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A 3R's Research: Rapid Assessment of New Indigenously Made Ventilators in Acute Respiratory Distress Syndrome (ARDS) Induced Rabbits by Non-invasive Method

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ABSTRACT

Background: Laboratory animal models were frequently used to assess the efficiency of newly made ventilators. Assessment of efficiency of these ventilators may be useful for human patients affected with respiratory distress or COVID-19.

Methods: Rabbits were randomly divided into four groups *viz*., negative control, positive control (ARDS induced), induced ARDS and co-treated with referral ventilator (VR), ARDS induced and co-treated with experimental ventilator (VE). The groups were assessed by measuring the BGA, vitals, tidal volume (VT) and other ventilator parameters.

Result: SpO_2 of control group (98.75±0.25) did not reveal any significant difference with VR -1 hr (97.75±0.63), VR-3 hrs (97.75±0.95), VE-1 hr (96.75±1.11) and VE-3 hrs (97.25±0.85). While comparing with other groups, ARDS group (61±7.69) revealed a highly significant decrease ($p<0.01$) in the SpO_2 level. PEEP of control and other groups were found to be equal (4±0.00).

Key words: 3R's, Acute respiratory distress syndrome, Rabbit, Ventilator.

INTRODUCTION

Globally ~80% of the COVID-19 virus infected patients exhibited mild symptoms, 14% suffered with acute respiratory distress syndrome (ARDS) and pneumonia, 5% suffered from septic shock and organ failure (mostly respiratory failure) and in 2% cases it was found to be fatal. In ARDS, radiography shows bilateral pulmonary infiltrates on chest with impaired oxygen levels in blood (Cross and Matthay, 2011; Christoph and Carsten, 2015). Mechanical ventilators are the only support and can replace spontaneous breathing in ARDS patients. The ventilators are critical life-support devices that provide assistance to respiratory therapy to millions of patients every day. Before introducing any type of new ventilators, it has to be tested in animal models which provide practical means to investigate the risks and complications of the newly constructed assisted ventilation. Rabbit is the simplest laboratory animal model frequently used to test drugs and ventilators (Dellaca and Farre, 2009; Dukic, *et al*., 2016). The current experiment was to evaluate and compare the functions of experimental ventilator (VE) with well-established referral ventilator (VR) tested on acute respiratory distress syndrome (ARDS) induced rabbits. These non-invasive experimental procedures were supporting the principle of 3Rs (Replacement, reduction and refinement) research leads to reduction of animal sufferings.

MATERIALS AND METHODS

Experimental animal and study design

Experiments were performed in accordance with ethical guidelines and approved by the Institutional Animal Ethics Committee (IAEC) (IAEC approval No. 105/SA/IAEC/2020). Sixteen adult New Zealand White strain rabbits (body weight between 2.50 to 3.00 kg) were purchased from CPCSEA approved vendor; Post Graduate Research Institute in Animal Sciences (PGRIAS), Kattupakkam, Chennai. Before the start of experiment, rabbits were acclimatized in 12 hours light and 12 hours dark cycle for 7 days. Animals had free access to water and lab animal feed. Rabbits were randomly divided into four groups. Group-I (Control) kept as untreated control (n=4). Group-II (ARDS) rabbits were induced with ARDS (n=4). ARDS was induced with 0.9% saline infusion and repetitive lavage in lung for 6 times *via* endotracheal tube with infant feeding

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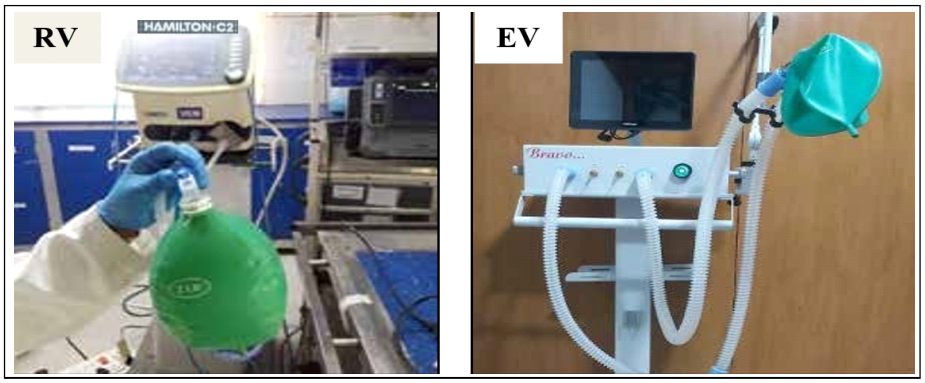


Fig 1: Test lung was performed prior to ventilation with referral and experimental ventilators.

tube at the dose rate of 30 ml/kg b. wt. (Kosutova *et al.*, 2018) in semi-upright right and left lateral positions of the animal and was immediately suctioned. Group III (VR) rabbits (n=4) were induced with ARDS and co-treated with referral ventilator (Hamilton-C2) (VR). Group III rabbits were compared with Group IV (VE) rabbits (n=4) which are ARDS induced and co-treated with experimental ventilator (Bravo ICU ventilator) (VE). The groups were assessed by measuring the BGA, vitals, tidal volume (VT) and other ventilator parameters. All the animals were kept under constant observation throughout the entire period of study.

