2'-AMP and 3'-AMP Inhibit Proliferation of Preglomerular Vascular Smooth Muscle Cells and Glomerular Mesangial Cells via A_{2B} Receptors

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ABSTRACT

Studies show that kidneys produce 2',3'-cAMP, 2',3'-cAMP is exported and metabolized to 2'-AMP and 3'-AMP, 2'-AMP and 3'-AMP are metabolized to adenosine, 2',3'-cAMP inhibits proliferation of preglomerular vascular smooth muscle cells (PGVSMCs) and glomerular mesangial cells (GMCs), and A_{2B} (not A₁, A_{2A}, or A₃) adenosine receptors mediate part of the antiproliferative effects of 2',3'-cAMP. These findings suggest that extracellular 2',3'-cAMP attenuates proliferation of PGVSMCs and GMCs partly via conversion to corresponding AMPs, which are metabolized to adenosine that activates A_{2B} receptors. This hypothesis predicts that extracellular 2'-AMP and 3'-AMP should exert A_{2B} receptor-mediated antiproliferative effects. Therefore, we examined the antiproliferative effects (cell counts) of 2'-AMP and 3'-AMP. In PGVSMCs and GMCs, 2'-AMP and 3'-AMP exerted concentration-dependent antiproliferative effects. 3'-AMP was equipotent with and 2'-AMP was 3-fold less potent than 5'-AMP (prototypical adenosine precursor). In PGVSMCs,

the effects of 2'-AMP and 3'-AMP were mimicked by adenosine, and 8-[4-(((4-cyanophenyl)carbamoylmethyl)oxy)phenyl]-1,3-di(*n*-propyl)xanthine (MRS-1754) (A_{2B} receptor antagonist) equally blocked the antiproliferative effects of 2'-AMP, 3'-AMP, and adenosine but less effectively blocked the effects of 2',3'-cAMP. Similar results were obtained in GMCs except that MRS-1754 also incompletely blocked the effects of 3'-AMP. We conclude that in PGVSMCs, 2'-AMP and 3'-AMP are antiproliferative, the antiproliferative effects of 2'-AMP and 3'-AMP are mediated nearly entirely by adenosine/A_{2B} receptors, and some of the antiproliferative effects of 2',3'-cAMP are independent of adenosine/A_{2B} receptors. Similar conclusions apply to GMCs except that 3'-AMP also has actions independent of adenosine/A_{2B} receptors. Because A_{2B} receptors are renoprotective, 2'-AMP and 3'-AMP may provide renoprotection by generating adenosine that activates A_{2B} receptors.

Introduction

Studies using high-performance liquid chromatography-tandem mass spectrometry demonstrate the production and release into the extracellular compartment of 2',3'-cAMP, a positional isomer of 3',5'-cAMP, by intact rat kidneys (Ren et al., 2009). Moreover, experiments in intact rat kidneys show

that extracellular 2',3'-cAMP is metabolized to extracellular 2'-AMP and 3'-AMP and that these AMPs are further metabolized to extracellular adenosine (a mechanism called the extracellular 2',3'-cAMP-adenosine pathway) (Jackson et al., 2009).

More recent studies indicate that extracellular 2',3'-cAMP has biological effects on kidney cells (Jackson et al., 2010). For example, extracellular 2',3'-cAMP inhibits the proliferation of preglomerular vascular smooth muscle cells (PGVSMCs) and glomerular mesangial cells (GMCs). Mechanistically, this inhibition is probably in part due to conversion of 2',3'-cAMP to adenosine, a conclusion supported by the observations that the antiproliferative effects of 2',3'-cAMP are accompanied by the metabolism of 2',3'-cAMP to 2'-AMP and 3'-AMP, which are

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ABBREVIATIONS: PGVSMC, preglomerular vascular smooth muscle cell; GMC, glomerular mesangial cell; MRS-1754, 8-[4-(((4-cyanophenyl)carbamoylmethyl)oxy)phenyl]-1,3-di(*n*-propyl)xanthine; SCH58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; VUF-5574, *N*-(2-methoxyphenyl)-*N'*-[2-(3-pyridinyl)-4-quinazolinyl]-urea; DMEM/F12, Dulbecco's modified Eagle's medium Nutrient Mixture F-12.

