Tissues

Liver was dissected, thoroughly washed with ice-cold 0.9% Nacl, weighed for determination of relative liver weight= (liver weight/mice weight (100) and sliced into two parts. One part was kept in 10% neutral buffered formalin for the histopathological examination, and the second was homogenized (10% w/v) using phosphate buffer (pH 7.4).

2.6. Oxidative stress Markers

The liver tissue homogenate was used for estimation of hepatic enzyme activities of catalase (CAT), glutathione *S*-transferase (GST) and concentration of malondialdehyde (MDA) and Total Thiol. Estimation of GST: the formation of theadduct due to conjugation of GSH with 1-chloro-2, 4-dinitrobenzene (CDNB); the absorbance was observed

at 340nm (*Habig et al., 1974).* Catalase: 3 mL buffered H_2O_2were, mixed by the sample (10µl), read for 1min at 250nm (Xu et al., 1997). MDA, in brief, to 2 ml of tissue extract were added to 1 ml of 20% trichloroacetic acid and 2 ml of 0.67%(w/v) thiobarbituric acid, the contents were heated in a tube for 10 min in a boiling water bath, cooled and then centrifuged to precipitate out the protein. The color developed in the supernatant was measured by colorimetric method at 530nm (Mesbah et al., 2004). Total Thiol of liver tissue was performed by the method of Sedlak and Lindsay (1968), briefly, 250µl of liver homogenate were mixed with 750 µl of Tris buffer pH 8.2(0.2 M).Followed by addition of 50 µl of dithionitrobenzoic acid (DTNB) then completed to 5ml with 3950 µl of absolute methanol. The color was developed for 15 min. at room temperature and measured at 412 nm.

2.7. **Flow cytometry**

The autophagy was detected Using a propriety fluorescent autophagosome marker (λex = 333/λem = 518 nm) of autophagy assay kit (Sigma Aldrich, USA). The autophagosome detected reagent was diluted with Dulbecco's Modified Eagle Medium at 1/10, and the solution was added to the EAC cells (were pulled from intraperitoneal cavity of mice) at 4µl per well and incubated at 37^0C with 5%CO_2 for 30min.The excess dye was removed by washing with buffer twice and cells were observed (λ ex = 360/ λ em = 520 nm) with Attune flow cytometer (Applied Biosystem, USA).

2.8. **Histopathological investigation**

Autopsy samples were taken from the liver of mice in different groups and fixed in 10% neutral buffered formalin for twenty four hours, prepared for histopathogical examination and stained by Hematoxylin and Eosin (H&E) with magnification power (X 80) according to Bancroft and Gamble (2008**)**.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was used to assess significant differences among treated groups. The Tukey Test was used to compare all groups with each other's and showed the significant effects of treatments. The criterion for statistical significance was set at P≤ 0.05 (GraphPadInStat Software). Data were expressed as Mean ± Standard Error, n=10 (number of mice) for each group. Small letters indicate significant differences between the indicated group versus control or other groups.

3. RESULTS

3.1. Effect of ATO and/or CIS on body weight gain and relative liver weight of the experimental groups

Table (1) showed that, GP2 (EAC only) has a significant increase (P < 0.001) compared to all groups in body weight gain. Groups 3 (ATO only), 4 (CIS only) and 5 (ATO & CIS only) showed a significant decrease (P < 0.001) in body weight gain than all groups. Groups 6 (EAC treated with ATO), 7 (EAC treated with CIS) and 8 (EAC treated with ATO & CIS) showed a significant increase (P < 0.001) than groups 1, 3, 4 and 5. On the other hand, GP8 showed a significant decrease (P < 0.001) in body weight gain than groups 6 and 7.

The relative liver weights (g/100 g body weight) of GP2 showed a significant decrease (P < 0.05) compared to all groups. Groups 3 and 4 showed a significant increase (P < 0.05), (P < 0.01) respectively, in relative liver weight as compared to GP1 (Naïve) (Table 1).While, GP8 showed a significant decrease (P < 0.01) compared to groups 3 and 4.

