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Alterations in Kidney of Albino Rat due to Acrylamide Exposure and the Possible Protective Role of l-arginine (Biochemical, Histological, Immunohistochemical and Molecular Study)

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ABSTRACT

Acrylamide (ACR): is an industrially conjugated reactive molecule that initiates cellular toxicity, induced oxidative stress and classified as a probable human carcinogen. L-arginine (L-Arg) produces nitric oxide (NO): that involved in vascular regulation, immune activity, and able to eliminate intracellular pathogens. This work conducted to evaluate nephrotoxicity of ACR and the possible protective role of L-Arg at biochemical, histopathological, immunohistochemical and molecular levels. Forty male rats were divided equally into four groups; **control**: received the ordinary water and diet. **ACR**: Animals were given a dose of ACR (50mg/kg/day): dissolved in water. **ACR + L- Arg**: Animals given the same dose of ACR together with L-Arg dissolved in water. **L-Arg**: Animals administered L-Arg by a dose of 200 mg/kg/day dissolved in water. The exposure period was a month. Kidneys were removed and prepared for histopathological, immunohistochemical studies (caspase3). Also, kidney specimens taken for real-time-PCR technique to measure p53 expression and comet assay. Blood samples were collected for kidney function detection. Animals exposed to ACR showed several histopathological lesions including glomerular shrinkage, loss of PCT brush borders, degeneration of renal epithelia, deposition of hyaline casts and necrotic areas in the renal parenchyma. significant increase in p53 expression, significant increase in caspase 3 activity in renal cells and increase in creatinine levels. However, urea levels recorded a significant decrease. Animals treated with L-Arg together with ACR showed a marked improvement in all these parameters towards the normal status. The study suggested that the administration of L-Arg provided a protective potential against ACR-nephrotoxicity.

Keywords: Acrylamide, L-arginine, Kidney, P53, Comet assay, Caspase3.

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INTRODUCTION

Acrylamide is an industrially conjugated reactive molecule; it is used globally to synthesize polyacrylamide. Neurotoxicity, reproductive toxicity, genotoxicity, clastogenicity (chromosome-damaging effects). and carcinogenicity have been reported as human health risks due to ACR exposure.[1] ACR has been reported to be neurotoxic in experimental animals at the histological and immunohistochemical level.[2] ACR exposure was an occupational onset, also via smoking, drinking water and cosmetics. The highest concentrations of ACR have been recorded in potato and grains that are cooked at high temperature e.g., frying, grilling or baking.[3] The toxicity of ACR is attributed to its biotransformation to a more potent and highly reactive molecule that initiates cellular toxicity. The most important pathogenic pathway is the oxidative biotransformation of ACR by cytochrome P450 2E1 (CYP2E1).[4] ACR administration induced apoptosis as well as inflammation, resulting in tubular necrosis in kidney tissues.[5]

L-Arg is a semi-essential amino acid that involved in several areas of physiology of human, including production of nitric oxide (NO). which involved in vascular regulation, immune activity, and endocrine role. L-Arg is also involved in protein production, wound healing and fertility.[6] NO is also able to eliminate intracellular pathogens and block viral replication and NO derived from leukocytes showed anti-tumor effect. It also up-regulates tumor suppressor p53 gene.[7]

The present study was designed to test the possible protective effect of L-Arg against the produced nephrotoxicity of ACR in experimental animals at the cellular and sub-cellular level.

MATERIAL AND EMTHODS

Animals

Forty male albino adult rats (*Rattus norvegicus*). aged about 9-10 weeks and weighting 150 ±10g were used. Rats were purchased from the animal house, Faculty of Veterinary Medicine, Zagazig University, Egypt. Upon arrival, rats were housed in the animal housing facility (in hygienic plastic cages in a clean well ventilated and a temperature-controlled room. Animals were given a standard diet and filtered tap water *ad libitum*. The protocol was approved by the Institutional Animal Ethics Committee for Use and Care of Animals, Zagazig University (ZU-IACUC/1/F/101/2020).

Chemicals

ACR is a white, odorless and solid crystals at room temperature. Its molecular formula is C_3HNO (Sigma-Aldrich Chemical Company, St Louis, MO, USA).

L-Arg (Research-Lab Fine Chem Industries, Mumbai, India).

