

Bivalirudin exerts antiviral activity against respiratory syncytial virus-induced lung infections in neonatal mice

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Respiratory syncytial virus (RSV) is the most common cause of small airways inflammation in the lungs (bronchiolitis) in neonates and immunocompromised adults. The deregulation of cellular and plasma components leads to increased morbidity and mortality. The activation of the clotting cascade plays a key role in the progression of disease severity during viral infection. The current investigation studied the effect of bivalirudin (BR) on the progression and cellular effects of RSV-induced infection in the neonatal mice model. Mice (5–7 days old) were inoculated intranasally with RSV with or without BR administration ($2 \text{ mg kg}^{-1} \text{ day}^{-1}$, *i.v.*) for 2 weeks. Tissue histopathology, inflammatory signalling genes such as TLR, and cytokines were analyzed. The results showed pneumocytes exhibiting nuclear pyknosis, cellular infiltration in lung tissue and increased lung titers in RSV-infected mice compared to the control. Furthermore, RSV-infected mice demonstrated altered clotting parameters such as D-dimer, soluble thrombomodulin, and increased inflammatory cytokines IL-5, 6, IFN- γ , IL-13, and CXCL1. Additionally, the mRNA expression analysis displayed increased levels of IL-33, TLR3, and TLR7 genes in RSV-infected lung tissue. Further, to delineate the role of micro RNAs, the qRT-PCR analysis was done, and the results displayed an increase in miR-136, miR-30b, and let-7i. At the same time, the down-regulated expression of miR-221 in RSV-infected mice compared to the control. BR treatment reduced the cellular infiltration with reduced inflammatory cytokines and normalized clotting indices. Thus, the study shows that RSV infection induces specific changes in lung tissue and the clotting related signalling mechanism. Additionally, BR treatment significantly reduces bronchiolitis and prevents the severity of the infections suggesting that BR can possibly be used to reduce the viral-mediated infections in neonates.

Keywords: respiratory syncytial virus, neonates, mice, bivalirudin, inflammatory cytokines

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Respiratory syncytial virus (RSV) is the most prominent cause of respiratory tract infections among infants (1, 2). It causes bronchiolitis in infancy, and the symptoms include recurrent wheezing and asthma. In many children, these symptoms are also associated with the onset of asthma (3) and could be life-threatening when it complicates viral pneumonia (4). RSV affects infants and children majorly and also elderly and immunocompromised individuals among adults (5).

RSV infections are caused due to the exposure of the individuals' lungs to highly polluted air, allergens from pets, fungi bacteria and they influence the quality of the lung functions and are prone to this infection (6). The severity of RSV infection has been global and there are no vaccines or effective medicines available for treatment except for the regular antivirals (ribavirin) and bronchodilators (7) in managing RSV bronchiolitis.

Innate immunity induced in the case of viral infections occurs through the activation of Toll-like receptors (TLRs), which recognize the viral particles and activate the production of cytokines and antigen presentation to mount an adaptive immune response in the host (8). Hence, many TLR agonists have been used as a therapeutic strategy in the fight against viral infections in animal models for influenza and RSV (9).

RSV-mediated disease development involves pulmonary inflammation combined with inflammatory cytokines secretion, the homeostatic imbalance between coagulation and fibrinolysis, and altered lung physiology with significant lesions (10). Insights into host responses have driven us to finalize our candidate molecule, bivalirudin in our proposed therapeutic strategy against RSV infection in mice. Bivalirudin (BR), a 20-aminoacid peptide derived from hirudin (11), is a direct thrombin inhibitor and is used in the treatment of thrombosis (12) as an alternative to heparin. It has antiviral properties in being effective against the influenza virus (13) and RSV bronchiolitis (14). It is potent in controlling the hemostatic and inflammatory responses (15). Hence we treated the neonatal mice infected with RSV, with BR and studied the responses of the drug candidate in controlling the pulmonary infection of RSV with parameters including inflammatory cytokines secretion, pulmonary inflammation, and pulmonary thrombosis.