3.2. Effect of ATO and/or CIS on Ehrlich volume and viability of EAC cells

Ehrlich volume of GP6 (ATO treated group) was significantly decreased (P < 0.01) than that of GP2 (EAC only), while GP7 (treated with CIS) showed a highly significant decrease (P < 0.001) in Ehrlich volume than that of GP2. Also, GP8 (EAC- treated with both ATO & CIS) showed a reduced volume of Ehrlich (P < 0.001) than that of GP2, which in turn reflected the additive effect of the combination of ATO and CIS on the Ehrlich volume (Table 2).

Viability of EAC cells in GP6 and GP7 were significantly decrease (P < 0.01) than that of GP2, while GP8 showed a highly significant decrease in viability of EAC cells (P < 0.001) as compared to GP2 that confirmed also the additive effect of both ATO and CIS (Table 2).

3.3. Effect of ATO and/or CIS on autophagic activity of EAC cells by flowcytometer

Table (2) showed that, GP2 (EAC only) has a significant increase (P<0.001) than all groups in fluorescence intensity of autophagosome marker. On the other hand, GP8 (EAC treated with ATO & CIS) showed a significant decrease (P<0.01) in fluorescence intensity of autophagosome marker than that GP7 (EAC treated with CIS) (Fig. (1), also, due to the additive effect of both ATO and CIS.

Table (1): body weight gain (g) and relative liver weight (g/100g body weights) in the different treated groups

Groups	(GP I) Naive	(GP 2) EAC only	(GP 3) ATO only	(GP 4) CIS only	(GP 5) ATO&CIS	(GP 6) EAC treated with ATO	(GP 7) EAC treated with CIS	(GP 8) EAC treated with ATO&CIS
Body weight gain	$0.1\pm0.1^{a,b}$	$10.96\pm1^{a,d}$	$-1.3\pm0.8^{d,g,}$	$-1.5\pm0.5^{d,j,l}$	$-2.8\pm0.6^{d,m}$	$6.2\pm0.7^{a,d,g,j,m,q}$	$4\pm0.95^{a,d,g,j,m}$	$2.1\pm0.5^{d,i,l,m,q}$
Relative Liver Weight	$5.04 \pm 0.3^{b,c}$	$3.43 \pm 0.4^{c,d,f}$	$6.69 \pm 0.2^{c,d,h}$	$6.8 \pm 0.3^{b,d,k}$	5.76 ± 0.2^{d}	$4.87 \pm 0.3^{f,h,k}$	5.9 ± 0.4^{d}	$4.95 \pm 0.3^{f,h,k}$

Table (2).Effect of ATO and/or CIS on Erlich volume (ml), Viability of EAC cells (%) and fluorescence % of flowcytometry,Resultsof EAC bearing mice

parameters	(GP2) EAC only	(GP6) EAC treated with ATO	(GP7) EAC treated with CIS	(GP8) EAC treated ATO&CIS
Ehrlich volume(ml)	$8.6\pm0.9^{a,b}$	$5.8\pm0.33^{b,e}$	$1.57\pm0.2^{a,e,h}$	$4.86\pm0.26^{a,h}$
Viability of EAC cells (%)	$96.83\pm1.2^{a,b}$	71.6 ± 3.9^{b}	71.4 ± 7.9^{b}	57.14 ± 4.9^{a}
Fluorescence % (relevant to EAC control group)	$100\pm3.4^{a,b}$	66.12 ± 2.94^{a}	$78,7\pm3.2^{b,i}$	$62.3\pm2.85^{a,i}$

Table (3).: Hepatic oxidative stress parameters (GST) (µmole/min/g liver),catalase (CAT) (mmole/min/g liver), malondialdehyde (MDA), (nmole/g liver) and total thiol (µmole/g liver)in the different treated groups

