The effects of different inhibitory pathways of prostaglandin E₂ biosynthesis on renomedullary interstitial cells in rats: a multidisciplinary study

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Abstract

Renomedullary interstitial cells (RMICs) are the most dominant cell type in inner renal medulla. Their most distinct characteristic is the presence of multiple lipid droplets in their cytoplasm. These lipid droplets are believed to be the storage units for precursors of prostaglandins (PGs), prostacyclin and medullipin. Especially prostaglandin E₂ (PGE₂) is synthesized by RMICs in kidney. PGs are produced by three key steps: 1) Arachidonic acid (AA) release from membrane phospholipids by the action of phospholipase A₂ (PLA₂); 2) Formation of prostaglandin H₂ (PGH₂) from AA by the action of cyclooxygenases (COXs); 3) Specific PG synthesis metabolism from PGH₂. PG biosynthesis can be regulated via activation or inhibition of these steps. In this study, we examined the effects of PGE₂ inhibition in different steps on RMIC function, the number of lipid droplets, medullary hyaluronan (HA) content and cell viability. We formed four groups (n=8): First group was control and treated with intraperitoneal (ip) 0.9% saline. Second group in which we inhibited AA release from membrane phospholipids was injected with ip dexamethasone (DEX) (2 mg/kg, 10 days); third group was treated with ip indomethacin (IND) (1 mg/kg, 10 days) to inhibit non-specific COX at the stage of PGH₂ formation from AA; and the fourth group was injected with ip celecoxib (CXB) (1 mg/kg, 10 days) to examine selective cyclooxygenase-2 (COX-2) inhibition.

We dissected renal medulla of the sacrificed animals after 10 days to analyze with light and electron microscopy. We counted the lipid droplets in 50 random RMICs for each animal (x6.000 magnification) in electron microscopy. Our morphometric analysis showed that the number of lipid droplets was significantly decreased in DEX group and was significantly increased in IND and CXB groups when compared to control. In addition, medullary HA content and CD44 immunoreactivity were significantly increased in all groups when compared to control. When we analyzed cell viability, we found that RMIC apoptosis was significantly higher in PGE₂ inhibited groups when compared to control. Besides this, 24-hour urine values collected on the 10th day were significantly increased in dexamethasone and indomethacin groups; but in celecoxib group the values were similar to control. These results indicate that lipid granules may be numerical and functionally influenced from PGE₂ changes, these granules may be storage units of AA, functional changes in RMICs by PGE₂ may influence HA quantity of medullary interstitium and urine volume, and finally PGE₂ inhibition may lead to RMIC apoptosis.

Keywords: Kidney, Non steroidal anti-inflammatory drugs, Prostaglandin E₂, Renomedullary interstitial cells, Cyclooxygenase-2

Introduction

The kidney has both pro- and anti-hypertensive activities [1,2].

Renomedullary interstitial cells (RMICs) are the most abundant cell type in the inner renal medulla and they have several char-

acteristic features. Their most distinctive characteristic is the presence of multiple lipid droplets in their cytoplasm. The investigators suggest that RMICs are secretory cells because of their well-developed rough endoplasmic reticulum (RER), Golgi complex and the content of multiple lipid droplets [3]. Therefore, they are referred to as the lipid-containing type 1 interstitial cells, which are the principal cells in the inner medulla [4]. In 1969, Bohman and Maunsbach isolated lipid droplets from RMICs and found that they had a vasodepressor activity. The content of these lipid granules are believed to be storage units for precursors of prostaglandins (PGs), prostacyclin and medullipin [1]. The amount and the size of lipid droplets are shown to be involved in the changes of diuretic stages, water-salt balance, renal perfusion pressure (RPP) and medullary blood flow (MBF) [1]. RMICs are often arranged in rows between the loops of Henle and vasa recta, with their long axes being perpendicular to those of adjacent tubules and vessels, thus resembling the rungs of a ladder [3]. This anatomic arrangement suggests that RMICs may play an important role in maintaining urinary concentrating ability via preventing the axial diffusion in concentration gradient [5,6]. Studies demonstrate that the transplants of fragments from the renal medulla or of the cultured RMICs reverse renoprival hypertension [1,7-13]. The biochemical analyses of these medullary transplants show the presence of prostaglandins (PGs), triglycerides, cholesterol esters and free fatty acids [14]. At the end of these studies, three types of substances with potential antihypertensive properties are derived from the kidney medulla [12]:

- 1) Prostaglandins (PGs)
- 2) Antihypertensive neutral renomedullary lipid (ANRL)
- 3) Antihypertensive polar renomedullary lipid (APRL).