further metabolized to adenosine (Jackson et al., 2010), and the antigrowth effects of 2',3'-cAMP are attenuated by antagonism of A_{2B} receptors with 8-[4-[(4-cyanophenyl)carbamoylmethyl]-oxy]phenyl]-1,3-di(*n*-propyl)xanthine (MRS-1754), but not by antagonism of A_1 receptors with 1,3-dipropyl-8-cyclopentylxanthine, A_{2A} receptors with 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH-58261), or A_3 receptors with *N*-(2-methoxyphenyl)-*N'*-[2-(3-pyridinyl)-4-quinazolinyl]-urea (VUF-5574) (Jackson et al., 2010). In addition, previous studies demonstrated that adenosine, via A_{2B} receptors, inhibits growth of vascular smooth muscle cells and GMCs (Dubey et al., 1996b, 1998b, 2000, 2005). It seems likely then that extracellular 2',3'-cAMP inhibits growth of PGVSMCs and GMCs at least in part via the extracellular 2',3'-cAMP-adenosine pathway, which then activates antiproliferative A_{2B} receptors (solid arrows in Fig. 1). However, this hypothesis predicts that extracellular 2'-AMP and 3'-AMP should also exert antiproliferative effects that are blocked by antagonism of A_{2B} receptors, and this prediction has yet to be tested. Therefore, a major objective of this study was to further test the hypothesis shown in Fig. 1 (solid arrows) by examining the antiproliferative effects of 2'-AMP and 3'-AMP in the absence and presence of a selective and potent A_{2B} receptor antagonist.

Although extracellular 2',3'-cAMP probably inhibits growth of PGVSMCs and GMCs in part via the extracellular 2',3'-cAMP-adenosine pathway and via A_{2B} receptors (Jackson et al., 2010), it is conceivable that extracellular 2',3'-cAMP has antiproliferative effects that are not adenosine-mediated. In this regard, it is possible that 2',3'-cAMP exerts nonadenosine-mediated antiproliferative actions that are also independent of 2'-AMP or 3'-AMP (dashed arrows in Fig. 1); it is also conceivable that 2',3'-cAMP has antiproliferative actions that are due to nonadenosine-mediated effects of 2'-AMP or 3'-AMP (dotted arrows in Fig. 1). Therefore, another goal of

the present study was to explore the possibility that extracellular 2',3'-cAMP inhibits growth of PGVSMCs and GMCs independently of adenosine.

Materials and Methods

Animals. PGVSMCs and GMCs were harvested from adult male Wistar-Kyoto rats (Taconic Farms, Germantown, NY). The institutional animal care and use committee approved all procedures, and the investigation conforms to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Drugs. 2',3'-cAMP, 5'-AMP, 3'-AMP, 2'-AMP, and MRS-1754 [selective A_{2B} receptor antagonist (Jacobson and Knutsen, 2001)] were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture. Rat PGVSMCs and GMCs were harvested and cultured as described by us previously (Dubey et al., 1992; Inoue et al., 1998; Mokkapatti et al., 1998). All experiments were performed in cells in third to fifth passage.

Cell Proliferation Studies. Cell proliferation studies were conducted as described previously (Dubey et al., 1996a). In brief, PGVSMCs and GMCs were plated at an initial density of 5000 cells/culture in Dulbecco's modified Eagle's medium Nutrient Mixture F-12 (DMEM/F12) containing 2.5% fetal calf serum and were allowed to attach and proliferate overnight. The next day, cells were growth-arrested in DMEM/F12 containing 0.25% albumin for 24 h to synchronize cells. Then DMEM/F12 containing 2.5% fetal calf serum (along with the various treatments) was added to stimulate cell proliferation. Treatments were administered daily, and after 4 days the cell number was determined by counting cells with a Coulter counter.

Statistics. Data were analyzed by one- or two-factor analysis of variance, with post hoc comparisons using Fisher's least significant difference test if the main effects of the analysis of variance were significant. The criterion of significance was $p < 0.05$. All values in text and figures are means $\pm$ S.E.M.

