

Original Research Article

Involvement of angiotensin II and beta-adrenergic receptors in the regulation of autophagy in human endothelial EA.hy926 cell line

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Sent for review: 11 November 2019

Revised accepted: 27 January 2020

Abstract

Purpose: To investigate the role of angiotensin II (Ang II) and β adrenergic receptors (β ARs) in autophagy regulation in human endothelial EA.hy926 cell line.

Methods: The effect of pharmacological modulation of Ang II receptors and β ARs on the expression of LC3B-II and p62 proteins (autophagosome formation marker and autophagic flux marker, respectively) in the human endothelial EA.hy926 cell line were investigated by immunoblotting technique.

Results: Ang II-induced autophagy was characterized by increased LC3B-II and reduced p62 expressions. Candesartan, an AT1R agonist, significantly suppressed the effects of Ang II, while a selective AT2R antagonist, PD123319, inhibited the effect of candesartan. An AT2R agonist, CGP-42112A, also suppressed the Ang II-induced autophagy. Treatment with isoproterenol enhanced the expression of LC3B-II and reduced that of p62; these effects were suppressed upon cotreatment with propranolol (non-selective β AR blocker propranolol). A selective β 1AR agonist, dobutamine, reduced the expression of LC3B-II, and increased that of p62; the same was suppressed upon treatment with a selective β 1AR antagonist, metoprolol. A selective β 2AR agonist, salbutamol, resulted in increased expression of LC3B-II and reduced expression of p62. These effects were encountered upon treatment with selective β 2AR antagonist, ICI-118,551.

Conclusion: Based on the foregoing, it is evident that AT1Rs mediates Ang II-induced endothelial cell autophagy, while AT2Rs antagonizes the mechanism. β AR activation mediates isoproterenol-induced endothelial cell autophagy, which results from the balance of β 1ARs-mediated suppression and β 2ARs-mediated upregulation of autophagy in the endothelial cells.

Keywords: Autophagy, Angiotensin II type 1 receptors, Angiotensin II type 2 receptors β adrenergic type 1 receptors, β adrenergic type 2 receptors endothelial cells

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INTRODUCTION

Autophagy is a regulated intracellular process in which damaged organelles and misfolded proteins are recycled in a lysosome-dependent

manner [1]. It is an essential mechanism for the maintenance of cellular homeostasis and stress response [2]. In autophagy, it is important to decipher the role of autophagosome formation markers, such as microtubule-associated light

chain-3 (LC3) and beclin 1, and the autophagy flux marker, p62. Autophagy plays a vital role in maintaining the structural and functional integrity of the endothelial cells [3]. The role of autophagy includes nitric oxide production during starvation [3], vasoreactivity independent of the changes in contractile apparatus, maintenance of metabolic homeostasis [4], regulation of pro-inflammatory and pro-thrombotic functions [5], endothelial cell maturation, senescence, migration and angiogenesis [6].

Alterations in the mechanistic pathway of endothelial cell autophagy is involved in the endothelial dysfunction associated with diabetes mellitus [7] and aging [8]. However, an exploration of the literature review indicates very little information regarding the factors affecting autophagy in the vascular endothelial cells, which may be helpful in preserving the endothelial functions and reducing the cardiovascular risks.

It is reported that angiotensin II (Ang II) induces autophagy in the rat cardiomyocytes [9], vascular smooth muscle cells [10], and podocytes [11]. Porrello *et al* demonstrated that the stimulation of the Ang II type 1 receptor (AT1R) increases autophagy, whereas Ang II type 2 receptor (AT2R) subdues it in neonatal cardiomyocytes [12]. The AT1R also mediates mechanical stress induced autophagy in the cardiomyocytes [13]. Isoproterenol-induced β AR stimulation is also known to trigger autophagy in cardiac fibroblast cells [14]. The stimulation of type 2 β AR (β_2 AR) provokes autophagy in the hepatic cells [15] and cardiac fibroblasts [14]. Type 1 β AR (β_1 AR) stimulation by anti β_1 AR autoantibodies causes a reduction in autophagy in the cardiomyocytes resulting into cell death followed by the reduced cardiac function [16]. However, despite extensive clinical experience with pharmacological agents that act through the Ang II receptors or β AR and their effects on the endothelial cell autophagy has not been studied. Thus, the present study aims to investigate the role of subtypes of Ang II receptor and β AR in mechanistic regulation of autophagy in the human endothelial EA.hy926 cell line using their pharmacological modulators.