Cell culture studies demonstrate that PGs, especially prostaglandin E_2 (PGE_2) are synthesized by RMICs in the kidney [3,7,12,13]. PGs are produced by three key steps: 1) Arachidonic acid (AA) release from membrane phospholipids by the action of phospholipase A_2 (PLA_2); 2) Formation of prostaglandin H_2 (PGH_2) from AA by the action of cyclooxygenases (COXs); 3) Specific PG synthesis metabolism from PGH_2 . PG biosynthesis can be regulated via activation or inhibition of these steps. Various researchers work with PGE_2 stimulators and inhibitors in their renal medulla studies; however, we are unable to find a research on inhibition before AA release in the literature. We believe that using pre-AA release inhibitors in addition to COX inhibitors better clarify the characteristic of lipid droplets and whether their activity in PGE_2 synthesis continues via other pathways or not. Selective cyclooxygenase-2 (COX-2) inhibitors (coxibs) and several non-selective COX-inhibiting nonsteroidal anti-inflammatory drugs (NSAIDs) block endogen PG synthesis. However, studies suggest that, this inhibition leads to RMIC apoptosis [15-20]. Furthermore, hyaluronan (HA) which is a negatively charged glycosaminoglycan (GAG) found in renal interstitium and in the synthesis by RMICs, may play an important role in maintaining of water homeostasis

[21,22]. The high concentrations of HA in the kidney medulla support tubules and blood vessels and it is important for urine concentration [22]. HA also has a wondrous renal water handling capacity which is important for maintaining the concentration gradient and affects water transport in renal medulla [21-23].

In this study, we examine the effect of inhibition of PGE_2 synthesis in different pathways on RMIC function, the number of lipid droplets, medullary HA content and cell viability. The glucocorticoids inhibit PLA_2 enzyme in AA release stage from membrane phospholipids. Thus, they reduce the formation of all the eicosanoids (PGs, prostacyclins, thromboxanes and leukotrienes). For this reason, we inhibit AA release with PLA_2 inhibition by using dexamethasone (DEX) which is a glucocorticoid. AA released via PLA_2 is converted into PGG_2 by being presented to COX (prostaglandin H synthase, PGHS). Then, COXs generate intermediate endoperoxide PGG_2 which is a substrate for terminal synthases by reducing PGG_2 . We inhibit PGE_2 formation from PGH_2 in two different pathways via COX. For this study, we use indomethacin (IND) which is a non-selective COX inhibitor (COX-1 and COX-2 inhibition), and celecoxib (CXB) which is a selective COX-2 inhibitor.

We aim to analyze the morphological changes and the number of lipid droplets of RMICs in different PGE_2 inhibition groups. Furthermore, we investigate how medullary HA content, amount of urine and cellular viability from PGE_2 inhibition are affected. Finally, we search for an answer: Are the lipid droplets the storage units for PG precursor AA?

Methods

Thirty-two adult male Wistar albino rats weighing 180-200g (Experimental Animals Reproduction and Research Laboratory, Istanbul University Cerrahpasa Medical Faculty, Turkey) were housed in individual cages in a temperature- and humidity- controlled room with a 12-h light/dark cycle. They were fed with standard rat chow and had free access to tap water. Rats were divided into four groups ($n=8$). Control group was daily injected with 1 ml ip 0.9% NaCl solution during 10 days. Second group was daily injected with ip 2 mg/kg dexamethasone during 10 days. Third group was injected with ip 1 mg/kg indomethasine during 10 days and the last group was injected with ip 1 mg/kg celecoxib during 10 days. All rats were sacrificed under anesthesia at the end of the study (10th day) according to the regulation of animal ethical committee of Istanbul University.

Biochemical analyses

24 h urine samples were collected on the 1st and 10th days from animals. Urine volumes were measured using micropipets and pH values were measured using pH-indicator strips (Merck; pH:0-14). The blood samples were collected on the 10th day from animals via intracardiac-puncture method. Samples were taken in heparinisetubes and centrifuged in 1500 rpm for 15 minutes. Serum samples collected and PGE_2

levels were determined by ELISA method.

