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Assessment of the Effect of Glucocorticoid Injections on the Expression of Apoptotic Active CASP3 and ALDH1A1 Renal Cell Markers in New Zealand Rabbits

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ABSTRACT

Acute kidney injury (AKI) is a suspected renal insult which may develop from different causes, one of these causes is the administration of high doses of glucocorticoids for long period of time as dexamethasone, which has a wide variety uses in different types of illnesses, inflammatory or immunological problems.

To examine the effect of different doses of dexamethasone in producing immunohistochemical changes on renal tissues in a different period of administration using cellular markers (Active CASP 3 and ALDH1A1) in rabbits.

Eight groups of white New Zealand female rabbits were used, seven animals in each group. The first and second (G1 and G2) groups served as control groups. G3 and G4 groups are treated groups injected with (0.5 and 1.5 mg/kg body weight of dexamethasone respectively) for 10 days. G5 and G6 were also treated groups where they were treated with (0.5 and 1.5 mg/kg body weight of dexamethasone respectively) for 15 days. G7 and G8 considered as follow up groups for G4 and G6 groups where they left without treatment for another 10 days for both groups. The renal tissue examined in all groups histologically by using H & E stain, and immunohistochemistry by Active CASP3 and ALDH1A1 markers.

Moderate expression of Active CASP 3 in most of treated and follow up groups with strong expression of ALDH1A1 in most of treated and follow up groups in comparison to control groups (G1 and G2) which was statistically significant.

Dexamethasone produces a pseudo acute kidney injury that healed after discontinuation of drug which is confirmed by expression of active CASP 3 and ALDH1A1 immunohistochemical markers.

Keywords: Kidney, ALDH1A1, Active CASP3, immunohistochemistry, rabbit.

Introduction

There is a lot of proof that dexamethasone has a long history as a corticosteroid medicine (1). It is a strong synthetic glucocorticoid drug for a wide range of inflammatory and immunological problems (2). Dexamethasone's major metabolic target is the liver (3), and acute high doses of it led to hepatic steatosis and mild to moderate aortic arteriosclerosis (4). However, the effect of glucocorticoids on other organs is also reported (5,6,7). The influence of glucocorticoids on the various tissues is achieved by the presence of the glucocorticoid receptor (GR) in these tissues, and one of them is the renal tissues, where the glucocorticoids produce their physiological and pharmacological action. These receptors regulate the glucocorticoid receptor expression genes, and this could be a stimulatory or inhibitory effect (8).

Dexamethasone has an effect on the synthesis of proteins and ribonucleic acids (RNA) by binding effectively to receptor complexes in the cell nucleus. Therefore, macromolecular synthesis may be required even in the case of catabolic or inhibitory steroid actions (9).

In several tissues, such as muscle, skin, and lymphoid cells, the catabolic activities of glucocorticoids resulted in a notable reduction in protein synthesis and an increase in protein and RNA degradation (10).

Glucocorticoids frequently increase lipolysis in adipose tissues and block the absorption of amino acids and glucose. Glucocorticoids promote the healing of wounds, blood eosinophils and lymphocytes, bone matrix, and the inhibition of immunologic and inflammatory reactions. These hormones raise levels of protein and glycogen and activate several enzymes, but they only partially repress some hepatic activities (11).

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The ability of the liver to create more glucose through gluconeogenesis—which takes substrate from other catabolic processes—has grown. Glucocorticoids work together to produce hyperglycemia, a negative nitrogen balance, and fatty loss. Nevertheless, the effects of glucocorticoids are counteracted by other hormones, as insulin, which is raised in response to hyperglycemia and partially reverses certain metabolic abnormalities (12). Glucocorticoids facilitate or augment some actions of other hormones. Cyclic adenosine three ',5'-monophosphate production (cyclic AMP) is usually increased by the latter. Cyclic AMP and glucocorticoids frequently have comparable mechanisms for conserving glucose. Glucocorticoids, which resemble cyclic AMP and other hormones, may have a greater role in the cellular and tissue development of the fetus than previously believed (13). Because of their numerous anti-inflammatory benefits, glucocorticoids are frequently used at higher dosages rather than for their impact on protein and glucose metabolism (14).