Groups	(GP I) Naive	(GP 2) EAC	(GP 3) ATO only	(GP 4) CIS only	(GP 5) ATO&CIS	(GP 6) EAC treated with ATO	(GP 7) EAC treated with CIS	(GP 8) EAC treated with ATO& CIS
GST	$4.59\pm0.09^{a,c}$	$3.43\pm0.1^{a,d,e}$	$2.66\pm0.2^{a,g}$	$2.11\pm0.3^{a,j}$	$5.47\pm0.05^{c,d,g,j,m}$	$2.48\pm0.1^{a,e,m,p}$	$2.23\pm0.2^{a,d,m,s}$	$5.01\pm0.2^{d,g,j,p,s}$
CAT	$425\pm5.9^{a,b}$	$213.9\pm1.9^{a,d,e}$	$394.9\pm7.8^{b,d,g,h}$	$363.4\pm4.8^{a,d,h,j}$	$496.5\pm4.1^{a,d,g,j,m}$	$182.5\pm3.6^{a,e,g,j,m,p,r}$	$158\pm3^{a,d,g,j,m,r,s}$	$351.2\pm4.9^{a,d,g,m,p,s}$
MDA	$19.3\pm0.3^{a,b,c}$	$58.5\pm2.3^{a,d,f}$	$31.6\pm1.6^{b,d,g}$	$31.5\pm2.6^{b,d,j}$	$10.39\pm0.3^{c,d,g,j,m}$	$72.5\pm1.7^{a,d,g,j,m,p}$	$73.5\pm3.3^{a,d,g,j,m,s}$	$48.4\pm1.2^{a,f,j,m,p,s}$
Total Thiol	86.9 ± 3.7^{a}	$61.4\pm2^{a,d,f}$	$62.6\pm1.9^{a,g,i}$	$61\pm1.6^{a,j,k}$	$72.9\pm1.4^{a,f,i,k,m}$	$43.2\pm2.2^{a,d,g,j,m,r}$	$41.8\pm2.4^{a,d,g,j,m,t}$	$53.9\pm1.2^{a,m,r,t}$