Experimental design

Forty rats were equally divided into four main groups named; control, received the ordinary water and diet. ACR, Animals were exposed by gavage to a dose of ACR (50mg/kg/day). dissolved in water according to Tyl and Friedman.[8] ACR+ L- Arg, Animals were given the same dose of ACR after treated by L-Arg where L-Arg administrated one week before starting the application of ACR for a protective purpose. L-Arg, Animals were given

L-Arg by a dose of 200 mg/kg/day according to Arellano-Mendoza.[9] dissolved water for gavage. The doses of ACR and /or L-Arg were freshly prepared just before use. The exposure period was one month for all experimental groups.

Biochemical analysis

Urea was determined according to the enzymatic method and creatinine determined by the method of Jaffé[10]. by Roche Hitachi modular system

Examination of renal histopathology by hematoxylin and eosin (H&E) staining

Kidneys were removed carefully from control and experimental animals, fixed in 10 % neutral formalin, dehydrated, cleared in xylene and then embedded in molten paraffin wax (58 ^{0}C). Paraffin blocks were sectioned at 5 µ and allowed for staining with hematoxylin and eosin stain.[11]

Immunohistochemistry

Caspase-3 activity was performed to indicate cell apoptosis where the kidney sections were deparaffinized, incubated in 3 % H_2O_2 for 15 minute and placed in PBS. Rabbit anti-caspase-3 (diluted to 1:1000, Abcam, Ltd., USA) was used as biotinylated primary antibodies. The sections were incubated with peroxidase-conjugated goat anti-rabbit IgG (1:1000) for 30 minutes. Diaminobenzidine (DAB) was applied as a chromogen to visualize the immune reaction for 10 minutes.[12]

Assessment of P_{53} level expression

Total RNA was isolated from rat kidney according to the manufacturer's protocol (QIAGEN, RNeasy Mini Kit), Nano-Drop spectrophotometer used to measure the concentration and purity of RNA, RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription kit (Thermo fisher Scientific, USA). to produce cDNA. RT-PCR was performed using a 2X RT Gene Expression Master Mix (Thermo fisher Scientific). The cDNA was used as a template to determine the relative expression of the apoptosis-related gene (p53). using Step One Plus real time PCR system (Applied Bio system, USA). The p53 primer was designed by Primer 5.0 software. The thermal cycling conditions were as follows, initial denaturation at 95°C for 10 min, 40-45 cycles of amplification of DNA denaturation at 95°C for 15second, annealing at 60°C for 30 s, extension at 72°C for30s. At the end of the last cycle, the temperature was increased from 63 to 95°C for melting curve analysis. The housekeeping GABDH was used to normalized target gen (P53). and analyzed by the comparative threshold cycle (C_t). method. Results were reported as the fold change in gene expression between samples ($2^{-\Delta\Delta Ct}$ method).[13.]

Table 1: Forward and reverse primers sequences

Gene	Forward primer (5' ------ 3')	Reverse primer (5' ------ 3')
P53	TAACAGTTCCTGCATGGGCGGC	AGGACAGGCACAAACACGCACC
GABDH	TGCTGGTGCTGAGTATGTCG	TTGAGAGCAATGCCAGCC

Comet assay

the comet assay, or single cell gel electrophoresis (SCGE), is a commonly- used technic for detection, analyzing and measuring DNA damage in individual cells. Tissues were homogenized in chilled homogenization buffer, pH 7.5, containing 75 mM NaCl and 24 mM Na_2EDTA to obtain a 10% tissue solution. A Potter-type homogenizer was used, and kidney samples were kept on ice during and after homogenization. We used a modified alkaline comet assay protocol according to Sasaki et al.[14.]

Statistical analysis

The data was calculated by a one-way ANOVA with Tukey's multiple comparisons test using SPSS, IMB version 24. The results were expressed as mean ± standard deviation where P-value <0.05.

RESULTS

Biochemical results

The effects of ACR toxicity on serum biochemical parameters as urea and creatinine were given in Table (2) that recorded significant decreases in blood serum urea of ACR-exposed animals compared to control ones. The treated group (ACR+ L-Arg) recorded a non-significant decrease as compared to ACR-exposed animals to reveal in the toxic effect of ACR relative to control ones. Serum creatinine levels in ACR-exposed animals recorded a significant increase compared to controls. However, there was non-significant increase in case of treatment L-Arg together with ACR as compared to control ones.

Histopathological results

Control animals showed the normal histological structure of renal tissue (Fig.1-a).