Dexamethasone is a synthetic chemical derivative of glucocorticoid hydrocortisone that is used to treat inflammatory diseases in many animal species as well as metabolic diseases in ruminants (e.g., ketosis). To manage chronic processes, dexamethasone is typically administered for several weeks or even months at a time. Once the situation is under control, it is crucial to taper the dose to a chart every three days. This is because the body does not generate its own hormones; instead, it recognizes the presence of these hormones. Over time, the patient's adrenal glands will atrophy to the point where they are unable to react to any stressful circumstance after the medicine is stopped. It could also lead to a cardiovascular crisis. The medication's every-other-day administration keeps the body's own adrenal glands functioning (9).

Acute kidney injury (AKI) is a common insult in medicine, and this may be obtained from many factors, and one of these factors is the consumption of drugs. According to Perazella and Rosner, dexamethasone is considered to produce a pseudo acute kidney injury (AKI) that may occur due to blocking of the creatinine secretion by the tubules and so elevated creatinine level in these patients must be considered or may be due to a disturbance in the hemodynamic environment of the renal tissue and hence an acute kidney injury will develop (15).

A family of highly conserved proteins known as caspases is mainly engaged in inflammation and apoptosis. They are produced as inactive zymogens with an N-terminal prodomain, one large subunit, and one small subunit. When the prodomain is eliminated by splitting the zymogen at a particular aspartic acid residue, the dormant zymogen becomes fully active. The tetramer contains two heterodimers that make up the activated caspase protein. These are the proteases that break a variety of proteins to initiate the process of cell death because they have cysteine residues at the catalytic site. They cleave lamins, cytoskeletal proteins, focal adhesion kinase (FAK), and other proteins, which cause the apoptotic cell to separate from its neighbors, alter cell shape, disassemble the nuclear membrane, and shrink the nucleus. DNA fragmentation is induced by a particular DNase called Caspase Activated DNase (CAD), which is activated by caspase (16).

Aldehyde dehydrogenase one family member A1, or ALDH1A1, this gene encodes a member of the aldehyde dehydrogenase family of proteins. In the primary pathway of alcohol metabolism, aldehyde dehydrogenase comes after alcohol dehydrogenase. The liver has two main isozymes of aldehyde dehydrogenase, cytosolic and mitochondrial, which are characterized by different kinetics, subcellular localization, and electrophoretic mobility. These isozymes are encoded by different genes. Encoded by this gene is the cytosolic isozyme. Research on mice suggests that this gene may be important in controlling the metabolic reactions to a high-fat diet by virtue of its function in the metabolism of retinol (17).

In this study, we examined the effect of different doses of dexamethasone in producing immunohistochemical changes on kidney tissues in a different period of administration using cellular markers (Active CASP3 and ALDH1A1).

Materials and Methods

Study Design: For this investigation, we employed healthy white female New Zealand rabbits weighing between 1000 and 1250 grams, housed them in separate plastic cages, and fed them freely throughout the study period. There were seven animals in each of the eight groups:

1. The first group served as the control group and received normal saline for ten days (G1).

2. The second group served as control group and received normal saline for 15 days (G2).

3. The third group received treatment daily for ten days with an intramuscular injection of dexamethasone sodium phosphate (Amriya pharmaceutical industries/ Egypt as 8 mg / 2 ml ampoules) at a dose of (0.5 mg/kg of body weight (b.w.) equal to 0.1 ml/kg b.w (G3).