EXPERIMENTAL

Experimental groups and induction model

C57BL/6 mice (5–7 days old) were obtained from Jackson Laboratory, Harbor, USA. All animal research experiments were carried out as per the guidelines provided by the animal ethical committee. All regulations and protocols were followed after obtaining prior permission from the Institutional Animal Care and Use Committee (Ethics committee XET132543, Dated: 2019) for the procedures implemented in the present study. The animals were housed in pathogen-free cages kept at 20–25 °C with 50–70 % relative humidity (RH). All mice were fed with clean tap water and commercial mouse chow. RSVA2 were obtained from ATCC and was propagated in HEp-2 cells for the RSV infection, as per the previous publications (16). RSV stock solution at the concentration of 5×10^6 TCID₅₀/0.035 mL was prepared. Mice were anaesthetized with ketamine/xylazine solution (Sigma Aldrich, K113) (0.04 mL/0.020 kg b.w.) and 0.035 mL of virus dilution was inoculated intranasally into its nostrils using a 0.1 mL pipette made to reach the lungs (17).

For experiments, mice were separated into 4 groups ($n = 8$ in each group) as follows: the normal control group animals were administered with normal saline (NaCl 0.9 %) (control group); mice infected with RSV were denoted as an infected group (RSV group); while mice administered with BR (obtained from Sigma Aldrich; purity $\geq 97\%$) with the dosage of 2×10^{-6} kg/kg, *i.v.* alternate days for 2 weeks just before RSV infection was kept as a group (RSV + BR group). Mice received BR alone was separated as a drug control group (BR group). At the end of 2 weeks, the animals were sacrificed by cervical decapitation, and lung tissues were collected for the histological analysis. BALF and blood were collected for cytokine analysis. Relative copy numbers of RSV infection were evaluated using the PCR method as described in previous publications (17).

Histological analysis

Lungs have been fixed in formalin, processed and embedded in paraffin blocks. 5 μm sections were made using a microtome, stained with hematoxylin and eosin (using H&E Staining Kit ab245880, Abcam, USA). Histology inflammatory response was scored in a blinded manner, according to the degree of inflammation from 0–4 ranging: no inflammation, mild moderate and severe was assigned to each section and the average of the scores was used for the analysis purposes.

Determination of clotting factor analysis

For assessing the involvement clotting factor, the estimation of D-dimer (Cusabio technology LLC, USA), thrombin time (TT), soluble thrombomodulin, and tissue-plasminogen activator inhibitor-1(PAI-1) was determined using commercial assay kits (Abcam).

Assessment of broncho-alveolar lavage fluid (BALF)

BALF from anaesthetized mice was obtained by insertion of a cannula into the exposed trachea was carried out. The lungs were lavaged with three aliquots (0.5 mL) of sterile saline that was recovered through the cannula. Estimation of inflammatory cytokines was carried out in BALF. The estimation of cytokines such as IL-5, 6, CXCL1, CCL11 (eotaxin), IL-13, and IFN- γ was estimated using marketable ELISA kits as per the manufacturer's instruction (Wuhan Fine Biotech Co., Ltd., China).

Reverse transcription-PCR

For the analysis of inflammatory genes associated with the RSV and BR administration, the total RNA was isolated from neonatal lung tissues using the RNeasy Mini Kit (Qiagen, USA, Cat. No.: 74004) and using the One-Step RT-PCR Kit (QIAGEN, USA, Cat. No.: 210212), and customized primers (Table I), the mRNA of specific genes were elucidated. On the other hand, expression analysis of selected microRNA was performed using miR-specific primers. Quantitative detection of miR-136, miR-30b, let-7i, and miR-221 was performed using TaqMan MicroRNA Reverse Transcription Kit, TaqMan miR specific assays and snoRNA assays (ThermoFischer scientific, USA). The PCR assays were carried out according to the manufacturer's instructions. The Ct values obtained were compared with housekeeping genes to determine the gene expressions by the comparative Ct method ($\Delta\Delta\text{CT}$). The results were expressed as a fold increase of the gene of interest.

Statistical analysis

Outcomes were assessed statistically using GraphPad Prism 6 (GraphPad Software, USA). The data obtained are presented as mean \pm SD ($n = 3$) and analysed by one-way analysis of variance (ANOVA), followed by a post-hoc test for multiple comparisons. Values of $p < 0.05$ were considered to be significant; the comparisons of individual p values between groups are indicated in the figure legends.