Animals exposed to ACR exhibited several histopathological lesions including shrinkage of the glomerular tuft with widened Bowman's space. The renal tubules become vacuolated and lost their brush borders. Also, degenerative changes could be observed in their epithelial lining followed by rupture of the cells, necrosis, congestion of the interstitial blood vessels which led to hemorrhage, enlarged hyper-chromatic nuclei (pykontic nuclei) and nucleoli. There was accumulation of hyaline casts in the renal cortical elements, deposition of proteinaceous materials in the lumen of the tubules (Figs1-b,1-c). Kidney sections of ACR and L-Arg treated animals revealed less prominent histopathological alterations compared to ACR-exposed animals where L-Arg caused a good protection that was noted in the form of normal glomeruli and convoluted tubules (Fig.1-d). The renal tissue of L-Arg administrated rat showed a histological pattern nearly similar to those of control ones (Fig.1-c).

Immunohistochemical results

The immunohistochemical examination of caspase3 expression in ACR-exposed animals exhibiting a high immune reactivity for caspase3 in damaged renal elements as compared to control (figs.2-a,2-b). However, treating animals with L-Arg showed a down regulation in the immune expression of caspase3 in the cytoplasm of renal cells (fig.2-c). While the immune expression of caspase3 in kidney section of L-Arg-administered exhibited a weak (+) immune reactivity nearly similar to that of control (fig.2-d). These immunohistochemical findings are summarized in Table (3).

Real-time PCR investigation results

For investigation of P53 tumor suppressor gene expression levels, quantitative real-time-PCR was used to quantify the RNA expression level in kidney tissue for the experimental groups. ACR-exposed ones recorded a high significant increase in the expression of P53 gene (1590.73± 106.64) compared to control ones and L-Arg group recorded a low significant increase (166.72± 28.03) as compared to control. However, L-Arg administration with ACR recorded non-significant increase compared to control ones (Fig.3), table (4).

Comet Assay results

ACR- exposed animals recorded a significant increase in the tail DNA and tail- DNA moment in the kidney tissues compared to control rats (Fig.4-b). However, The DNA fragment of ACR with L-Arg treated animals recorded an obvious decrease in both tail DNA and tail moment equaling, in response to L-Arg treatment (Fig.4-c) where L-Arg administrated ones recorded DNA damage nearly similar to those of control (Fig4-d)Table(5)

DISCUSSION

Acrylamide is one of the important environmental toxicants occurring in heated food products. Exposure of humans and animals to ACR *via* their diet is currently one of the most serious global health problems. Dietary antioxidants have been given attention as possible protective agents and as a health food supplement against toxicity induced by dietary contaminants. The present work suggested the possible protective potential of L-Arg against ACR nephrotoxicity in male albino rats.

Concerning the biochemical status of urea and creatinine, the present investigation revealed that ACR- exposed animals recorded a significant decrease in serum urea compared to that of control. These results are line with those of Özturan-Özer et al.[15] and Shler et al.[16] since urea is biosynthesized in the liver from ammonia which derived from tissue or dietary proteins.[17] The decreased urea levels may be a result of impaired urea synthesis due to hepatic insufficiency due to liver damage.[16] ACR caused a significant decrease in uric acid as ACR stops urate absorption and so leading to urate diuresis. This will end in minimizing serum uric acid levels. A low serum urate level results from decreased production or increased excretion and since xanthine oxidase(the enzyme responsible for conversion of oxypurines to uric acid)is found in a large amount in the liver and the mucosa of the small intestines, the enzyme's rate of formation may decrease due to liver necrosis.[18] This decrease in serum urea levels may be attributed to extracellular dehydration following a net loss of sodium which is likely due to kidney loss in its characteristics by a decrease in its weight.[19] this decrease in serum urea which proved by the current study in contrast to Abdel-Daim et al who suggested that ACR administration increases serum urea, creatinine, uric acid, and renal proinflammatory cytokine levels, while also inducing lipid peroxidation and DNA damage.[20]

However, ACR +L-Arg co-administration gave a non-significant decrease in serum urea compared to control ones as production of NO through the administration of L-Arg reduces necrosis and apoptosis.[21] where NO binds to the heme moiety of guanylate cyclase and increases its activity by 400-fold by catalyzing the conversion of guanosine triphosphate to cyclic guanosine monophosphate.[22] so, liver the formation rate of xanthine oxidase wasn't affected and didn't affect urea levels.