Light microscopical analyses

Renal medullar tissues were fixed in 10% neutral buffered formalin dehydrated with graded ethanol and embedded into paraffin. The sections of 5µm thickness were placed onto poly-*L*-lysine-coated slides. Histological determination of renal medullar HA content was carried out by Alcian blue staining (pH: 2.5). The evaluation of the Alcian blue staining was done utilizing H-SCORE from renal papilla and inner medulla, using randomly selected 5 different fields for each slide, x40 magnification.

Immunohistochemical analyses

The sections of 5 µm thickness were placed onto poly-*L*-lysine-coated slides. After deparaffinization and rehydration samples were placed in citrate buffer (pH6), antigen retrieval was performed by treating the samples in a microwave oven at 750 W for 5 min three times, and then slides were washed in PBS. Immunohistochemistry procedure was performed using standard streptavidin-biotin-peroxidase method. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide (Dako, Glostrup, Denmark). Each section was then incubated for 15 minutes at room temperature with blocking solution to block cellular peroxidase activity. After blocking, sections were incubated with primary antibodies mouse anti-rat CD44 diluted 1:100 (Life Span Biosciences, USA) and rabbit anti-human, rat, mouse active caspase-3 diluted 1:100 (Millipore) for 1 hour at room temperature, then washed with PBS. Specific staining was performed with biotinylated universal secondary antibody and horse radish peroxidase streptavidin-complex. Immunoreactivity was developed using amino-ethyl-carbazole as chromogen (Invitrogen). The sections were counter-stained with Mayer's hematoxylin and mounted with glycerol gelatin (Sigma). All incubations were performed in a moist chamber at room temperature. As for negative control, distilled water was performed instead of primary antibody.

Electron microscopical analyses

After necropsy, the left kidney medulla was immediately divided into 1mm³ pieces for transmission electron microscopy. They were firstly fixed in 3% glutaraldehyde in a 0.1 M phosphate buffer solution (PBS), post-fixed by 1% OsO₄ prepared in the same buffer solution, dehydrated and embedded into araldite medium. Semi-thin sections were cut into 1 µm thickness by glass knives to help of the ultra microtome and stained with 1% toluidine blue (prepared with 1% borax in bidistilled water). The sections were examined under a binocular light microscope using immersion objective. Ultra-thin sections were obtained in 50 nm thickness onto copper grids (200 meshes) with the same microtome, stained with uranyl acetate and lead citrate and they were investigated by transmission electron microscope (Jeol JEM 1011).

Morphometric studies

We obtained ultra-thin sections from one animal in each experimental group and counted lipid droplets in randomly chosen 50 different RMICs with EM micrographs (x6000 magnification). Data was statistically evaluated.

H-Score and semi-quantitative evaluations

The evaluations of the alcian blue staining and immunohistochemical staining were done utilizing H-SCORE. From each randomly selected slides per animal, five different fields were evaluated. Stainings were scored in a semi-quantitative fashion that included the intensity of specific staining in sections. The evaluations were recorded as values between 0-300 of five intensity categories: 0 (none), 0-75 (weak but detectable above control), 75-150 (distinct), 150-225 (dense), 225-300 (intense). Two observers blinded to the experimental groups performed the H-SCORE. For each tissue, obtained an average H-SCORE value. (0:negative; 1:weak positive; 2:positive; 3:dense positive; 4:very dense positive and presented in [Figure 1](#) and [Table 1](#)).

Statistical analyses

Statistical analyses of 1st and 10th days urine volumes, 1st and 10th days urine pH values, PGE₂ values in blood serum, HSCORE values of alcian blue staining and immunohistochemistry analyses and the number of lipid droplets were compared by One-Way ANOVA tests. Statistical calculations were performed using Sigma Stat for Windows, version 3.0 (Jandel Scientific, San Rafael, CA). Comparisons were made versus the inhibition groups as a control. Probability values of less than 0.05 (p<0.05) were considered significant; values are presented as mean ±SEM.