EXPERIMENTAL

In vitro cell culture

The human endothelial EA.hy926 cell line was provided as a gift from Dr. C. J. Edgell, the University of North Carolina, Lineberger Comprehensive Cancer Centre, Chapel Hill, United States [17]. The cells were cultured in complete Dulbecco's Modified Eagle Medium

(DMEM, Himedia, India) supplemented with 10 % fetal bovine serum (FBS, Himedia, India) and 1 % Penicillin/Streptomycin (Himedia, India). It was incubated at 37 °C in 5 % CO₂ until they reached 80 - 90 % confluence. The cultured cells were then randomly exposed to following treatments: Ang II, candesartan, PD1232319, CGP-42112A, isoproterenol, propranolol, dobutamine, metoprolol, salbutamol and ICI-118,551 as described below under respective assay.

Cell viability assay

Cytotoxicity of the human endothelial EA.hy926 cells in response to the pharmacological modulators was determined by 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. The principle behind the assay is directly dependent on the metabolic reduction of MTT. A total of 2 x 10⁴ cells were seeded in 96 well plates and were left overnight to adhere. The cells were then exposed for 24 h to following treatments; 1 nM to 10 μ M: Ang II (Sigma, USA), candesartan (Sigma, USA), PD123319 (Sigma, USA), CGP-42112A (Sigma, USA), isoproterenol hydrochloride (Sigma, USA), propranolol hydrochloride (Sigma, USA), dobutamine hydrochloride (Sigma, USA), metoprolol tartrate (Sigma, USA), salbutamol (Sigma, USA), and ICI-118,551 (Adooq bioscience, USA).

After the treatment, the cells were then incubated with MTT for 2-3 h (0.5 mg/L) followed by the addition of 150 μ L of dimethylsulphoxide to each well. A microplate reader (BioTek, USA) was used to record the changes in absorbance at 570 nm. The data is presented as the percent cell viability normalized to their respective controls.

Immunoblotting

The cell lysates containing 40 μ g of the total proteins were fractionated on a 12 % SDS-PAGE. The fractionated proteins were transferred to a polyvinylidene fluoride membrane. The blots were then blocked at room temperature with a solution consisting of 5 % bovine serum albumin in Tris-buffered saline (TBS) containing 0.1 % Tween 20 for 2 h. Following which the membranes were incubated overnight with the diluted antibodies: anti-LC3B (1:1000 dilution; Cell Signalling Technology, USA), anti-p62 (1:1000 dilution; Sigma, USA) and anti-beta actin (1:10000 dilution; Sigma, USA).

The membranes were then incubated for 2 h with the respective HRP-conjugated mouse or rabbit secondary antibodies and visualized using ECL

western-blotting detection reagent. The intensity of the immunoblots was analysed with myImageAnalysis software version 2.0 (Thermo Scientific, USA).

Statistical analysis

The results are expressed as mean ± standard error of the mean (S.E.M). In order to investigate the variability amongst the groups, one-way ANOVA was employed. Bonferroni's test was used for the post hoc analysis when the *F*-test of variance had achieved the necessary level of statistical significance (i.e., *p* < 0.05). The statistical testing was done using the Graph Pad prism software version 5.00.28 (GraphPad Software, Inc, San Diego, CA, USA). A level of the probability less than 0.05 was considered significant.

RESULTS

Angiotensin II upregulated endothelial cell autophagy via the AT1R stimulation

Angiotensin II (1 nM – 10 µM) exposure for 24 h did not affect the viability of the human endothelial EA.hy.926 cell line (Figure 1 A). Candesartan showed no sign of toxicity at a concentration of 1nM to 1 µM, but its 10 µM showed a significant reduction in cell viability (Figure 1 B). Angiotensin II at 1 µM concentration significantly increased LC3B-II and decreased p62 (Figure 1 C). It was observed that co-treatment with the AT1R blocker, candesartan (1 µM) significantly inhibited the Ang II (1 µM)-enhanced expression of the LC3B-II (Figure 1 D). Reduction in p62 expression by Ang II was also significantly suppressed on cotreatment with candesartan at 100 nM and 1 µM.