As regards serum levels of creatinine, the current experiment ACR-exposed animals recorded a significant increase and this in line with those of other studies.[23-25] on contrast, Shler et al. reported that creatinine is an excretion product of muscle activity, which circulates in blood.[16] Its elimination is exclusively renal, so there is a direct correlation between creatinine levels and renal function. Most creatinine that is eliminated by the kidneys is freely filtered in renal glomeruli, and a small fraction is filtered by the tubular component, which is a good indicator of renal-glomerular function.[19] Dietary L-Arg supplementation prevented the decrease of glomerular filtration rate, didn't affect serum urea and creatinine and minimized nephrotoxicity of some nephrotoxic.[26]

Concerning the histopathological alterations, the present work revealed that ACR-exposed animals showed congestion of the glomerular capillary tuft, lost their brush borders, infiltration of inflammatory cells, vacuolar degenerative, focal hemorrhages, necrosis of tubular in renal parenchyma. These observations are consistent with those.[23,27,28.]

Other findings may be attributed these histological changes to the fact that kidneys are the main way of excretion of ACR and its metabolites which reach blood stream and extracted in the urine where about 40% to 70% of the dose of ACR which reach blood stream was extracted in the urine in 24 hours in male albino rats, which may indicate that kidney can perform its function to some extent during the toxicity by ACR.[29,30] ACR intoxication induced electrolyte imbalance which has been attributed to the derangement of renal function resulting from interference with ions transport across the renal tubules.[31] ACR altered membrane integrity and fluidity in the cells of adult rats through the generation of ROS. Their enhanced production led to membrane disruption and oxidation of poly unsaturated fatty acids after ACR treatment as revealed by a marked elevation in kidney MDA and index of lipid peroxidation. Oxidative injury may cause molecular disorganization of lipids resulting in increased membrane permeability and leakage of cellular enzymes into blood.[32]

Those histopathological aspect could be due to the accumulation of free radicals as the consequence of the increased H_2O_2 products and MDA levels in the kidney tissue of ACR treated rats.[32] The treatment of L-Arg in the present work showed a negligible change in the histological pattern of kidney as compared to control ones. The possible protective effect in case of ACR+L-Arg groups may be due to L-Arg is a semi-essential amino acid that acts as a precursor for NO synthesis and many biologically important compounds that support cellular homeostasis. Nitric oxide was able to decrease oxidative stress by hunting superoxide radicals and stoppage of free radical release in lipid membrane thus, reducing the inflammatory factors.[34] L-Arg significantly reduced lipid peroxidation and increased GSH content in the heart tissue of exhaustively exercised rats so it decreased kidney damage by synergistically increasing cellular levels of GSH.[35,36.]

Exogenous administration of L-Arg has been shown to protect the kidney against toxic or ischemic injury.[37] In chronic renal failure L-Arg supplementation improved kidney functions, decreased systolic blood pressure and decreased inflammatory cytokine levels including IL-1α, IL1-β, IL-6 and TNF-α.[38,39.]

Concerning the immunohistochemical observations of Caspase-3 is a key protease that is triggered in the apoptotic stages.[40] In the present study, ACR exposure enthused apoptosis and significantly increased renal caspase-3 expression and this in agreement with other studies.[28] where ACR induced in ROS formation, the lipid peroxidation, and the GSH depletion that leaded to oxidative stress hypothesis, decline in MMP and lysosomal membrane labialization where the oxidative stress caused damage to mitochondrial and lysosomal membranes as a result of the increased ROS formation, which leads to an MPT-mediated caspase-3 activation and cell death.[41] However, L-Arg administration with ACR minimized the effect of ACR on caspase3 activity as L-Arg is a donor of No which is the most effective free radical scavenger that ACR produced as concluded by Pedrycz et al.[42] L-Arg decreased the mRNA expression levels of caspase-3.[43] L-arginine supplementation inhibited by decreasing the ratio of Bax to Bcl-2 and the decreased Bax/Bcl-2 ratio inhibited cytochrome C release and caspase-3 activation and consequently decreased apoptosis.[44]