RESULTS AND DISCUSSION

The present study aims to evaluate the beneficial role of BR in neonatal RSV-infected mice. Mice infected with RSV showed a significant increase in smooth muscle mass, with pneumocytes exhibiting nuclear pyknosis and cellular infiltration in lung tissue compared to control. At the same time, mice co-administered with BR demonstrated a significant ($p < 0.05$) decrease in smooth muscle mass (Fig. 1a,b). Moreover, the viral copy number showed changes towards the less multiplication in BR surmise that the RSV infection was on hold that could reduce the mortality in immune-compromised mice (Fig. 1c).

Furthermore, the clotting parameters were determined as shown in Fig. 2. The results established a significant upsurge in the levels of D-dimer ($p < 0.05$), TT ($p < 0.05$), soluble thrombomodulin ($p < 0.05$), and tissue-plasminogen activator inhibitor-1 ($p < 0.05$) in RSV-infected mice. The clotting parameters were found to be less affected in mice administered with BR compared to infected animals (Fig. 2).

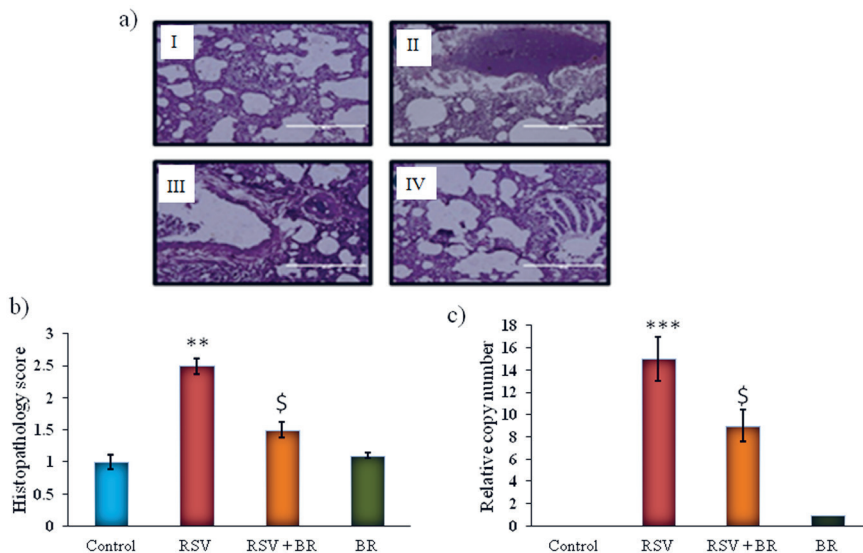


Fig. 1. a) Lung tissue histology (I-IV), where I represents control lung tissue, II represents lung tissue after RSV treatment, III represents lung tissue after RSV and BR treatment, and IV represents lung tissue after BR treatment, b) inflammation score, c) relative copy number of virus in the lung tissues between experimental group and control (* shows $p < 0.05$).

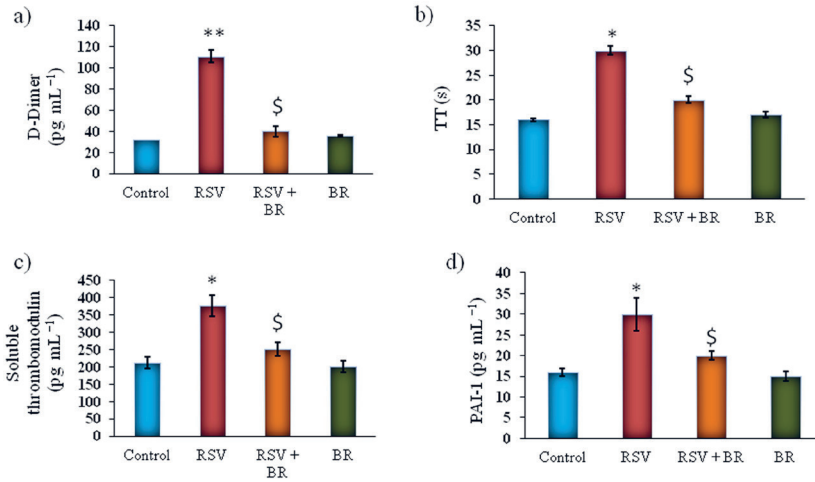


Fig. 2. a) Levels of D-dimer between experimental groups and control, b) thrombin levels between experimental groups and control, c) soluble thrombomodulin levels between experimental groups and control, d) tissue-plasminogen activator inhibitor-1 between experimental groups and control (* shows $p < 0.05$).