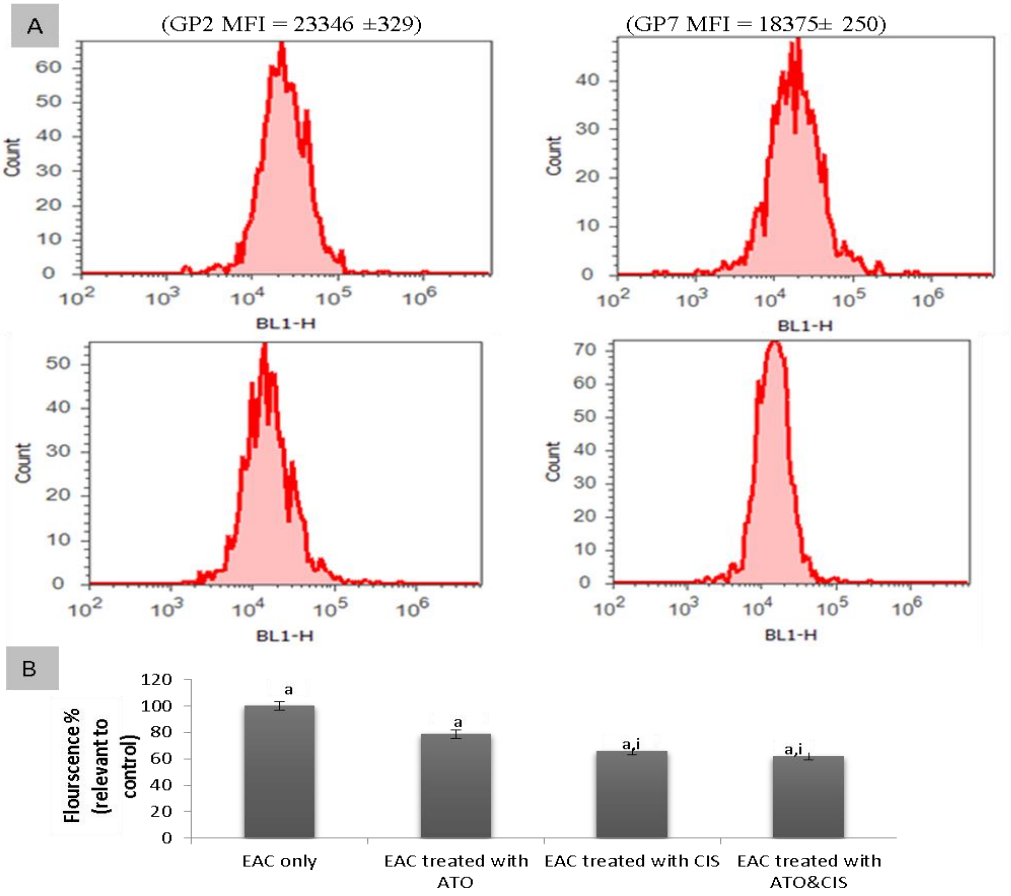

Fig. 1: Flow cytometric analysis of autophagic activity of EAC cells after treatment by ATO and/or CIS. (A)Flow cytometry histogram showing mean ± SEM fluorescence intensity (results are the mean of three independent experiments) (BL1-H Blue Configuration Channel), (B) comparison between groups

3.4. Effect of ATO and/or CIS on oxidative stress parameters of liver cells

Table (3) showed that, Hepatic glutathione S – transferase (GST) activity was significantly decrease (P< 0.001) in GP2 (EAC only), GP3 (ATO only), GP4 (CIS only), GP6 (EAC treated with ATO) and GP7 (EAC treated with CIS) when compared to GP1 (Naïve). On the other hand , GP5 (ATO & CIS) was slightly significant increase (P < 0.05) in GST activity as compared to groups1, but it showed highly significant increase (P < 0.001) when compared to 3 ,4,6 & 7. On the other hand, GP8 (EAC treated with ATO & CIS) showed a significant increase (P< 0.001) in GST activity as compared to groups 1,6 and 7.

Hepatic catalase (CAT) activity showed significant increase (P < 0.001) in GP1 (Naive) than all groups (Table 3). GP5 (ATO &CIS) showed a significant increase (P < 0.001) in CAT activity when compared to GP3 (ATO only) and GP4 (CIS only). Groups 3, 4 and 8 (EAC treated with ATO & CIS) induced significant increase (P < 0.001) in CAT activity in comparison with GP2.On the other hand, GP6 (EAC treated with ATO) (P < 0.01) and GP7 (EAC treated with CIS) showed a significant decrease (P < 0.001) in CAT activity as compared to GP2 but GP8 was significantly increase in CAT activity as compared to GP2. GP8 was significantly increased (P < 0.001) in CAT activity in comparison with groups 6 and 7.

Lipid peroxidation products (MDA) of liver in all groups (except GP5 (ATO & CIS)) showed a significant increase (P< 0.001) when compared to GP1 (Naïve). On the other hand GP5 was significantly decreased (P < 0.001) in MDA level than all groups. GP8 (EAC treated with ATO &CIS) was slightly significant decrease (P < 0.05) in MDA level when compared to GP2. Also, GP8 showed highly significant decrease (P < 0.001) in MDA level as compared to GP6 (EAC treated with ATO) and GP7 (EAC treated with CIS) (Table 3).

Total thiol in liver was significantly decreased (P< 0.001) in all groups as compared to GP1 (Naïve). GP6

(EAC treated with ATO) and GP7 (EAC treated with CIS) showed a significant decrease ($P < 0.001$) in total thiol level as compared GP2 (EAC only). While, GP5 (ATO &CIS) induced a significant increase ($P < 0.05$) in total thiol level as compared to GP2. On the other hand, GP5 was significantly increase in total thiol level as compared to GP3 (ATO only) ($P < 0.05$) and GP4 (CIS only)($P < 0.01$). As the other, GP8 (EAC treated with ATO &CIS) showed a significant increase in total thiol level when compared to GP6 (EAC treated with ATO)($P < 0.05$) and GP7 (EAC treated with CIS)($P < 0.01$) (Table 3).