Results

We first investigated the concentration-dependent effects of 2'-AMP and 3'-AMP on proliferation of PGVSMCs and GMCs. As shown in Fig. 2, both 2'-AMP and 3'-AMP significantly, profoundly, and concentration dependently attenuated the proliferation of PGVSMCs (Fig. 2A) and GMCs (Fig. 2B). Of importance, 3'-AMP was as potent and efficacious in this regard as the prototypical adenosine precursor, 5'-AMP. Although 2'-AMP was approximately 3-fold less potent than 3'-AMP and 5'-AMP, nonetheless, 2'-AMP was an effective inhibitor of PGVSMC and GMC proliferation (Fig. 2, A and B, respectively). 2'-AMP, 3'-AMP, and 5'-AMP did not affect cell morphology or trypan blue exclusion, indicating that the AMPs did not alter cell viability.

Next, we examined whether the effects of 2'-AMP and 3'-AMP in PGVSMCs could be accounted for by adenosine acting via A_{2B} receptors. The ability of 2'-AMP (30 μ M) to inhibit PGVSMC proliferation was abolished by MRS-1754 (100 nM) (Fig. 3A), and the ability of 3'-AMP (30 μ M) to inhibit PGVSMC proliferation was nearly abolished (Fig. 3B). In contrast to 2'-AMP and 3'-AMP, although the antiproliferative effects of 2',3'-cAMP (30 μ M) were reduced by MRS-1754 (100 nM), there remained a considerable antiproliferative effect of 2',3'-cAMP even in the presence of MRS-1754 (Fig. 3C). MRS-1754 (100 nM) inhibited nearly all of the antiproliferative actions of adenosine (Fig. 3D), indicating

Fig. 1. Our previously published work (Jackson et al., 2009, 2010) indicated that 2',3'-cAMP exerts antiproliferative effects on PGVSMCs and GMCs at least in part by the mechanism shown by the solid arrows. The dashed and dotted arrows represent possible additional mechanisms. PDE, phosphodiesterase; NT, nucleotidase.

Fig. 2. Line graphs summarize the concentration-dependent effects of 2'-AMP, 3'-AMP, and 5'-AMP on cell number in PGVSMCs (A) and GMCs (B). Values are means $\pm$ S.E.M. of n PGVSMC and GMC cultures (S.E.M.s are smaller than symbol size). a, $p < 0.05$ for "0" levels of AMPs compared with all other concentrations of all other AMPs; b, $p < 0.05$ for 2'-AMP compared with corresponding concentrations of 3'-AMP and 5'-AMP; c, $p < 0.05$ for 3'-AMP compared with corresponding concentration of 5'-AMP.

that the concentration of MRS-1754 was adequate to antagonize the A_{2B} receptor in PGVSMCs.

Subsequently, we examined whether the effects of 2'-AMP and 3'-AMP in GMCs could be accounted for by adenosine acting via A_{2B} receptors. The ability of 2'-AMP (30 μ M) to inhibit GMC proliferation was abolished by MRS-1754 (100 nM) (Fig. 4A), and the ability of 3'-AMP (30 μ M) to inhibit GMC proliferation was attenuated but clearly present (Fig. 4B). In contrast to 2'-AMP, although the antiproliferative effects of 2',3'-cAMP (30 μ M) were reduced by MRS-1754 (100 nM), there remained a considerable antiproliferative effect of 2',3'-cAMP even in the presence of MRS-1754 (Fig. 4C). As in PGVSMCs, in GMCs, MRS-1754 (100 nM) inhibited nearly all of the antiproliferative actions of adenosine (Fig. 4D), indicating that the concentration of MRS-1754 was adequate to antagonize the A_{2B} receptor in GMCs.

The data in Figs. 3 and 4 were used to statistically compare the percent reversal (antagonism) by MRS-1754 of the antiproliferative effects of adenosine, 2',3'-cAMP, 2'-AMP, and 3'-AMP. As shown in Fig. 5, in PGVSMCs (Fig. 5A) the antagonism by MRS-1754 of the antiproliferative effects of the purines was 1) nearly complete for adenosine, 2'-AMP, and 3'-AMP, 2) not statistically significantly different for adenosine versus 2'-AMP versus 3'-AMP, and 3) statistically significantly less for 2',3'-cAMP versus those of the other

three purines (adenosine, 2'-AMP, and 3'-AMP). As shown in Fig. 5B, similar results were obtained in GMCs with the exception that although MRS-1754 antagonized the effects of 3'-AMP more so than the effects of 2',3'-cAMP, the antagonism by MRS-1754 was greater against adenosine than against 3'-AMP.