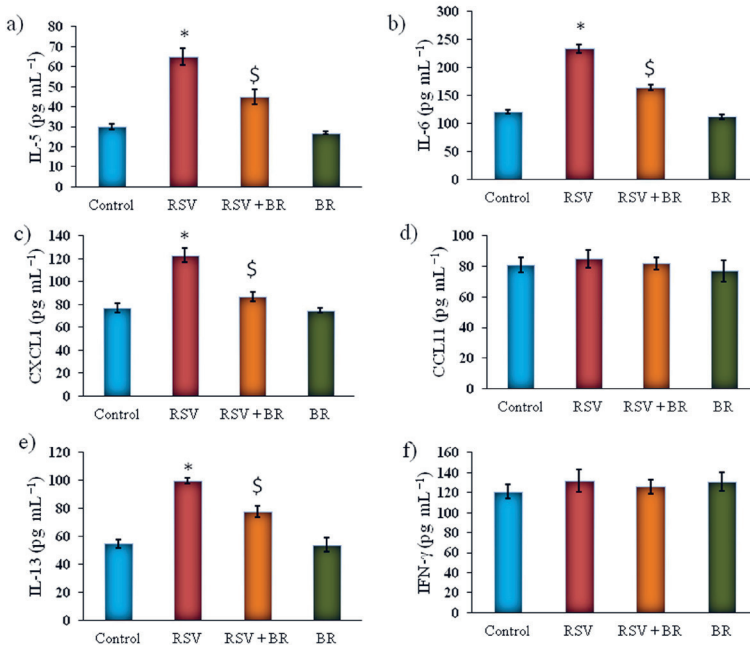


Fig. 3. Cytokine expression analysis of: a) IL-5, b) IL-6, c) CXCL1 (keratinocyte chemoattractant), d) CCL11 (eotaxin), e) IL-13 and f) IFN- γ , respectively, between experimental and control groups (* shows $p < 0.05$).

We then investigated the levels of cytokines as shown in Fig. 3. In the present investigation, mice infected with RSV demonstrated a substantial ($p < 0.05$) increase in the levels of cytokines, like IL-5, IL-6, CXCL1 and IL-13 compared to control. Furthermore, increased levels of cytokines were attenuated by BR treatment (Fig. 3). On the other hand, no significant changes were observed in CCL11 (eotaxin), and IFN- γ expression in any of the groups.

Fig. 4 demonstrates the inflammatory markers corroborated in RSV infection with or without BR administration. The results demonstrated a significant ($p < 0.05$) increase in the IL-1 β , IL-33, CCL5, TLR-3, TLR-7, and CXCL2 in RSV mice lung tissues compared to control. However, it was observed that increased levels of these indicator molecules were attenuated by BR treatment (Fig. 4).

Studies have proved that the onset of micro RNAs is the predictor of the prognosis of various cellular functions. To observe the potential regulation of miRs in RSV infection, in this study, we performed a qPCR analysis of miR-136, miR-30b, let-7i, and miR-221. A significant increase in the levels of miR-136, miR-30b and let-7i was observed in RSV-infected mice, and a reduced expression level of miR-221 was observed. On the contrary, expression levels of miR-136, miR-30b, let-7i, and miR-221 did not change in the BR group, suggesting that these molecular clues could be the regulators of RSV-mediated signalling (Fig. 5).

In our present study of RSV infection in neonatal mice, the ability of BR to inhibit the effects of RSV infection by reducing inflammation and clotting was investigated. Importance of thrombin has been highlighted here because many of the viruses, particularly those that affect the respiratory tract like RSV, influenza virus and human metapneumovirus have exploited thrombin in their pathogenicity although by different mechanisms (18). Thrombin has been implicated in the viral replication of RSV in the host and the concomitant inflammation in the lungs (19). BR, a thrombin inhibitor, has been used in mice challenged with RSV infection and monitored their progress in lung improvement with a significant reduction in lung viral replication and lung inflammation.