Results

Biochemical findings

In control and experimental groups, urine volumes (ml/24 hours) and pH (0-14) values are shown in [Table 1](#) and [Figure 1](#). 1st day urine volumes were not significantly different from each other (p>0.05). 10th day urine volumes were significantly different between control and experiment groups (p=<0.001). Urine volumes were found to increase in DEX and IND groups, while they were similar in CXB groups when compared with control ([Table 1](#) and [Figure 1](#)). The pH values of 1st and 10th day urine samples were not statistically significant (p>0.05) ([Table 1](#) and [Figure 1](#)). We validated our experiment by measuring PGE₂ levels by ELISA, in blood serum taken on the 10th day. In all inhibitions groups, PGE₂ levels were shown to be significantly lower than control (p=<0.05) ([Table 1](#) and [Figure 1](#)).

Light microscopic findings

Alcian blue dyes non-sulphatated acid mucopolysaccharides and acetic mucins. In this study, we used this dye to determine HA, a non-sulphatated GAG in interstitial field [24]. HA levels were shown to be significantly elevated in both areas in inhibition groups when compared to control (p<0.05) ([Table 1](#)).

Table 1. All data were given in the table.

GROUPS	1 st day urine volume (ml/24h)	10 th day urine volume (ml/24h)	1 st day urine pH (0-14)	10 th day urine pH (0-14)	PGE ₂ levels (pg/ml)	H-SCORE Values of Alcian Blue Staining		H-SCORE values of Cas-3 and CD44 immunopositivity		Number Of Lipid Droplets
						Papilla	Inner Medulla	Cas-3	CD44	
CNT	8,85	9,65	10,5	10,5	5,285	2	2	2	2	4,9925
DEX	13,2	21	11	10,5	0,406	3	3	3	3	1,85
IND	13,45	17,15	10,5	10	1,115	4	4	3	4	9,02
CXB	9,3	8,8	10,5	10	1,690	4	4	3	3	7,9625

(Figure 1 and 2). Semi-thin sections were dyed with toluidine blue and examined with immersion objective (x100). Increased lipid droplets were observed in groups where PGE_2 cannot be produced because of the COX inhibition after AA step (IND, CXB) (Figure 2).

Figure 2. The Alcian blue staining in renal papilla and inner medulla (Magnification: x40). The semi-thin sections dyed with toluidine blue (Magnification: x100). Arrows indicated the increase lipid droplets in COX inhibited groups (IND and DEX).

Immunohistochemical findings

Cells that show a red reaction were accepted as immune positive (Figure 3). In Cas-3 staining there was significant difference between groups ($p < 0.05$) (Table 1 and Figure 1). PGE_2 inhibition was shown to increase apoptosis when compared to control ($p < 0.001$) (Figure 3). H-SCORE values determined for CD44 were significantly different between inhibition and control groups ($p < 0.05$) (Table 1 and Figure 1). PGE_2 inhibition was shown to increase CD44 receptor numbers when compared to control ($p < 0.001$) (Figure 3).

Figure 3. Immunohistochemical determination of Caspase-3 and CD44 in all groups (Magnification x100). Cas-3 and CD44 immunoreactivity are shown to be increased in all inhibition groups while compared with control ($p < 0.001$).

Ultrastructural findings

In control group, RMICs were found to be located between ascending and descending loops of Henle, capillaries and collecting tubules in longitudinal sections (Figure 4). RMICs were shown to have a polygonal shape in this group, with multiple cytoplasmic inclusions (Figure 4). Cytoplasmic density was normal. Most interestingly, osmophilic lipid granules of various sizes were present in the cytoplasm (Figure 4). Nucleus was usually round or eliptic, with condensed heterochromatin peripherally (Figure 4). In some of the analyzed cells,

Figure 4. Electronmicrographs of RMICs in all groups. CNT: A) The longitudinal sections of RMICs between ascending and descending loops of Henle, capillaries and collecting tubules (x6,000). B) Circled area in A. SER has a normal appearance (x12,000). C) A RMIC in control group with normal appearance (x15,000). DEX: A) A RMIC with decreased lipid droplets (x6,000). B) Circled area in A. Lysosomes filled with granular material (arrows) (x25,000). C) RMICs were deprived of organelles. Empty SER cisternae and degeneration of SER membrane (x12,000). IND: A) Note the increased number of lipid droplets in RMIC cytoplasm (x6,000). B) Circled area in A. Note the enlarged SER and PNCs. Arrow shows lipid droplets in the process of degeneration to form myelin structures (x20,000). C) An apoptotic RMIC (x6,000). CXB: A) A RMIC from CXB group (x6,000). B) Circled area in A. Mitochondria show myelin pertrophy. Note the close relation of mitochondria with enlarged SER cisternae (x20,000). C) Note the increased number of lipid droplets and organelles in RMICs (x6,000). Red arrow shows an apoptotic RMIC. SER: Smooth endoplasmic reticulum. White stars: RMICs. Red stars: Apoptotic RMICs.