At the molecular level, the ACR-exposed rats recorded an increase in p53 expression since ACR induce apoptosis and p53 is a good marker of nuclear apoptosis in response to DNA damage since ACR induces the production of free radicals which cause oxidative stress in the cellular DNA and this is confirmed by previous studies.[45,46.] The genotoxicity of ACR may be attributed to its bio transformation to a highly reactive glycidamide which directly reacts with DNA molecule forming DNA-glycidamide adducts. So, the present results supported and confirmed previous studies of Morris and Lin et al.[47,48] In addition to the formation of DNA adducts ACR exposure causes a profound carcinogenesis-related loss of histone H4K20 methylation.[49] In the current study L-Arg played a protective role against ACR effect on DNA where L-Arg is a NO donor. NO also inhibits DNA damage mediated by reactive oxygen species (ROS). and inhibits hydroxylation reactions.[50] The synthesis of polyamines and NO from L-Arg may be responsible for its DNA repair effect.[51] Polyamines can stabilize DNA and promote protein synthesis, whereas physiological levels of NO enhance intracellular cyclic guanosine monophosphate (cGMP). content and stimulate DNA synthesis in cells, including endothelial cells and tumors.[52]

CONCLUSION

L-Arg administration effectually had a protective role against ACR nephrotoxicity and genotoxicity so, L-Arg can be provided in food supplementation.

CONFLICT OF INTEREST

There is no conflict of interest.

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Table 2: Serum urea and creatinine levels in control and experimental groups.

Groups / Parameters	Control	ACR	ACR+ L-arg	L-arg
Urea (mg/dl)	35.96 ± 6.68	25.94 ± 1.82*	27.90 ± 6.89	31.10± 2.88
Creatinine (mg/dl)	0.56 ± 0.026	0.63 ± 0.037*	0.59 ± 0.015	0.53 ± 0.056

where * values significant ($p<0.05$).

Table 3: Immunohistochemical findings and their scores in kidney tissue

	Control	ACR	ACR+L-Arg	L-Arg
Caspase3	+	+++	++	+

weak (+), moderate (++), and severe (+++)

Table 4: Relative expression levels of P53 gene in control and experimental groups

Groups	Fold change (RQ) means ± S. D
control	1.00 ± 000
ACR	1590.73± 106.64*
ACR +L-Arg	127.14± 26.90
L-Arg	166.72± 28.03*

where * Denotes values were significant ($p<0.05$).

Table 5: Comparison of data between measurements of DNA damage among different experiment groups

Groups	Untailed %	Tailed%	Tail Length µm	T DNA %	Tail moment
Control	5	95	1.72	1.66	2.86
ACR	13	87	3.15	3.24	10.21
ACR+L-Arg	10	90	2.98	3.12	9.30
L-Arg	3	97	1.39	1.45	2.02