Discussion

Very little is known about the pharmacology of 2'-AMP or 3'-AMP. However, the discovery that 2',3'-cAMP is endogenously generated and can be metabolized to 2'-AMP and 3'-AMP (Jackson et al., 2009; Ren et al., 2009) provides strong motivation to explore thoroughly the pharmacology and physiology of these neglected AMPs. The existence of 2',3'-cyclic nucleotide 3'-phosphodiesterase, an enzyme that can metabolize 2',3'-cAMP to 2'-AMP (Sprinkle, 1989), along with the important recent discovery by Rao et al. (2010) of six different phosphodiesterases that metabolize 2',3'-cAMP to 3'-AMP, stimulates additional enthusiasm for investigating the pharmacology and physiology of AMPs generated from 2',3'-cAMP.

In the current report, we focused on the pharmacological effects of 2'-AMP and 3'-AMP on proliferation of PGVSMCs and GMCs. The incentive for these experiments is that previously published data (Jackson et al., 2010) indicated that 2',3'-cAMP inhibits PGVSMC and GMC proliferation in part via adenosine A_{2B} receptors and that the mechanism seems to be 2',3'-cAMP $\rightarrow$ 2'-AMP + 3'-AMP $\rightarrow$ adenosine $\rightarrow$ A_{2B} receptor activation $\rightarrow$ antiproliferative effect. This model predicts that 2'-AMP or 3'-AMP ought also to inhibit PGVSMC and GMC proliferation and the antiproliferative effects of the AMPs should be at least partially blocked by antagonism of A_{2B} adenosine receptors. Indeed, the present experiments confirm that both 2'-AMP and 3'-AMP potently and efficaciously inhibit proliferation of both PGVSMCs and GMCs via, at least in part, A_{2B} receptors.

Our previous study regarding the antiproliferative effect of 2',3'-cAMP demonstrates that a portion of the antiproliferative action of 2',3'-cAMP, as assessed by thymidine incorporation, is not reversed by antagonism of A_{2B} receptors with MRS-1754 (Jackson et al., 2010). This result suggests that either 2',3'-cAMP has nonadenosine-mediated effects or that some of the antiproliferative effects of the adenosine generated from 2',3'-cAMP are mediated in part by A_1 , A_{2A} , or A_3 adenosine receptors. However, the second possibility is ruled out by our findings that the antiproliferative effects of 2',3'-cAMP are not affected whatsoever by antagonism of A_1 , A_{2A} , or A_3 adenosine receptors (Jackson et al., 2010). In addition, our previous studies showed that the antiproliferative effects of adenosine are accompanied by increases in intracellular levels of 3',5'-cAMP (suggestive of A_{2B} receptor signaling), are inhibited by antisense oligonucleotide-induced down-regulation of A_{2B} receptors, and are not mimicked by selective A_1 , A_{2A} , or A_3 receptor agonists (Dubey et al., 1996b, 1997, 1998a,b, 2000, 2001, 2005, 2010). Thus, we are left with the possibility that 2',3'-cAMP has effects that are independent of adenosine.

The results of the present study confirm, using cell counting (a more reliable index of antiproliferative effects), that although some of the antiproliferative effects of 2',3'-cAMP are mediated via adenosine acting on A_{2B} receptors, indeed a

Fig. 3. Bar graphs illustrate the effects of 30 μ M 2'-AMP (A), 30 μ M 3'-AMP (B), 30 μ M 2',3'-cAMP (C), and 30 μ M adenosine (ADO) (D) on PGVSMC cell number in the absence and presence of 100 nM MRS-1754 (MRS). Values are means $\pm$ S.E.M. of n PGVSMC cultures.

portion of the effects are not mediated by adenosine. In this regard, we observe that concentrations of MRS-1754 that nearly abolish the antiproliferative effects of adenosine are statistically less effective in preventing the effects of 2',3'-cAMP. Because the concentration of MRS-1754 used in the present study effectively prevents the antiproliferative actions of adenosine, the lack of full reversal of 2',3'-cAMP-induced growth inhibition is not due to inadequate concentrations of MRS-1754. This result is consistent with the fact that the K_i of MRS-1754 for A_{2B} receptors is approximately 2 nM (Jacobson and Knutsen, 2001; Kalla et al., 2009), and the current study uses a concentration of 100 nM (50-fold greater than the K_i). Thus, the present study confirms that 2',3'-cAMP has adenosine-independent actions.