Activation of AT2Rs suppressed angiotensin II-induced endothelial cell autophagy

No significant cytotoxic effect was observed on endothelial cell line upon 24 h exposure to either PD123319 (an AT2R antagonist, 1 nM to 10 µM) (Figure 2 A) or CGP-421112A (an AT2R agonist, 1 nM to 10 µM) (Figure 2 B). Candesartan at a concentration of 1 µM inhibited the effect of Ang II on the expression of LC3B-II and p62. This effect of candesartan was significantly suppressed upon the AT2R blockade by PD123319 (1 µM, Figure 2 C). There was observed a significant decrease in the LC3B-II expression and increase in p62 expression in the Ang II treated cells upon cotreatment with CGP-42112A (1 µM and 10 µM, Figure 2 D).

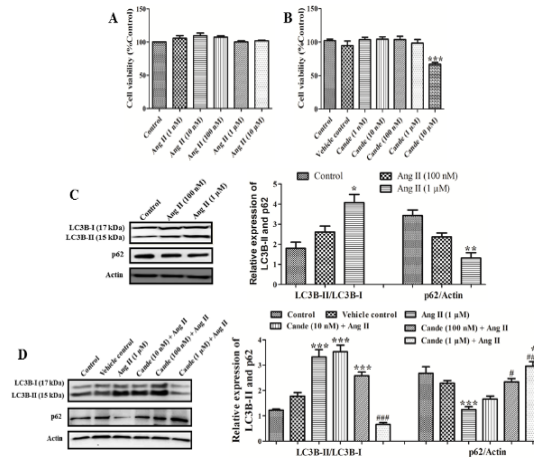


Figure 1: Effect of Ang II (A) and candesartan (B) on the cell viability of human endothelial EA.hy926 cell line. C represents the effect of Ang II on the relative protein expression of LC3B-II (autophagosome formation marker) and p62 (autophagic flux marker). Effect of candesartan on co-treatment with the Ang II-altered expressions on markers of autophagy (LC3B-II and p62) in human endothelial EA.hy926 cell line (D); **p* < 0.05, ***p* < 0.01, ****p* < 0.005, vs. Control, and #*p* < 0.05, ###*p* < 0.005 vs. Ang II treated cells (n = 5)

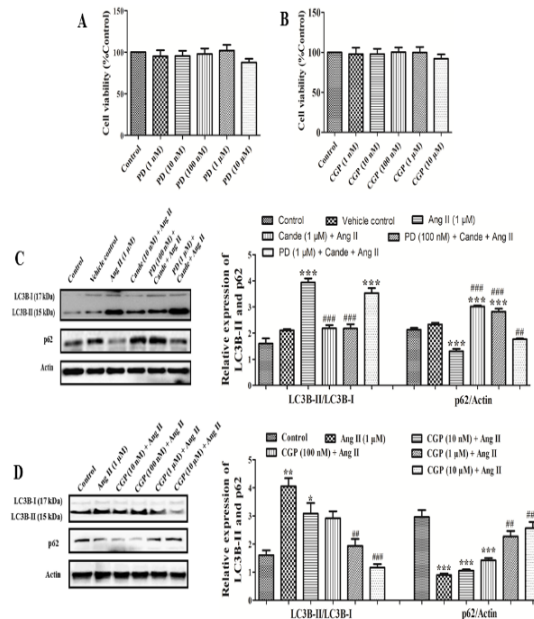


Figure 2: Effect of PD123319 (A) and CGP-42112A (B) on the cell viability of human endothelial EA.hy926 cells. Effect of AT2R receptor blockage by PD123319 following co-treatment with candesartan (C), and the effect of AT2R receptor activation by CGP-42112A (D) on the expression of LC3B-II and p62 in Ang II treated human endothelial EA.hy926 cell line; **p* < 0.05, ***p* < 0.01 and ****p* < 0.005 when compared with the control group and ###*p* < 0.01 and ####*p* < 0.005 when compared with the Ang II treated cells (n = 5)

βAR activation modulated endothelial cell autophagy

Treatment with either isoproterenol (Figure 3 A) or propranolol (Figure 3 B) for 24 h showed no toxicity in human endothelial EA.hy926 cells. Isoproterenol (100 nM to 10 μM) increased the expression of LC3B-II, while it decreased that of p62 in a concentration-dependent manner (Figure 3 C). Co-treatment with propranolol, a non-selective βAR blocker, at a concentration of 100 nM - 10 μM, (Figure 3 D) significantly reduced the expression of LC3B-II, and increased the expression of p62 in isoproterenol treated endothelial cells.