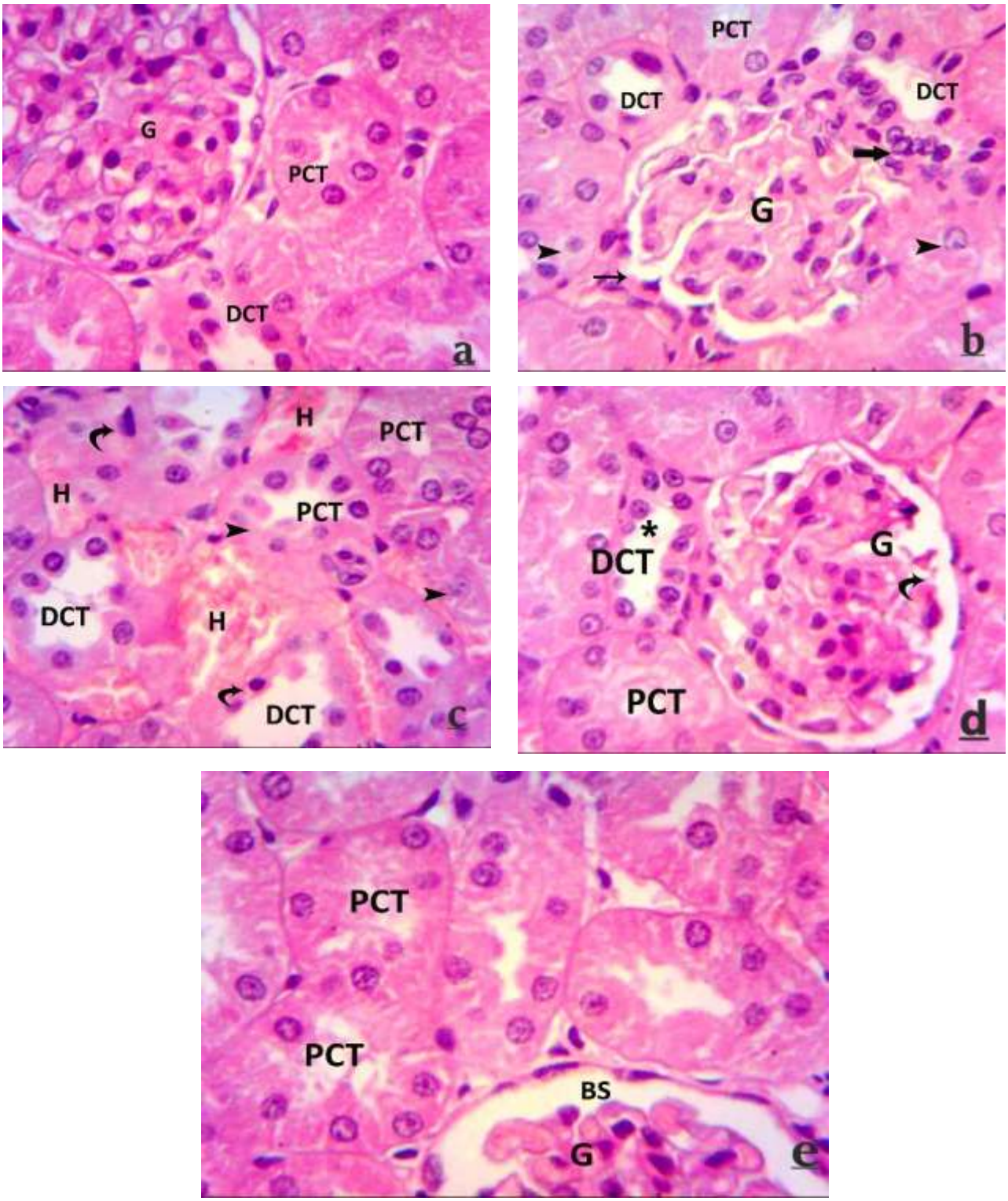

Fig 1: (a) Section of kidney of control rat stained with H&E (X1000) showing a normal histological pattern with glomeruli (G) and proximal convoluted tubules (PCT) and distal convoluted tubules (DCT). **(b)** Section of kidney of ACR- exposed rat showing shrunken glomeruli (G) :with degeneration of parietal layer (thin arrow), necrotic tubular epithelium of renal tubules (arrowhead) and leucocytic infiltration cells (thick arrow). **(c)** Section of kidney of ACR-exposed rat with higher magnification showing necrotic areas (arrowhead), extensive extravasated hemorrhage (H), deteriorated renal tubules of distal type (DCT) and pyknotic nuclei (curved arrow). **(d)** Section of Kidney of ACR-exposed rat treated with L-Arg showing some sort of improvement in the histological pattern of the glomeruli (G) with wide
Bowman's space (curved arrow) and normal renal tubules (PCT, DCT) with well-representative macula densa (*). **(e)** Section of kidney of L-Arg administered rat demonstrating a histological pattern nearly similar to that of control animals.