outer nuclear membrane was observed to enlarge and form perinuclear cisternas (PNC) (Figure 4). Round shaped mitochondria with less crista and very dense matrix were observed (Figure 4). Lysosomes were consisting of lysosomal material (Figure 4). SER and perinuclear location of Golgi complex was well determined in cells (Figure 4). In some of the samples, RER was also present. Free ribosomes were also observed in some samples (Figure 4).

In DEX group, cellular lipid droplets in RMICs were significantly decreased (Figure 4). Heterochromatin was increased and cells were deprived of organelles (Figure 4). ER cisternas were usually empty (Figure 4). Most specific property of these groups was endothelial hypertrophy (Figure 4). Lysosomes were filled with granular material (Figure 4). Mitochondrion was rarely observed (Figure 4).

In IND group, lipid droplets in RMICs were significantly increased (Figure 4). Heterochromatin was increased and heterogenously located in the nucleus (Figure 4). ER cisternas were enlarged, containing granular material (Figure 4). PNCs were also enlarged with granular material (Figure 4). Lipid droplets stacked in cytosol, since they were not used, were marked to be degraded, engulfed in lysosomes, and then formed myelin structures (Figure 4). In this group, the frequency of apoptotic cells were highly increased (Figure 4).

In CXB group, lipid droplets in RMICs were significantly increased (Figure 4). Cells were euchromatic and rich in organelle, like an active cell (Figure 4). ER was well developed and enlarged ER cisternas were present (Figure 4). Also developed Golgi system had a perinuclear location. Mitochondria were observed to be hypertrophic (Figure 4). As in the previous group, this group also had increased apoptotic cells (Figure 4).

Morphometric findings

The number of lipid droplets in randomly selected 50 RMICs was counted in ultra-thin sections prepared for electron microscopy analysis, x6000 magnification. Data was analysed by One Way ANOVA (Table 1) (Figure 1). In the DEX group, where AA formation via PLA₂ was inhibited, number of lipid droplets was significantly decreased when compared with control ($p < 0.001$) (Table 1 and Figure 1). Contrarily, number of lipid droplets were shown to be significantly increased in IND and CXB groups, compared to control ($p < 0.001$) (Figure 1).

Discussion

Kidneys are known to show pro- and anti-hypertensive activity. The pro-hypertensive effect of kidney is regulated by locally active vasopressor systems, including renin-angiotensin system; while the anti-hypertensive effect is thought to depend on vasodepressor agents [1,13].

Muirhead et al. showed that renal cortical fragment transplantation does not inhibit malign hypertension development, while transplantation of renal medullar fragments of RMICs in culture overcomes renoprival hypertension [7,9-13,25]. Biochemical analyses revealed that these transplants contain

abundantly PGs, triglycerides, cholesterol esters and free fatty acids [14].

Renal medulla is the primary synthesis location for PG synthesis. Tissue culture studies on RMICs revealed that these cells are specialized in PG synthesis [1,13]. It is well known that the most specific property of RMICs is the numerous lipid droplets they contain [1,3,8]. The number and size of these lipid droplets change according to the species and physiological state [1]. The number and size of lipid droplets are reported to decrease in the 24 hours following dehydration [1]. In the water-restored rats, the number and size of lipid droplets increased [1]. Although there is not a clear connection identified between lipid droplets and diuretic state of the animals, further studies should be performed to confirm this. The content of these lipid droplets is also not completely known but they are thought to store pivotal products of PGs [1]. The relation between RMIC lipid droplets and renal medullar antihypertensive effect depends on these findings.

Our study also stems from the hypothesis that lipid droplets could be storage units for AA; and we examined the effect of PGE₂ inhibition at different steps on cellular lipid droplets. It is known that PGs are produced in 3 steps via renal COXs (COX-1 and COX-2) by releasing AA from membrane phospholipids [26]:

1. AA release from membrane phospholipids by PLA₂.
2. Production of PGH₂ from AA by COX.
3. Specific PG synthesis metabolism.