3.5. Histopathological findings

In GP1 (Naïve), there was no histopathological alterations and the histological architecture of the central vein and hepatocytes in the parenchyma was normal (Fig. 2-a). However, in GP2 (EAC only), group of neoplastic cells (nc) embedded in the portal area surrounding the dilated portal vein (Fig. 2-b). In GP3 (ATO only),focal necrosis with inflammatory cells infiltration in hepatic parenchyma (Fig. 2-c).In GP4 (CIS only) showing focal lymphoid cells aggregation with hepatic parenchyma adjacent the central vein with diffuse Kuffer cells proliferation (Fig. 2-d). But, in GP5 (ATO &CIS) showing sever dilation and congestion in portal vein with few leucocytes infiltration and fibroblastic cells proliferation in portal area (Fig. 2-e). On the other hand, in GP6 (EAC treated with ATO), showing vacuolar degeneration in diffuse manner all over the hepatocytes in the parenchyma (Fig. 2-f).In GP7 (EAC treated with CIS) showing diffuse Kuffer cells proliferation in between the hepatocytes (Fig. 2-g). In GP8 (EAC treated with ATO plus CIS) showing focal extravagated red blood cells in between the hepatocytes (Fig. 2-h).

4. DISCUSSION.

Chemotherapeutic drugs are more effective when there are given in combination therapy. ATO is now an FAD (Food And Drug Administration) approved chemotherapeutic for treating all-trans retinoic acid (ATRA) resistant APL(acute promyelocytic leukemia) (Yan et al., 2016). And so we studied its effect in treating EAC model to see its anti-carcinogenic effect. Weight gain was used as parameter to quantity toxicity. The present data showed that the body weight gain of non-treated EAC mice (GP2) significantly increased compared with the other groups because the rapid and progressive accumulation of ascites tumor cells. Normal treated mice with ATO only (GP3), CIS only (GP4) and combined with each other (GP5) have insignificant effect on body weight gain when compared with untreated mice (GP1), suggesting that low ATO doses did not induce negative impact on animal body weight. However, higher doses and or prolonged exposure time demonstrated delirious effect on body weight (data not shown). However, side effects of ATO or CIS depended on dosage and duration because high dose from ATO or CIS was more toxic. Our data indicated that, ATO side effects are endurable. These results are agreed with Mondal et al.,(2018).Treatment of EAC mice with ATO (GP6) , CIS (GP7) and combination between ATO & CIS (GP8) resulted in a significant decrease in body weight gain when compared with non-treated EAC mice (GP2) that because ATO induced apoptosis so tumor volume decreased so body weight decreased in the previous groups. These result agreed with Zhao et al.,(2014). Also, CIS formed adducts with DNA and induction of apoptosis (Choi et al., 2015).

The relative liver weight of non-treated EAC mice(GP2), ATO(GP6) and CIS(GP7) treated groups showed a significant decrease as compared with the normal (GP1).As liver is a very active site of xenobiotic metabolism, it is the main site of arsenic intoxication, where arsenic methytransferase enzymes mediated the methylation process with S-adenomethionine as the methyl donor and GSH as an essential co-factor to convert ATO to dimetharsinous acid (DMA) through series of methylation reactions (Ahangarpour et al., 2017).And also, formation of GSH-CIS metabolite in liver is a major route for cellular elimination of CIS (Nagar et al., 2015).

The viability of cancer cells was significantly reduced in EAC treated with ATO (GP6), EAC treated with CIS (GP7) and a more significant decrease was observed in the combination group (EAC treated with ATO & CIS (GP8), further exhibited the significant decrease in the ascetic fluid volume collected after therapy in the these groups. This result may be due to activation of ATO to death associated protein kinase (DAPK 1 and DAPK2). DAPK are a family of five Ser/Thr kinase and required for the induction of cell death (Gade et al., 2014). DAPK 1 may require the activation of P^{53} apoptotic pathway. In other cellular setting, DAPK1 and DAPK2 have been linked to the activation of an alternate type of programmed cell death (autophagic cell death). One such mechanism involves the phosphorylation of beclin 1 which is required for autophagosome nucleation formation. It is also believed that DAPK2 may function upstream of