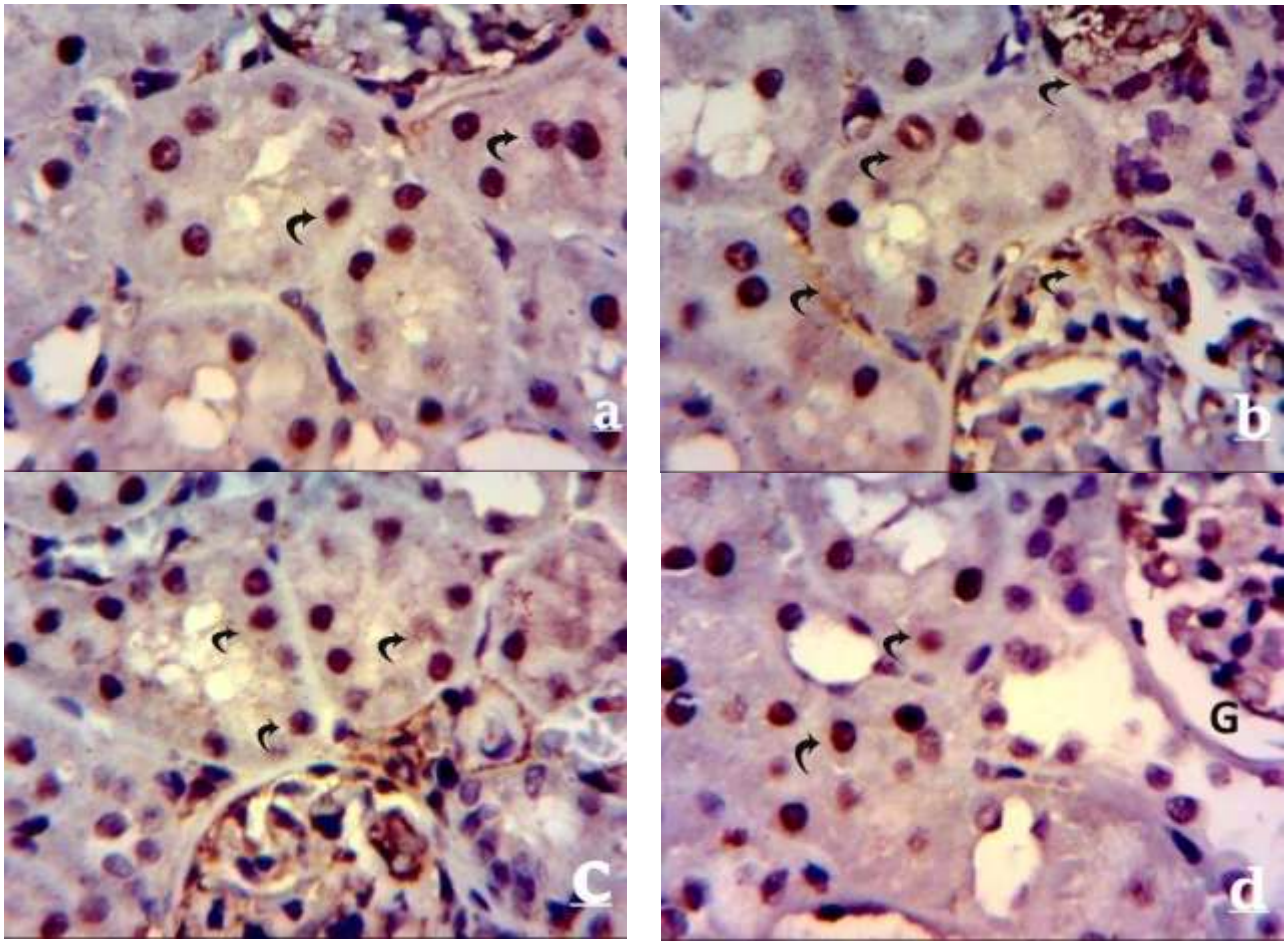

Fig 2: - (a) Immunostaining of kidney section of control animal with caspase3 staining (X1000) reveling a weak (+) caspase expression in the cytoplasm of all cells of the renal elements. **(b)** Immunostaining of kidney section of ACR-exposed animal with caspase3 staining reveling a relative strong (+++) caspase reaction in the cytoplasm of the damaged renal elements. **(c)** Immunostaining of kidney section of ACR-exposed animal treated with L-Arg and stained by caspase3 showing moderate (++) caspase reaction in the cytoplasm of the renal cells. **(d)** Immunostaining of kidney section of L-Arg administered animal with caspase3 staining reveling a weak (+) caspase expression nearly similar to that of control in the cytoplasm of all cells of the renal elements