PG production can be regulated via activation and inhibition of these steps. Various researchers used PGE₂ inhibitors and stimulators in their studies on RMICs; however, we could not find any inhibition performed pre-AA-release in our literature studies. We designed this study to clarify how this kind of inhibition together with COX inhibition, and the co-use of specific and non-specific COX inhibitors in experimental groups effect lipid droplets, and whether the activity of these cells during PGE₂ production process continues using other pathways or not. In our study, we used a glucocorticoid DEX, to inhibit PG synthesis in AA release step by PLA₂; a non-specific COX inhibitor IND (NSAID), to inhibit production of PGH₂ from AA step; and a specific COX-2 inhibitor CXB (coxib).

In DEX group, cellular lipid droplet numbers were significantly decreased when compared to control. Nucleus was rich in heterochromatin and the cells were deprived of organelles, as in inactive cells. Enlarged ER cisternas were mostly empty. We assume that these cisternas are empty because PGE₂ cannot be produced, since AA synthesis is inhibited. We also observed endothelial hypertrophy and capillary narrowing in this group.

In IND group, the number of cellular lipid droplets was significantly increased. We believe this increase is the result of non-processed (because of the lack of COX) and build-up AA in the cell. Heterochromatin was found to be increased and the cellular structure was diminished and degenerated. In addition, the cells had highly enlarged ER cisternas and

disturbed mitochondria and Golgi. We also observed defected lipid droplets and accumulation of these granules in lysosomes.

The number of lipid droplets showed a smaller increase in CXB group (specific COX-2 inhibition group) when compared to IND group, and that the cells in the CXB group have a richer organelle appearance suggests that PG synthesis continues, although minimally, via COX-1 pathway. The fact that PGE₂ levels measured in blood serum are higher in CXB group compared to IND supports this hypothesis. 10th day urine volumes, showing an increase in DEX and IND groups while CXB values were similar to control, also propose that COX-1 pathway has a role in PGE₂ synthesis in the CXB group. In all three groups where we inhibited PGE₂, the significant decrease in granule numbers of RMICs in pre-AA-release inhibited group by DEX, and the significant increase observed with COX inhibition after AA release by IND and CXB suggest that these granules may be storage units for AA.

It is proven by in vitro and in vivo studies that RMICs synthesize HA [23,27,28]. HA may be regulated depending on the water balance of the organism. Moreover, it has been reported that AVP decreased HA levels [22] by increasing hyaluronidase activity [29-31]. The primary cell receptor for HA is CD44 and it is also identified in RMICs [28]. It is known that this receptor plays a role in cell-cell clustering, matrix-cell interaction, cell migration and receptor-specific HA internalization/fractionation. CD44 is a transmembrane glycoprotein and consists of 4 functional domains. The change of these domains leads to change in the structure and thus, changes in HA binding and the interaction of CD44 with the cytoskeleton. In our findings, we determined a significant increase in medullar HA and CD44 levels in all PGE₂ inhibited groups compared to control, suggesting an inverse relation between PGE₂ inhibition and levels of renal medullar HA and CD44.

Cell viability is also related to PGE₂ inhibition. Studies showed that COX-2 inhibition results in apoptosis in cultured RMICs [15-20]. Aoudjit et al. reported an anti-apoptotic and protective role of COX-2-originated PGE₂ in glomerular epithelial cells, mediated by EP4 [32]. Furthermore, Küper et al. found that PGE₂ inactivates pro-apoptotic protein Bad and protects cell viability in renal medullar epithelial cells [33]. In parallel with these findings, we also determined a significant increase in RMIC apoptosis in PGE₂ inhibited animals when compared to controls. These results suggest that PGE₂ inhibition results in RMIC apoptosis.

Conclusion

We suggest that the changes in PGE₂ affects lipid droplets in RMICs quantitatively and qualitatively; these granules may be the storage units for AA; functional changes in RMICs via PGE₂ affects HA levels in medullar interstitium and urine volume; and finally, PGE₂ inhibition results in RMIC apoptosis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	SDD	HS	HE	LAE	IS
Research concept and design	✓	--	--	--	✓
Collection and/or assembly of data	✓	--	--	--	--
Data analysis and interpretation	✓	✓	✓	--	✓
Writing the article	✓	--	--	--	--
Critical revision of the article	✓	--	--	--	--
Final approval of article	✓	--	--	--	--
Statistical analysis	--	--	--	✓	--

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