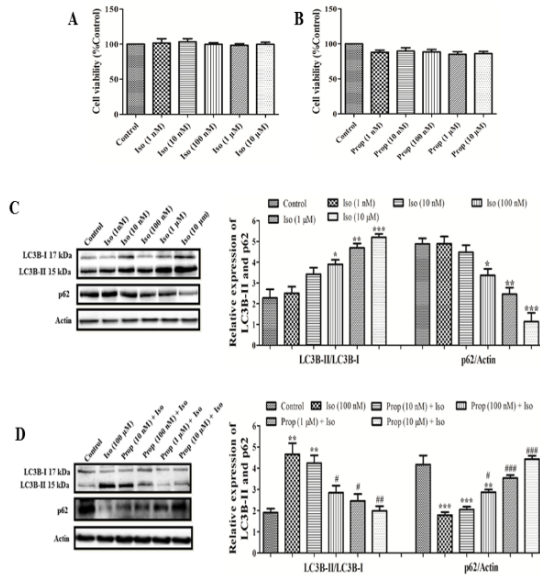


Figure 3: Effect of Isoproterenol (A) and propranolol (B) on the cell viability of human endothelial EA.hy926 cell line. Effect of isoproterenol on the relative protein expression of LC3B-II and p62 (C), and the effect of propranolol cotreatment on the isoproterenol altered expression of markers of autophagy (D) in human endothelial EA.hy926 cell line; ***p* < 0.01 and ****p* < 0.005 when compared to the control, #*p* < 0.05, ###*p* < 0.005, when compared to isoproterenol-treated cells (n = 5)

Activation of β₁AR suppressed autophagy in endothelial cells

No significant cytotoxic effect on human endothelial EA.hy926 cells was observed upon 24 h treatment with selective β₁AR agonist dobutamine (Figures 4 A) and selective β₁AR antagonist metoprolol (Figures 4 B) at a concentration of 1 nM to 10 μM. A significant decrease in the expression of LC3B-II and increase in the expression of p62 was observed upon treating the cells with dobutamine (1 μM and 10 μM, Figure 4 C). There was observed a

significantly enhanced LC3B-II and reduced p62 expression in dobutamine-treated (1 μM) cells cotreated with metoprolol (1 μM and 10 μM, Figure 4 D).

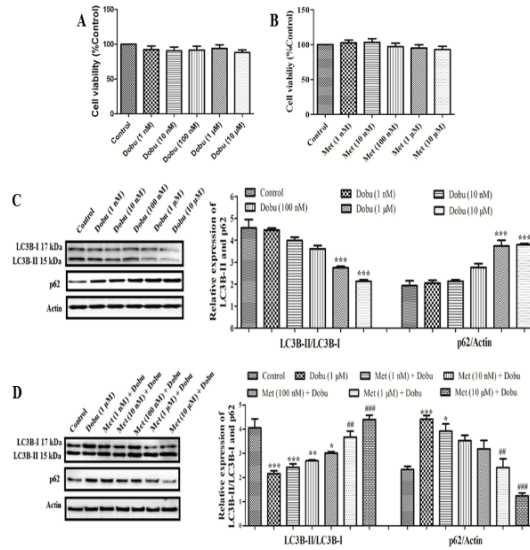


Figure 4: Effect of dobutamine (A) and metoprolol (B) on the cell viability of human endothelial EA.hy926 cells. Effect of dobutamine on the relative protein expression of LC3B-II and p62 (C), and the effect of metoprolol cotreatment on the dobutamine altered expression of markers of autophagy (D) in human endothelial EA.hy926 cells; **p* < 0.05, ***p* < 0.01 and ****p* < 0.005 vs. control and ##*p* < 0.01 and ###*p* < 0.005 vs. dobutamine-treated cells (n = 5)

Activation of β₂AR induces autophagy in endothelial cells

Upon treatment with salbutamol (Figure 5 A) or ICI-118,551 (Figure 5 B) at a concentration of 1 nM to 10 μM on EA.hy926 cells, MTT assay revealed no significant loss in cell viability. Salbutamol (100 nM and 1 μM) exposure for 24 h significantly increased the expression of LC3B-II and decreased p62 protein expression in the cell line (Figure 5 C). There was observed a significant reduction in the expression of LC3B-II and increase in p62 expression upon co-treating the salbutamol-treated (100 nM) cells with ICI-118,551 (100 nM - 10 μM, Figure 5 D).