4. The fourth group got the same treatment at (1.5 mg/kg b.w. equal to 0.4 ml/kg b.w.) for 10 days (G4).

5. The fifth group was injected with (0.5 mg/kg b.w.) of dexamethasone injection for 15 days (G5).

6. The sixth group was given (1.5 mg/kg b.w.) of dexamethasone ampoule for 15 days (G6).

7. The seventh group was regarded as ing thefollow-up group, which was injected with (1.5 mg/kg b.w.) of dexamethasone injection for ten days and then left without treatment for another ten days.

8. The eighth group was regarded as thefollow-up group, which was injected with (1.5 mg/kg b.w.) of dexamethasone treatment for 15 days and then left without treatment for another ten days.

The animals were put to sleep with chloroform 24 hours after the last dose. The kidneys were removed after the abdomen was dissected, fixed in a 10% neutral buffered formalin solution for 24 hours, dehydration and clearing were done, and then embedded in paraffin, and the resulting blocks were sectioned.

Histology: For each rabbit, three serial slices were cut of 4 micrometers thickness for each. The first slice was put on an ordinary slide and stained by haematoxylin/eosin stain to evaluate the histological changes.

Immunohistochemistry: The second & third slides were put on a positively charged slide for immunohistochemical staining analysis with anti-active CASP3 antibody (Primary antibody from Elabscience, Cat.No.: E-AB-22115) and anti-ALDH1A1 antibody (Primary antibody from ABNOVA, Cat.No.: MAB12300). A secondary antibody detection kit (Elabscience, Cat.No.: E-IR-R211, rabbit/mouse specific HRP/DAB) was used. Using xylene and gradually hydrating it, the slides were dewaxed. After applying pressure cooking using citrate buffer for 20 minutes, the antigen was fully recovered. After being diluted to a 1:200 ratio using background lowering dilution buffer (Abcam, code ab64211), the primary anti-active CASP3 and ALDH1A1 antibodies were allowed to warm up at room temperature for half an hour. Labeled streptavidin-biotin from an Abcam secondary detection kit was used to achieve detection, which was then followed by chromogen staining and DAB. The slides were promptly hydrated, mounted with DPX, and counterstained with hematoxylin [18].

Evaluation of The Immunohistochemical Staining: Without any prior information, all kidney tissue slides were assessed. The staining intensity and percentage for ALDH1A1 and Active CASP3 were estimated as follows:

There were four staining intensity scores: 0 for no staining, 1+ for faint staining, 2+ for moderate staining, and 3+ for high staining.

The percentage labelling the extent of staining was as follows: 0 represents nil, 1 represents less than 10% of the cell stained positively, 2 represents 10–50%, 3 represents 51–80%, and 4 represents more than 80% (19).

Statistical Analysis: The Statistical Packages for Social Sciences, or SPSS, V18, was used to perform statistical analysis. To calculate categorical variables, the percentage, mean, and range (min-max values) were examined. The independent sample t-test and the Pearson Chi-square test (X2-test) were used to validate the qualitative data.

Results

Histology: Sections of H & E showed changes include:

1. vacuolation of tubules.
2. deformation of cells lining the tubules.
3. oedema.
4. inflammatory cell infiltration.
5. vasodilatation and congestion.

The G3 group was the starting point for these alterations, which became more pronounced in the following groups as the dosage and length of therapy increased. The G7 and G8 groups (follow-up groups) also experienced these changes (Fig. 1).

Immunohistochemistry:

Staining Intensity Distribution for CASP3 & ALDH1A1

CASP3 Staining Intensity: Immunohistochemical expression intensity of CASP3 showed a moderate (2+) expression of 57% in G3 when compared to G1 which was the dominant result in G3 group, while CASP3 intensity dominance in G4 was a weak (1+) expression of 57% compared to G1 group. G5