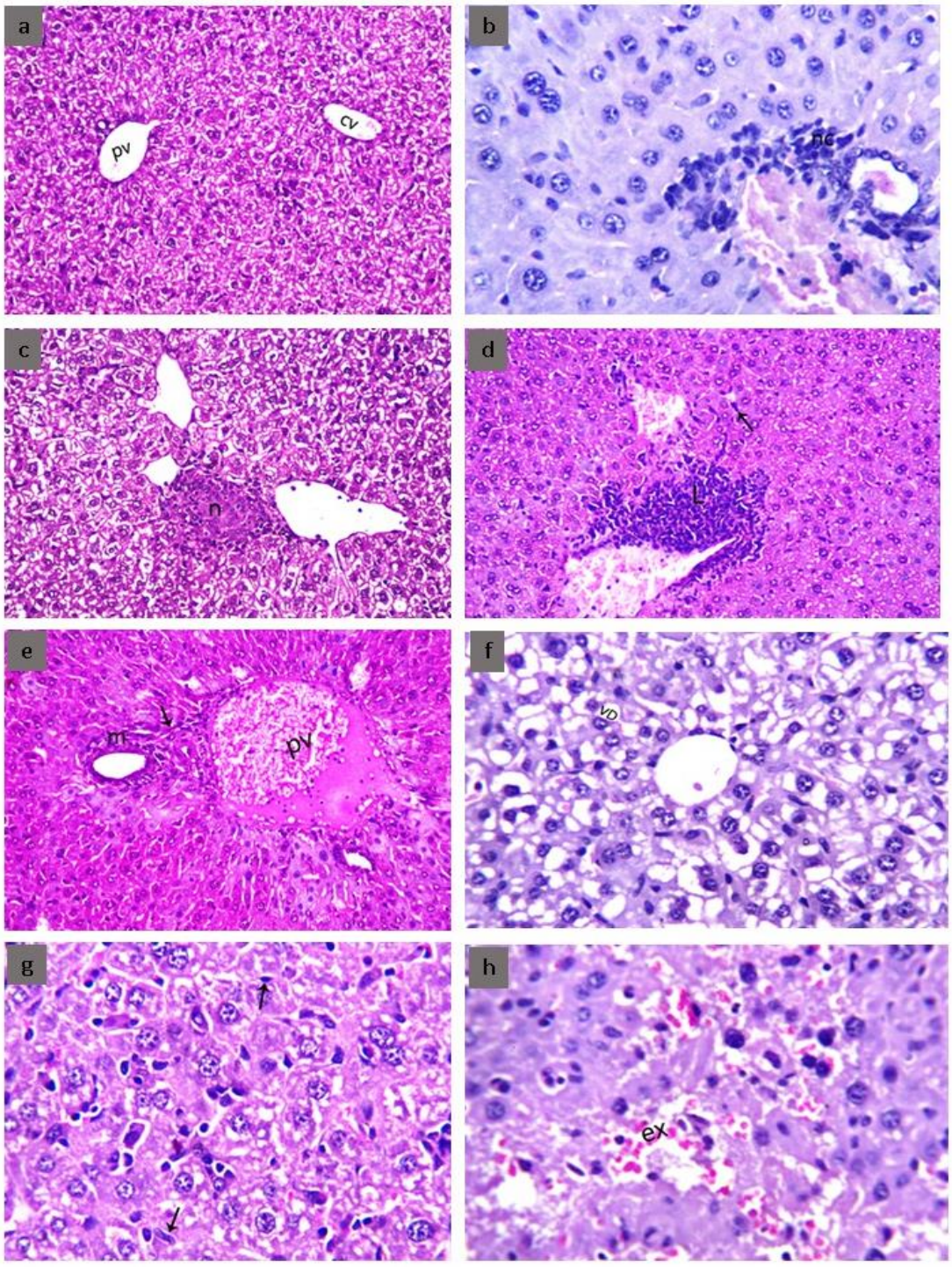

Fig. 2: Liver of mice, **a)** in normal control group (GP1) showing normal histological structure of the central vein(cv) and portal vein (pv) surrounding hepatocytes in the parenchyma . **b)** : in EAC bearing positive control group (GP2) showing group of neoplastic cells (nc) embedded in the portal area surrounding the dilated portal vein. **c)** in ATO control group (GP3) showing focal necrosis with inflammatory (n) cells infiltration in hepatic parenchyma. **d) :** in CIS control group (GP4) showing focal lymphoid (L) cells aggregation with hepatic parenchyma adjacent the central vein with diffuse kuffer cells proliferation. **e)**: ATO plus CIS control group (GP5) showing sever dilatation and congestion in portal vein (pv) with few leucocytes infiltration (m) and fibroblastic cells proliferation in portal area. **f)** : ATO treated group (GP6) showing vacuolar degeneration (VD) in diffuse manner all over the hepatocytes in the parenchyma. **g)** : CIS treated group (GP7) showing diffuse kuffer cells proliferation in between the hepatocytes. **h) :** ATO plus CIS treated group (GP8) showing focal extravasated red blood cells (ex) in between the hepatocytes.