DISCUSSION

A detailed understanding of the molecular aspect of autophagy pathway is evolving, but limited information is available about the cardiovascular cues influencing autophagy in the endothelial cells. The present study is the first to report about the involvement of the Ang II receptors and βARs in the regulation of endothelial cell autophagy.

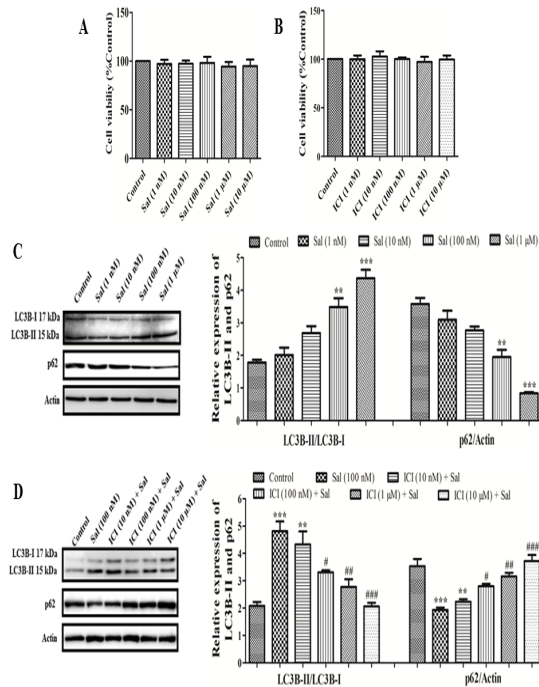


Figure 5: Effect of salbutamol (A) and ICI-118,551 (B) on the cell viability of human endothelial EA.hy926 cells. Panel (C) represents effect of salbutamol on the relative protein expression of LC3B-II and p62, and the effect of ICI-118,551 co-treatment on the salbutamol-altered expression of markers of autophagy in human endothelial EA.hy926 cells is represented in panel (D) ; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$ when compared to control and # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.005$ when compared to salbutamol-treated cells (n = 5)

Angiotensin II was found to induce autophagy in the endothelial cells via AT1R stimulation, while AT2R activation antagonized it. The activation of β_1 AR suppressed the endothelial cell autophagy; whereas, β_2 AR activation induced it. Angiotensin II, the vasoactive peptide of RAAS implicated in various vascular pathologies, including hypertension, has been documented to trigger autophagy in tissues of cardiovascular origin, namely cardiomyocytes [12], cardiac fibroblasts [18], vascular smooth muscle cells [19], and endothelial cells [20]. In line with the earlier studies, Ang II-treated endothelial cells showed increase in autophagosome formation and the autophagy flux as indicated by the increased LC3B-II and reduced p62 expression. There was observed suppression of Ang II-induced endothelial cell autophagy upon treatment with candesartan, a selective AT1R antagonist. This indicates that the AT1R activation mediates the pro-autophagic effect of Ang II on the endothelial cells.

Treatment with PD123319, a selective AT2R blocker, resisted the inhibitory effect of

candesartan on the Ang II-induced autophagy. This suggests that AT2Rs may also be involved in regulating the effect of Ang II on endothelial cell autophagy. The inhibitory effect of AT2R stimulation on the Ang II-induced endothelial cell autophagy was confirmed when cotreatment with CGP-4211A, a selective AT2R activator, suppressed the Ang II-induced endothelial cell autophagy. This finding goes in line with the previous study of Porello *et al.* reporting that the AT1R stimulation by Ang II increases the autophagosome formation in the neonatal rat cardiomyocytes, whereas AT2R overexpression in cardiac cells counteracts the effect of Ang II [12].