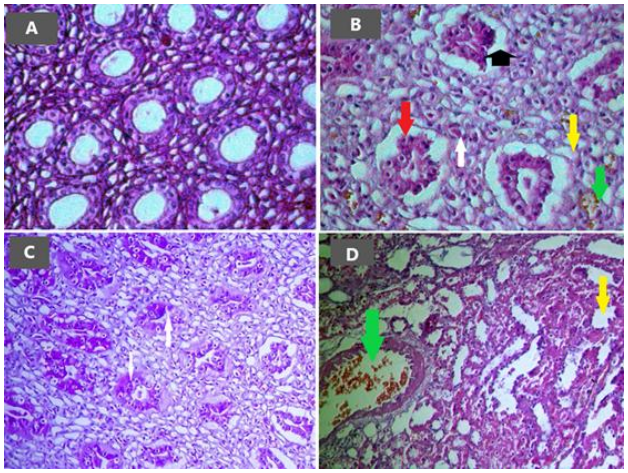

Fig. 1. Photomicrograph of rabbit kidney in control (A) and treated and follow up groups (B, C, D) showing vacuolation of tubules (black arrow), deformation of cells lining the tubules (red arrow), oedema (yellow arrow), inflammatory cell infiltration (white arrow), vasodilatation and congestion (green arrow) H&E (A,B,D 400X, C 100X)

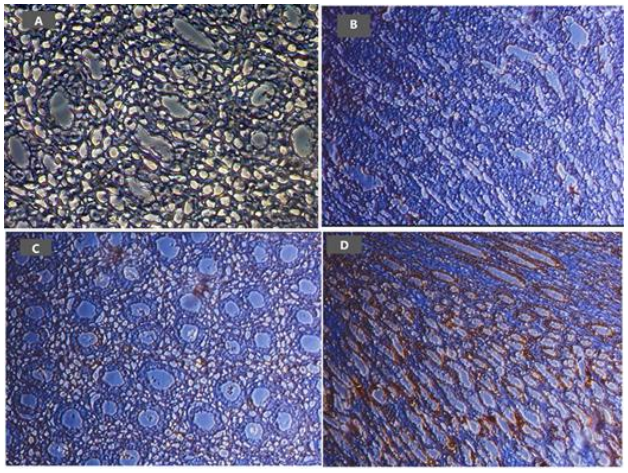

Fig. 2. Photomicrograph of rabbit kidney of the studied groups showing the immunohistochemical reaction of Active CASP3 marker intensity (brown pigmentation) as negative reaction (A), 1+ reaction (B), 2+ reaction (C) and 3+ reaction (D). Active CASP3 (100X)

and G6 showed a strong intensity (3+) of 86% for both in comparison to their control group G2. All these results are statistically significant (Fig. 2).

Follow-up groups G7 and G8 both express CASP3 moderately (2+) of 86% in G7 and 71% in G8 groups in comparison to G1 and G2 groups, respectively, and these results are statistically significant (p-value < 0.0001) as appeared in Fig. 3 and Table 1.

Aldh1a1 Staining Intensity: G1 and G2 groups showed a weak (1+) intensity of ALDH1A1 expression (86% for both); these are the control

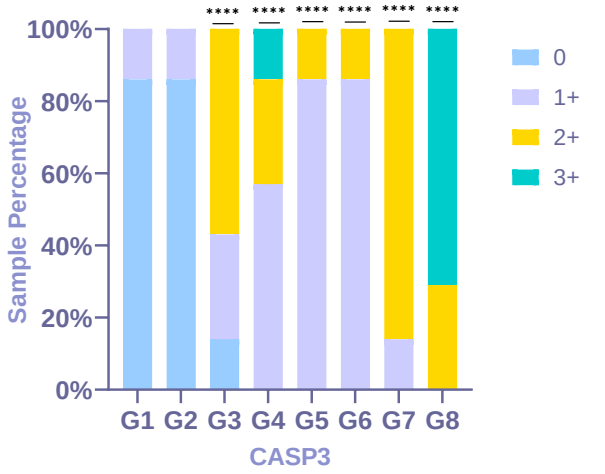

Fig. 3. Staining intensity distribution of CASP3 according to sample type.