The adenosine-independent actions of 2',3'-cAMP could be independent of the antiproliferative effect of its "downstream" AMPs or could be mediated by antiproliferative actions of 2'-AMP or 3'-AMP that are adenosine-independent. The present study demonstrates that in PGVSMCs, MRS-1754 abolishes the antiproliferative effects of 2'-AMP and nearly abolishes the antiproliferative effects of 3'-AMP. Of importance, there is no statistically significant difference between adenosine, 2'-AMP, and 3'-AMP with regard to re-

versal of their antiproliferative effects by MRS-1754. Thus, in PGVSMCs the evidence supports the conclusion that 2',3'-cAMP exerts an adenosine-independent antiproliferative effect that bypasses both 2'-AMP and 3'-AMP (dashed arrow in Fig. 1). On the other hand, in GMCs, although MRS-1754 reverses completely the antiproliferative effects of 2'-AMP, MRS-1754 is unable to block the effects of 3'-AMP as efficiently as the effects of adenosine or 2'-AMP. Moreover, MRS-1754 has even less effect on 2',3'-cAMP than on 3'-AMP. Thus, in GMCs, the evidence supports the conclusion that 2',3'-cAMP exerts an adenosine-independent antiproliferative effect that bypasses both 2'-AMP and 3'-AMP (dashed arrow in Fig. 1) but also exerts an adenosine-independent antiproliferative effect that is due to the nonadenosine-dependent effects of 3'-AMP.

The present study does not address the mechanisms of the nonadenosine-mediated antiproliferative component of 2',3'-cAMP or 3'-AMP. Studies by Azarashvili et al. (2009) demonstrated that in isolated mitochondria 2',3'-cAMP promotes the opening of mitochondrial permeability transition pores, and studies by Johnson et al. (1989) in detergent-solubilized and purified tissue preparations showed that 3'-AMP is a P site inhibitor of adenylyl cyclase. It is conceivable that these

Fig. 4. Bar graphs illustrate the effects of 30 μ M 2'-AMP (A), 30 μ M 3'-AMP (B), 30 μ M 2',3'-cAMP (C), and 30 μ M adenosine (ADO) (D) on GMC cell number in the absence and presence of 100 nM MRS-1754 (MRS). Values are means $\pm$ S.E.M. of n PGVSMC cultures.

reported effects of 2',3'-cAMP and 3'-AMP are involved in the nonadenosine-mediated antiproliferative actions of these purines. However, in the present study these compounds are applied extracellularly. Although cAMPs are actively pumped out of cells (Barber and Butcher, 1983; Deeley et al., 2006; Li et al., 2007; Cheng et al., 2010), because both 2',3'-cAMP and 3'-AMP are very hydrophilic, they are not likely to diffuse across cell membranes into cells. Because the effects on mitochondria and the P site of adenylyl cyclase would require an intracellular action of 2',3'-cAMP and 3'-AMP, it seems unlikely that these mechanisms are involved. Moreover, whereas activation of A_{2B} receptors stimulates adenylyl cyclase and inhibits proliferation; in contrast, interaction of 3'-AMP with the P site inhibits adenylyl cyclase, an effect not predicted to inhibit proliferation.