Sympathetic hyperactivity is another significant stressor affecting vascular health during cardiovascular diseases, the landmark of which is tachycardia due to the sustained β AR activation. In a recent study, isoproterenol was found to induce vascular oxidative stress and endothelial dysfunction via β_2 AR [21]. Given that the autophagy is a cellular stress response mechanism, which is also involved in regulating endothelial morphology and functions, the understanding of the effect of the β AR stimulation on autophagy in the endothelial cells becomes important.

In the present study, the isoproterenol treatment increased LC3B-II and reduced p62 expression in the endothelial cells, indicating upregulation of autophagy in the cells. Cotreatment with propranolol, a non-selective β AR, significantly suppressed the autophagy inducing effect of isoproterenol indicating that the β ARs could be involved in modulating the endothelial cell autophagy. The role of β_1 ARs and β_2 ARs in modulating endothelial cell autophagy was investigated by treating the cells with dobutamine (selective β_1 AR agonist) in absence or presence of metoprolol (selective β_1 AR antagonist) and salbutamol (selective β_2 AR agonist) in absence or presence of ICI-118,551 (selective β_2 AR antagonist), respectively [22].

Dobutamine treatment reduced the LC3B-II expression and increased p62 expression in the endothelial cells, which marks the inhibition of autophagy. The inhibitory effect of dobutamine on endothelial cell autophagy was suppressed upon cotreatment with metoprolol, which indicates β_1 AR activation exhibits anti-autophagic effect in the endothelial cells. This goes in line with the findings of Wang and his colleagues in 2013 and 2018, in which they revealed that β_1 AR activation by anti β_1 AR autoantibodies in the cardiomyocytes inhibits autophagy [16,23].

Treatment with salbutamol enhanced the expression of LC3B-II expression and reduced p62 expression in the endothelial cells autophagy, indicating induction of autophagy. The autophagy stimulatory effect of salbutamol was counteracted upon the selective β_2 AR blockade by ICI-118,551, which suggests proautophagic effect of β_2 AR activation in the endothelial cells. Similar findings were observed in a report published by Farah and colleagues in 2014, demonstrating the induction of autophagy by activation of β_2 ARs in the hepatic cells [15].

Earlier reports also demonstrated that the majority of the β AR population on the vascular endothelium is of the β_2 AR subtype, which may also be the reason for the predominance of the pro-autophagic effect upon β AR activation by isoproterenol in the endothelial cells [24]. Aranguiz-Urroz and colleagues (2015) observed upregulation of autophagy in the cardiac fibroblasts upon exposure to isoproterenol, which was blocked by the selective β_2 AR antagonist ICI-118,551, but not by atenolol (selective β_1 AR blocker). Thereby suggesting that β_2 AR activation mediates the autophagy stimulatory effect of isoproterenol in the cardiac fibroblasts [14]. From the above findings, it could be summarized that β_1 AR stimulation results in anti-autophagic effect, whereas β_2 AR activation has pro-autophagic effect in the endothelial cells.

CONCLUSION

Autophagy plays a crucial role in regulating the endothelial cell biology, damage, dysfunction, survival, and apoptosis, and thus, could be an important contributor in the development of cardiovascular diseases. However, knowledge about the proximal cues affecting autophagy in the endothelial cells is limited. The present study revealed that AT1R and AT2R act reciprocally to regulate Ang II mediated autophagy in the endothelial cells. AT1R mediates Ang II-induced autophagy, while AT2R antagonizes it. It was also observed that β_1 AR stimulation suppresses the endothelial cell autophagy, while β_2 AR activation triggers it. These novel findings aid in understanding the roles of Ang II receptors as well as β ARs in regulating autophagy in the endothelial cells that could be exploited therapeutically to lower cardiovascular disease risks.

DECLARATIONS

Acknowledgement

This work was supported by the grants from the CSIR-Central Drug Research Institute Network

project, THUNDER (no. BSC0102), UNDO (no. BSC0103) and Senior Research Fellowship to Moon Jain from Council of Scientific and Industrial Research, Government of India. This manuscript is allotted with CDRI communication no. 10017.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Moon Jain designed, performed experiments, compiled results and wrote the manuscript. Prasanna K Sahu performed experiments. Kashif Hanif designed the study, analyzed the data and wrote the manuscript.

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