G1= control (10 days), G2= control (15 days), G3= low dose (10 days), G4= high dose (10 days), G5= low dose (15 day), G6= high dose (15 day), G7= follow up (high dose, 10 days), G8= follow up (high dose, 15 days). ****= p-value < 0.0001

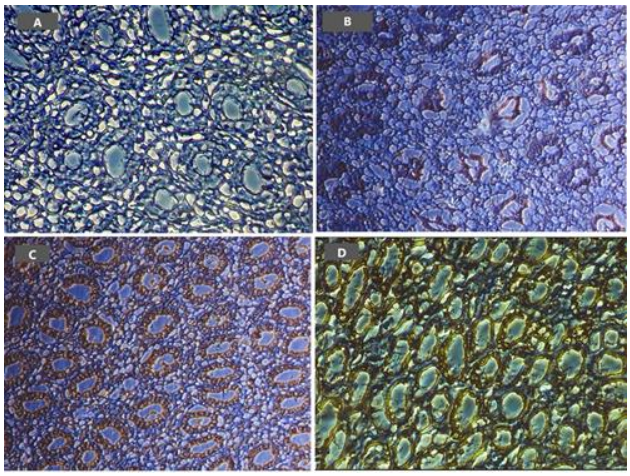

Fig. 4. Photomicrograph of rabbit kidney of the studied groups showing the immunohistochemical reaction of ALDH1A1 marker intensity (brown pigmentation) as negative reaction (A), 1+ reaction (B), 2+ reaction (C) and 3+ reaction (D). ALDH1A1 (100X)

groups. In the treated groups, the expression of ALDH1A1 in G3 and G4 group was moderate which was statistically significant. In G5 group, the intensity was moderate (2+), while group G6 had strong intensity expression (3+) and was significant statistically when compared to control groups. Furthermore, the follow up groups, G7 and G8, showed strong intensity of ALDH1A1 expression of statistically significant p value (Fig. 4, Fig. 5, and Table 1).

Staining Percentage for CASP3 & ALDH1A1

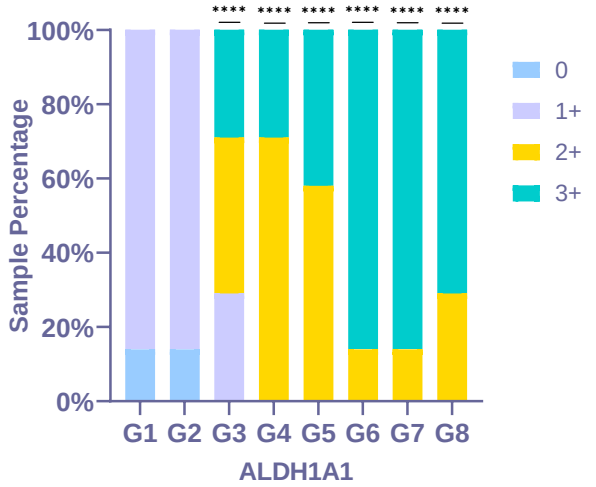

Fig. 5. Staining intensity distribution of ALDH1A1 according to sample type.

G1= control (10 days), G2= control (15 days), G3= low dose (10 days), G4= high dose (10 days), G5= low dose (15 days), G6= high dose (15 days), G7= follow up (high dose, 10 days), G8= follow up (high dose, 15 days). ****= p-value < 0.0001

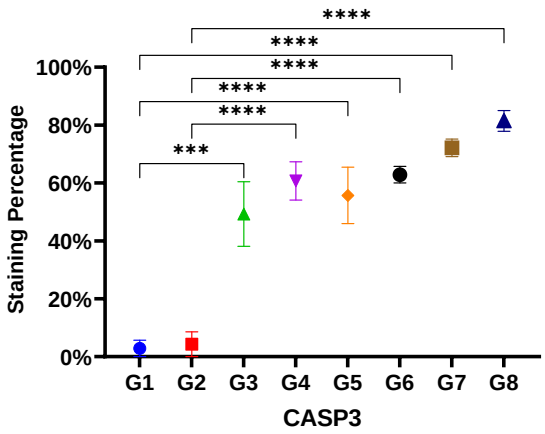

Fig. 6. Staining percentage of CASP3 according to sample type.