The successful establishment of the mouse model for RSV infection was monitored by the virus growth kinetics and has demonstrated the pulmonary lesions in the RSV-infected mice, and the virus titer is high. These lesions are due to the recruitment of the secretion of cytokines to cause inflammation (20). Simultaneously, neutrophils, monocytes, eosinophils towards the respiratory epithelium, and an increase in the infection on the pneumocytes has rendered them defunct with the morphonuclear changes (21). RSV infection has critically affected the cell morphology with distortions in the cytoskeleton and flattening of the apical ends. It is typical of the viral infection to invade the cytoplasm as a host response to the infection (22). Hence we observed an increase in the smooth muscle mass in the lungs of mice infected with RSV. RSV-infected cells show alterations in the structure of the nucleus due to nuclear pyknosis that is a characteristic feature of the immune system in preventing the spread of the virus to the neighbouring cells and the cells undergo apoptosis (21). Our histological studies have proven that the infection in the pneumocytes by RSV has been effective and has activated the immune mechanism. In the BR-treated mice group, the morpho-nuclear changes were reversed with the cells showing a recovery in their structure, and that has prevented the viral spread causing a reduction in the nuclear pyknosis, which is substantiated (23) by the reduction in the viral copy number due to the effective antigen presentation.

RSV infection causes changes in the blood clotting factors by increasing the coagulation activation, reducing the clotting time, increase in thrombin generation, inhibition of

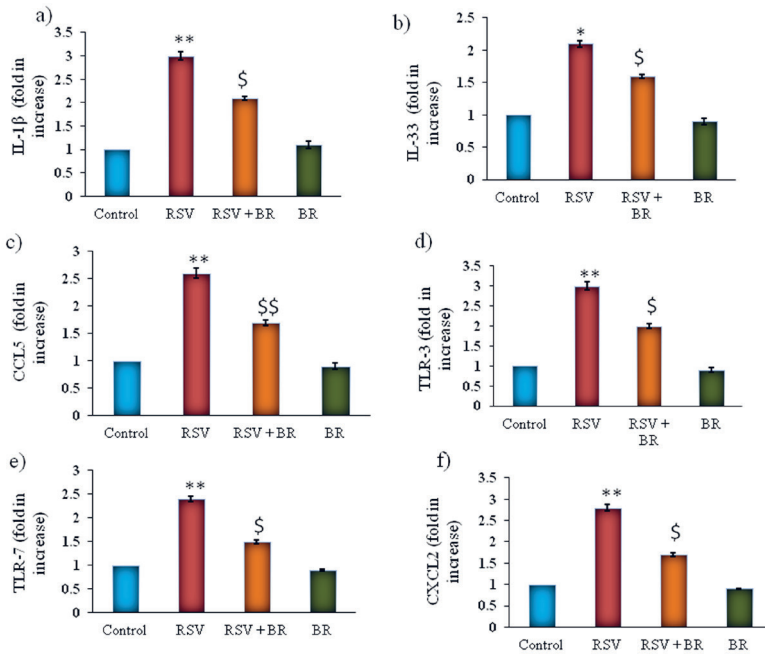


Fig. 4. qRT-PCR mRNA expression analysis of: a) IL-1 β , b) IL-33, c) CCL5, d) TLR-3, d) TLR-7 and e) CXCL2, respectively, between experimental and control groups (* shows $p < 0.05$).