As mentioned, our recent report showed that 2',3'-cAMP, 2'-AMP, and 3'-AMP are metabolized to adenosine in PGVSMCs and GMCs (Jackson et al., 2010), and our previous studies (Dubey et al., 1996b, 1998b, 2000, 2005) and the current study demonstrate that adenosine inhibits proliferation of vascular smooth muscle cells and GMCs via A_{2B} receptors. Thus, it is likely that any antiproliferative effects of 2',3'-cAMP, 2'-AMP, and 3'-AMP prevented by MRS-1754

are mediated by adenosine generated from these purines. Although it is logically possible that 2',3'-cAMP, 2'-AMP, and 3'-AMP could be acting in part as direct A_{2B} receptor agonists, this possibility is extremely unlikely. The pharmacology and structure-activity analysis of a vast number of adenosine analogs support the conclusion that the 2'-hydroxy and 3'-hydroxy moieties on the ribose ring of adenosine analogs are, with very few exceptions not involving negatively charge substituents, required for activity at adenosine receptors (Jacobson and Knutsen, 2001). However, rigorous testing of this hypothesis must await the availability of inhibitors that can block the conversion of 2',3'-cAMP, 2'-AMP, and 3'-AMP to adenosine.

Evidence is rapidly accumulating that renovascular A_{2B} receptors play an important role in the renal microcirculation. For example, A_{2B} receptors are strongly expressed in rat preglomerular microvessels (Jackson et al., 2002), and studies in A_{2B} receptor knockout mice provide striking evidence that adenosine (Grenz et al., 2007a,b) via activation of renovascular A_{2B} receptors (Grenz et al., 2008) mediates renal ischemic preconditioning and protects the kidney from ischemia/reperfusion injury. Moreover, renovascular A_{2B} receptors dampen the hypoxia-induced vascular leak in mouse

Fig. 5. Bar graphs directly comparing percent reversal (antagonism) by 100 nM MRS-1754 (MRS) of the antiproliferative effects of adenosine (ADO) (30 μ M), 2',3'-cAMP (30 μ M), 2'-AMP (30 μ M), and 3'-AMP (30 μ M) on PGVSMCs (A) and GMCs (B). Values are means $\pm$ S.E.M. of n PGVSMC cultures. a, $p < 0.05$ versus adenosine, 2'-AMP, and 3'-AMP; b, $p < 0.05$ versus adenosine and 2',3'-cAMP.

kidneys (Eckle et al., 2008), and recent studies using the rat in vitro blood-perfused juxtamedullary nephron technique show that A_{2B} receptors in the afferent arteriole cause profound vasodilation and counteract A_1 receptor-induced vasoconstriction (Feng and Navar, 2010). The fact that 2',3'-cAMP, 2'-AMP, and 3'-AMP can be metabolized to adenosine and can thereby activate renovascular A_{2B} receptors suggests that the 2',3'-cAMP-adenosine pathway not only may regulate vascular and glomerular structure via antiproliferative effects but may also participate in renal protection and regulation of renovascular tone.

In summary, the present study shows for the first time that 2'-AMP and 3'-AMP exert antiproliferative effects in both PGVSMCs and GMCs. In PGVSMCs, these effects are completely mediated by adenosine- A_{2B} receptor interactions. In GMCs, all of the antiproliferative effects of 2'-AMP are due to adenosine; however, in GMCs a small component of the antiproliferative effects of 3'-AMP is independent of adenosine. Finally, in both PGVSMCs and GMCs, 2',3'-cAMP has antiproliferative effects independent of 2'-AMP, 3'-AMP, and adenosine. Because mRNA turnover is a major endogenous source of 2',3'-cAMP (Jackson et al., 2009; Ren et al., 2009), increased mRNA metabolism stimulated by apoptosis (Del Prete et al., 2002) may inhibit PGVSMC and GMC growth and limit pathological remodeling in renal diseases via production of 2',3'-cAMP, 2'-AMP, and 3'-AMP. Moreover, part of the pharmacology of rapamycin, which stimulates mRNA turnover (Banholzer et al., 1997; Hashemolhosseini et al., 1998; Albig and Decker, 2001), may be due to effects of 2',3'-cAMP, 2'-AMP, and 3'-AMP. In this regard, rapamycin is commonly used for renal transplant patients because of its lower renal toxicity relative to that of other immunosuppressants, and inhibition of proliferation of

PGVSMCs and GMCs could be involved in this beneficial action.

Authorship Contributions

Participated in research design: Jackson and Dubey.

Conducted experiments: Gillespie.

Contributed new reagents or analytic tools: Dubey.

Performed data analysis: Jackson.

Wrote or contributed to the writing of the manuscript: Jackson and Dubey.

Other: Jackson acquired funding for the research.

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