G1= control (10 days), G2= control (15 days), G3= low dose (10 days), G4= high dose (10 days), G5= low dose (15 days), G6= high dose (15 days), G7= follow up (high dose, 10 days), G8= follow up (high dose, 15 days). ****= p-value < 0.0001, ***= p-value < 0.001

CASP3 staining percentage: The higher percent of CASP3 expression was in G8 group of animals, which is equal to 81.43% ± 9.44% (mean ± SD), and the lowest expression was in the G1 group represented 2.85% ± 7.55% (Fig. 6, Table 2).

Aldh1a1 Staining Percentage: ALDH1A1 was highly expressed in G3 group representing 87.86% ± 8.59%, while the lowest expression of the marker was obtained in G2 group equal to 31.43% ± 19.52% (Fig. 7, Table 2).

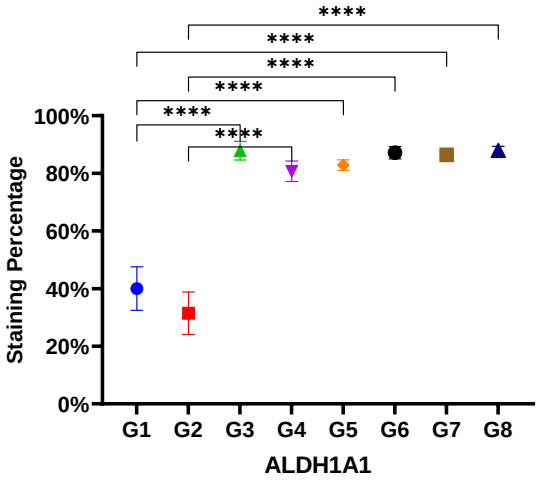

Fig. 7. Staining percentage of ALDH1A1 according to sample type.

G1= control (10 days), G2= control (15 days), G3= low dose (10 days), G4= high dose (10 days), G5= low dose (15 days), G6= high dose (15 days), G7= follow up (high dose, 10 days), G8= follow up (high dose, 15 days). ****= p-value < 0.0001

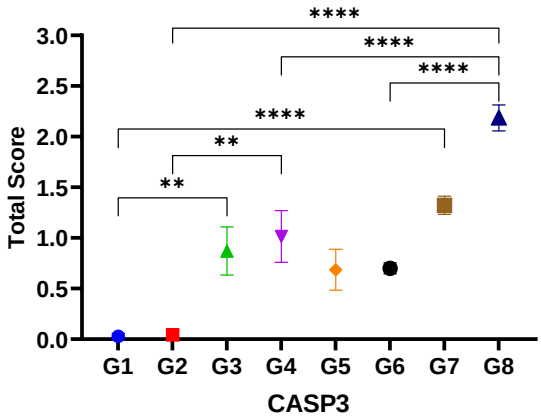

Fig. 8. Total Score of CASP3 according to sample type.

G1= control (10 days), G2= control (15 days), G3= low dose (10 days), G4= high dose (10 days), G5= low dose (15 days), G6= high dose (15 days), G7= follow up (high dose, ten days), G8= follow up (high dose, 15 days). ****= p-value < 0.0001, **= p-value < 0.01

Total score for CASP3 & ALDH1A1

Total Score For Casp3 Expression: The higher score of expression of CASP3 was obtained in the G8 group of 2.186 ± 0.338 (mean ± SD), while the lowest score was in the G1 group representing 0.028 ± 0.075 (Fig. 8, Table 3).