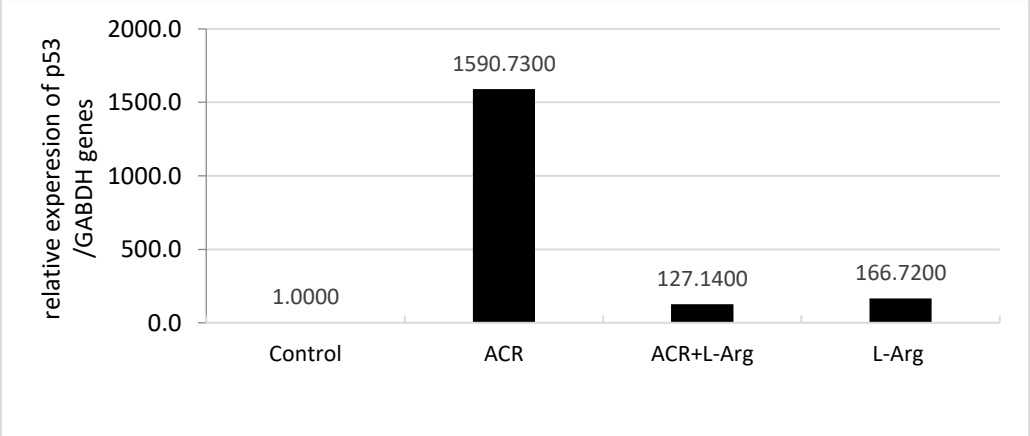

Fig 3: the expression levels of P53 gene among four experimental groups

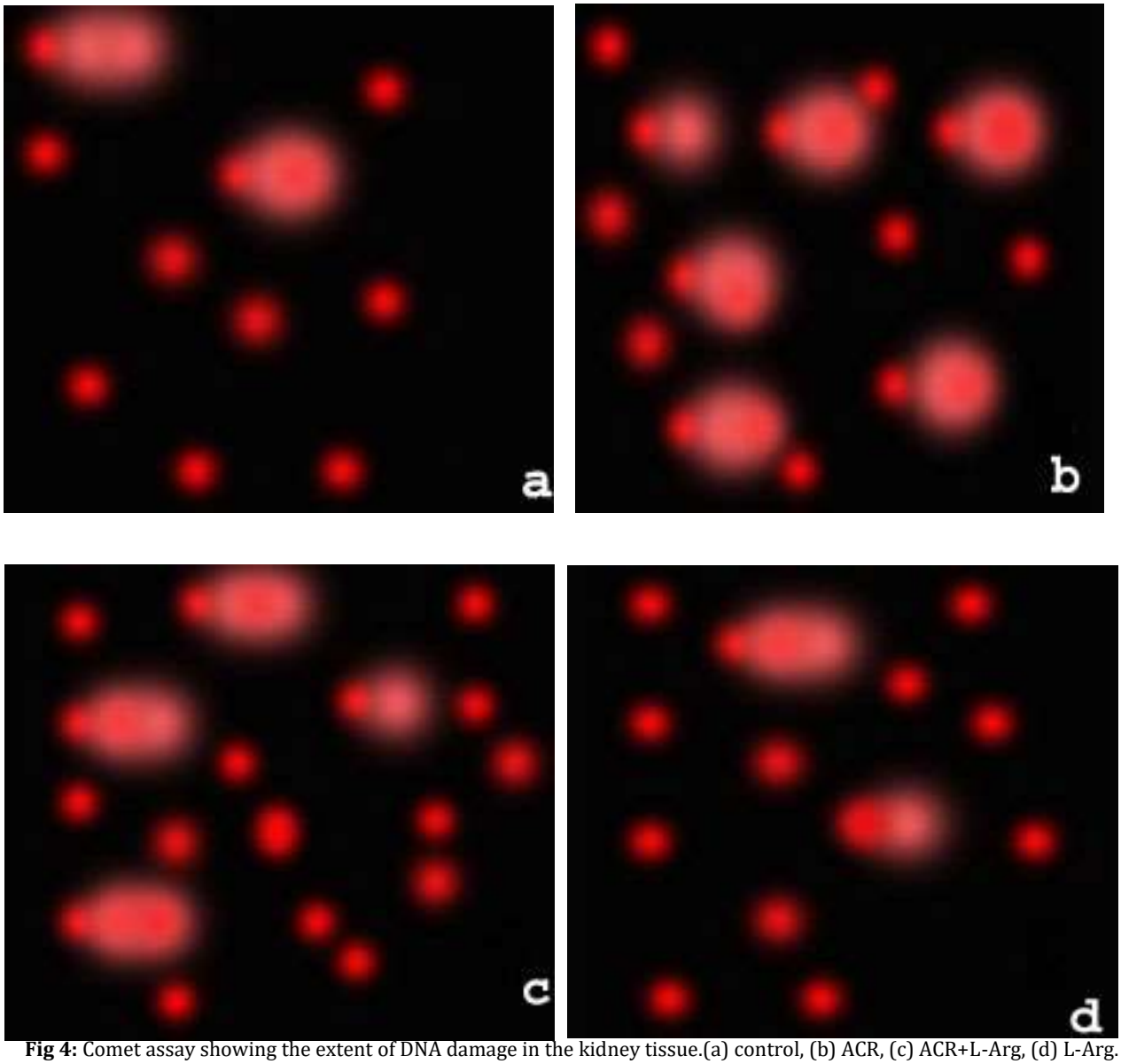

Fig 4: Comet assay showing the extent of DNA damage in the kidney tissue.(a) control, (b) ACR, (c) ACR+L-Arg, (d) L-Arg.