DAPK1 as the over expression of a dominant negative DAPK1 reduced DAPK2 induced cell death (Tur et al., 2017). So that our data showed unexpected significant decrease in autophagic activities (as revealed by a reduction in fluorescence intensity of autophagosome marker that determined by flow cytometry which in turn in disagreement with the result of beclin 1 that also decreased - data not shown) in treated groups (GP6, 7) and especially in combination group (GP8) when compared with EAC group (GP2).

From another point of view, if any step upstream of autophagosome formation is blocked, the number of all autophagic structures is decreased. In contrast, the blockade of any step downstream of autophagosome formation increases the number of Autophagosomes while decreasing the number of auto lysosomes (Iovanna and Vaccaro, 2010). Therefore, the determination of autophagosome number is insufficient for an overall estimation of autophagic activity. Rather, different methods often need to be used in concert to distinguish between basal levels of autophagy, induction of autophagy and suppression of upstream or downstream steps of autophagy.

There was a significant decrease of oxidative stress markers as Catalase, GST enzymes activity, total thiol while, a significant increase in MDA level in ATO (GP6) and CIS (GP7) treated groups as compared to EAC group (GP1).These result because ATO and CIS caused oxidative stress through generation of ROS, increase in lipid peroxidation, induction of DNA damage and reduction of GSH level (Kumar et al., 2014). On the other hand, combining both ATO and CIS in EAC treated group (GP8) decreased MDA level and augmented the level of total thiol and activities of CAT and GST enzymes. These findings indicated that treatment of ATO with CIS enhance the antineoplastic effect of low dose of CIS in an additive manner as shown in results.

Elevated oxidative stress causing autophagy promotes either cell survival or cell death and the fate of which depends up on the security of stress occurring with a particular disease (Michela et al., 2016), and this happened in group (GP8). According to the above results, oxidative stress is the main reason for cancer, indeed, the decrease of autophagy in GP8.

Relative to the relation between oxidative stress and autophagy in groups 6 and 7, oxidative stress predominates autophagy, these results in agreement with (Abdel-Hamid, et al., 2017) ,here free radicals due to oxidative stress resulted from treatment with Cisplatin (Gp 6) and ATO (Gp 7) increased free radical production which in turn elevate the oxidative stress parameters (Table 2) and decreased autophagy. However the combination treatment of both cisplatin and ATO (GP8) showed decrease in oxidative stress parameters accompanied with decrease in autophagy indicating the additive positive effect of both cisplatin and ATO combination in treatment of liver cancer due to EAC.

Histopathological examination, there was histopathological alterations in EAC control and treated groups. In EAC groups, these alterations may be due to the accumulation of hemorrhagic ascetic fluid within the peritoneal cavity in which the cells proliferate and move to invade the internal organs (Chakraborty et al., 2007). CIS caused severe damage in the liver, such as degenerative hepatocytes and moderate enlargement of sinusoids, which was observed by microscopic examination (Do Amaral et al., 2008). ATO caused vacuolar degeneration in diffuse manner all over the hepatocytes in the parenchyma. Ferzand et al.,(2008) indicated that chronic exposure to different inorganic arsenic compounds (arsenite, arsenic trioxide, or arsenate) produces characteristic pathology in the liver, including fatty infiltration, liver degeneration, inflammatory cell infiltration, and focal necrosis. On the other hand, in combination group (GP8) there was a slight amelioration in these histopathological findings. These result may be due to the liver is an important organ for various metabolic pathways and effect of any chemical or xenobiotic appears primarily in the liver (Wang et al., 2018).

5. CONCLUSION

In conclusion arsenic trioxide synergistically enhanced Cisplatin effect in an additive manner. Our result did not exclude the role of autophagy in antitumor effect of ATO. Further study is needed to evaluate autophagy as antitumor mechanism.

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