Total Score For ALDH1A1 Expression: The total score of ALDH1A1 was highest in the G6 group at 2.479 ± 0.288 (mean ± SD), whereas the lowest score obtained from G2 (0.314 ± 0.195) (Fig. 9, Table 3).

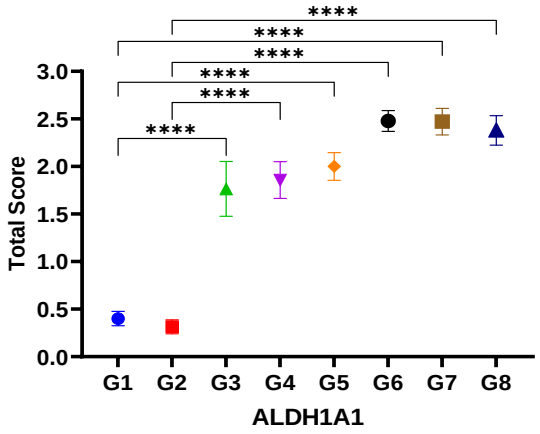

Fig. 9. Total Score of ALDH1A1 according to sample type.
G1= control (10 days), G2= control (15 days), G3= low dose (10 days), G4= high dose (10 days), G5= low dose (15 days), G6= high dose (15 days), G7= follow up (high dose, ten days), G8= follow up (high dose, 15 days). ****= p-value < 0.0001

Discussion

Dexamethasone, a corticosteroid drug, as we know, has different actions on various body organs (20); its effect sometimes is stimulatory, as in pancreas, kidney, and other organs (21), and inhibitory in others like liver (22), and even in the same organ; it produces both stimulatory and inhibitory actions depending on the dose used and on the length of the period of consuming dexamethasone treatment.

One of dexamethasone's inhibitory actions is the production of unfunctional cells that become apoptotic cells to overcome this negative dexamethasone effect. Apoptosis is a programmed cell death caused by many external and internal factors, and this can be performed either by extrinsic and/or intrinsic pathways.

One of the important methods used for the detection of apoptosis is the immunohistochemical reaction using active caspase-3 protein. Although caspase-3 is thought to have a role in the structural disintegration of cells going through apoptosis (23,24), caspase-independent cell death has been observed in the kidney (25) and can happen.

The caspases are divided into three groups: inflammatory caspases (Caspases 1,4,5), executioners (Caspases 3,6,7), and initiators (Caspases 2, 8, 9, 10). In humans, apoptosis is initiated by caspases 8 and 9, and the cell is committed to an apoptotic fate by caspase 3 (26).

Our results indicate that the expression of CASP3 started to increase as the dose of dexamethasone increased and as the duration became longer (15 days), which indicated that the rate of apoptotic cells was somewhat increased at the beginning, dexamethasone-induced apoptosis is confirmed by other researchers (21,27), Caspase activation, which down-streamed DEX/Gc receptor binding and PI-PLC/aSMase pathway stimulation, is required for this apoptosis (28).

The scenery of apoptosis still appeared in follow-up groups, although it was of moderate expression. This scenery is acceptable as the injured tissue of the kidney is still healing its cells from the damage that occurred due to dexamethasone, and so the programmed cell death is a reflection of the injury events.

The presence of renal damage was highlighted by ALDH1A1 expression. When synthetic glucocorticoids were administered, the renal tissue had a histological alteration that suggested damage (29). Injections of dexamethasone in the prenatal period produced fewer nephrons than controls, but they also markedly increased the excretion of salt and urine flow. Additionally, it increased urinary potassium excretion by 50% (30), but it had no direct effect on potassium secretion by single microperfused tubules.