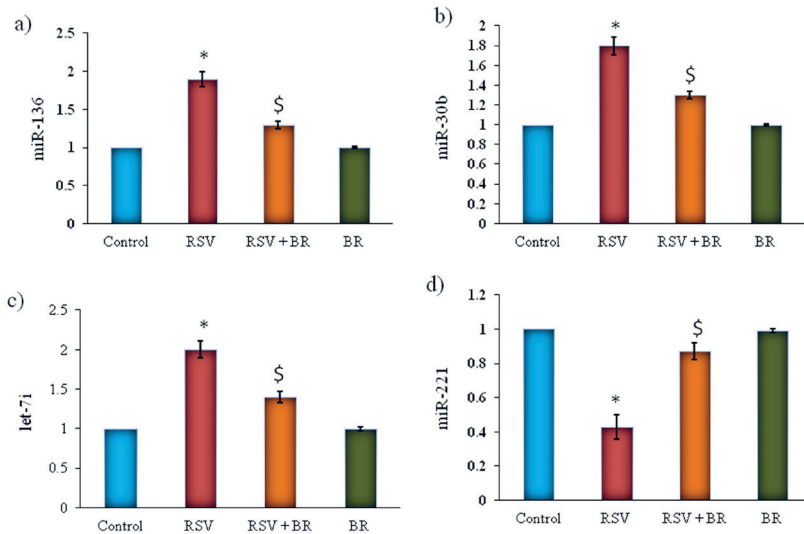


Fig. 5. Expression analysis of microRNAs: a) miR-136, b) miR-30b, c) let-7i and d) miR-221, respectively, between experimental and control groups (* shows $p < 0.05$).

fibrinolysis (24) and increase in the hemostatic coagulation markers of D-dimer (25) and plasminogen activator inhibitor-1 (PAI-1) (26). Our animal experiments have also indicated the acute phase of RSV infection with increased levels of these parameters that indicate the increased fibrinolysis and changes in the hemostatic system of the mice that showed a trend towards a procoagulant state after were infected with RSV (27). Mice treated with BR showed their hemostatic parameters have returned to near-normal levels with a reduction in the D-dimer, thrombin time (28) and soluble thrombomodulin indicating a reduction in the viral infection. They would be in the convalescent phase with the viral clearance has been initiated.

RSV infection was found to increase the expression levels of TLR3 and TLR7, as has been previously observed (23). The replication of RSV converts the single-stranded RNA to double-stranded form, and that is an effective ligand for TLR3 indicating that TLR3 is expressed higher than that of TLR7 in case of RSV infection and replication (26) as demonstrated in our results (Fig. 4d,e). BR treatment has effectively reduced the RSV replication as evidenced by the decreased expression of TLR3 and the corresponding decrease in TLR7 with the reduction in the inflammation.

The replicated viral nucleic acids would bind to TLR3 and TLR7 and would activate the NF-KB (30) and IRF-3 pathways to induce the expression of cytokines and chemokines (31) like IL-1 β , IL-6 (32) to mount effector immune response towards pathogen and lung inflammation. These cytokines have been observed to be linked to the development and maintenance of the airway infectious disease (33), disease severity (34) and its pro-inflammatory response in the lung (35). Besides, TLR7 activates the expression of IL-13 (36) specifying the role played by TLR7 in recognizing RSV infection and the ‘immune initiation’ (37).

Neutrophils have been the prominent immune cells recruited in the lungs due to their infiltration during RSV infection (38). They are recruited by the increased expression of chemokines such as CXCL1 and CXCL2 that are widely studied in mice for their role in RSV infection (39). They are highly expressed in the RSV-infected mice and may serve to exert the ‘neutrophil traps’ (40) to counter the pathogen spread but more studies are required to confirm the beneficiary effects of neutrophil recruitment to the lungs in RSV-infected animals. BR treatment has reduced the excessive neutrophil infiltration to the lungs by reducing the pathogenicity of the RSV infection.

At the molecular level, we have evaluated the role played by the micro RNAs in the pathogenicity of the RSV infection in mice. Micro RNAs expressed during the RSV infection can influence the functions of epithelial cells, macrophages, monocytes in mounting an innate response in the host. Our results have demonstrated that miR-30b, miR-136 and let-7i have been upregulated in the RSV-infected mice and are known to be participating in the upregulated expression of proinflammatory cytokines whereas miR-221 is known to be inhibiting the RSV infection by interfering in their replication is upregulated in the BR treatment mice contrary to the other miRs discussed here.

Our study has demonstrated that BR has antiviral activity against RSV by modulating the expression of proinflammatory cytokines, hemostatic responses in our mouse model for RSV infection. It has treated the mice from the acute infection by enhancing the lung capacity and reducing the viral titers and improving lung function by reducing inflammation. This has proven that BR is an effective therapeutic alternative in the treatment of RSV infection in mice and control RSV pathogenesis.

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