All these theories indicate the occurrence of renal injury by dexamethasone, but it is an AKI, as proposed by Perazella & Rosner (15). Strong positive expression of ALDH1A1 in the treated groups and follow-up groups was to overcome the renal injury obtained by dexamethasone, and as we noticed that the expression was stronger than CASP 3, according to Yu et al., aldehyde dehydrogenase-2 was able to significantly reduce regional ischemia/reperfusion injury in a number of organs, including the tissues of the kidney (31). This was achieved either by activating the Beclin-1 pathway through autophagy activation (32) or by inhibiting cell apoptosis (31). This is agreed with our results of enhancement of ALDH1A1 expression and becoming stronger as the CASP 3 expression had a moderate appearance in the treated groups and follow-up groups, whereas ALHH1A1 was of strong intensity expression in these follow-up groups as the healing process began. From all these findings, the theory that dexamethasone produces pseudo-acute kidney injury (AKI) (15) is confirmed and indicates that all these events of renal injury by dexamethasone using different doses and durations are temporary and need only time for the healing process to occur, and so we recommend to use a very higher doses

Table 1: Staining Intensity (chi-square) Versus Marker Type and Sample Type

Staining Intensity	Sample Type																P value
	G1		G2		G3		G4		G5		G6		G7		G8		
	CASP3	ALDH1A1	CASP3	ALDH1A1	CASP3	ALDH1A1	CASP3	ALDH1A1	CASP3	ALDH1A1	CASP3	ALDH1A1	CASP3	ALDH1A1	CASP3	ALDH1A1	
0	86 %	14 %	86 %	14 %	14 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %	
1+	14 %	86 %	14 %	86 %	29 %	29 %	57 %	0 %	86 %	0 %	86 %	0 %	14 %	0 %	29 %	0 %	<0.000
2+	0 %	0 %	0 %	0 %	57 %	42 %	29 %	71 %	14 %	58 %	14 %	14 %	86 %	14 %	71 %	29 %	1
3+	0 %	0 %	0 %	0 %	0 %	29 %	14 %	29 %	0 %	42 %	0 %	86 %	0 %	86 %	0 %	71 %	

Table 2: Sample & Marker Type In Relation To Staining Percentage (t-test)

Sample Type	IHC Marker	Staining percentage	
		Mean	SD
G1	CASP3	2.85%	7.55%
	ALDH1A1	40.00%	20.00%
G2	CASP3	4.28%	11.34%
	ALDH1A1	31.43%	19.52%
G3	CASP3	49.29%	29.50%
	ALDH1A1	87.86%	8.59%
G4	CASP3	60.71%	17.42%
	ALDH1A1	80.71%	9.32%
G5	CASP3	55.71%	25.73%
	ALDH1A1	82.86%	4.88%
G6	CASP3	62.86%	7.55%
	ALDH1A1	87.14%	5.66%
G7	CASP3	72.14%	8.09%
	ALDH1A1	86.43%	4.75%
G8	CASP3	81.43%	9.44%
	ALDH1A1	87.22%	3.93%

Table 3: Sample and Type of Marker Compared to Total Score (t-test)

Sample Type	IHC Marker	Total score	
		Mean	SD
G1	CASP3	0.028	0.075
	ALDH1A1	0.400	0.200
G2	CASP3	0.042	0.113
	ALDH1A1	0.314	0.195
G3	CASP3	0.871	0.629
	ALDH1A1	1.764	0.764
G4	CASP3	1.014	0.674
	ALDH1A1	1.857	0.509
G5	CASP3	0.685	0.533
	ALDH1A1	2.000	0.383
G6	CASP3	0.700	0.141
	ALDH1A1	2.479	0.288
G7	CASP3	1.321	0.237
	ALDH1A1	2.471	0.368
G8	CASP3	2.186	0.338
	ALDH1A1	2.379	0.410

and for a longer period of time to examine the possibility of a permanent injury of renal tissue.

Dexamethasone produces a pseudo-acute kidney injury that healed after discontinuation of the drug, which is confirmed by expression of active CASP 3 and ALDH1A1 immunohistochemical markers.

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Conflict of interest: none declared.

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