Procedure

i. Test lung

Test lung was performed prior to ventilation with referral (VR= Hamilton ventilator, model: Hamilton-C2) and experimental ventilator (VE= Bravo ventilator, model: Bravo ICU ventilator) (Fig 1). The already reported accuracy and clinical settings for Hamilton ventilator (RV) (Al Otaibi *et al.* 2019) were used for VR. The new indigenously made Bravo Ventilator (Experimental Ventilator) also had similar clinical settings and both the VR and VE were expected to have similar functions.

ii. Anaesthesia and clinical monitoring

Anaesthesia was induced by administration of intramuscular injection of a mixture of ketamine @ 30 mg/kg and xylazine @ 4 mg/kg in the gluteal region of the rabbits. After 10 minutes interval, midazolam @ 0.2 mg/kg by i/m was injected to achieve adequate muscle relaxation. After the palpebral reflex and pain reflex were abolished, the rabbits were intubated and monitored with a pulse oximeter and an electrocardiograph. The information of oxygen saturation in arterial blood, respiration rate and pulse rate were recorded. The experimental animals' bodies were covered by a heating blanket and their rectal temperature was maintained at 38 to 39°C (Rotta *et al.,* 1999; Mullhaupt *et al.,* 2017) (Fig 2).

iii. Haematology, Biochemical and blood gas analysis (BGA)

Arterial blood samples were collected for blood gases



Fig 2: Anesthesia and clinical monitoring.

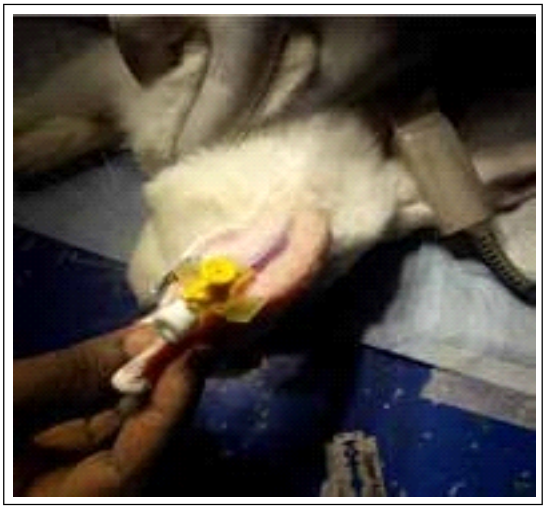


Fig 3: Collection of blood sample for hematology, biochemical and blood gas analysis (BGA).

analysis (BGA) using Epoc reader Blood gas analyser and estimation of haematological parameters (Auto haemo-analyser, Bevet 2800) and biochemical parameters (A15 Biosystems). Blood sampling for BGA in ventilated rabbits was recorded at one hour and three hours after inducing ARDS. The volume of sample necessary for single BGA

was 0.15 ml which was collected in a 1 ml heparin-rinsed insulin syringe from the auricular artery. All BGAs including the partial pressure of oxygen (PO_2), partial pressure of carbon dioxide (PCO_2), pH of the whole blood, oxygen saturation (SO_2) and concentration of bicarbonate ions (HCO_3-) were measured by a portable BGA analyser (cartridges' Epoc gas analyser) (Lord *et al.,* 2010) (Fig 3).

iv. Endotracheal intubation

After induction of anaesthesia, the rabbits were intubated by positioning them in left lateral recumbency and extending the neck of the rabbit. A lubricated No. 2.5 non-cuffed endotracheal tube was passed along the dorsal surface of the tongue and gently introduced into the trachea after the rabbit elicited cough reflex when the endotracheal tube tip touches the epiglottis. The correct position of endotracheal tube was assessed by radiography (Fig 4) (Kollef and Micek, 2010).

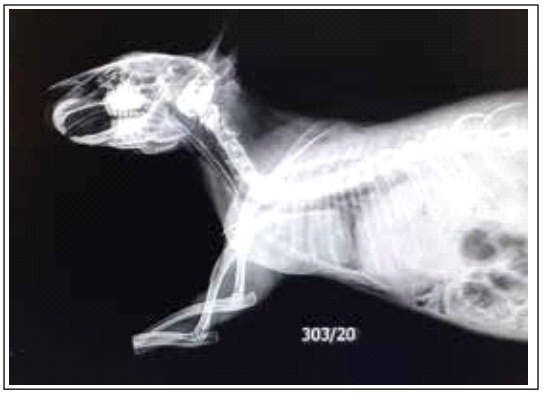


Fig 4: Endotracheal intubation in radiography.



Fig 5: Mechanical ventilation.

v. Mechanical ventilation

The experimental ventilator was periodically auto-calibrated (inbuilt) for the set ventilator parameters. The animals were intubated and stabilized on positive pressure ventilation with the following settings: FiO2 = 100%, Flow = 10 L/min, respiratory rate (RR) = 40 breaths/min, positive end-expiratory pressure (PEEP) = 4 cm H_2O, tidal volume (VT) targeted to 8 ml/kg (with the peak inspiratory pressure, PIP not exceeding 15 cm H_2O). Tidal volume (VT) was monitored continuously with a flow sensor connected to the endotracheal tube. Body temperature was continuously monitored with a rectal probe and covered with warmer blanket and it was also maintained by placing a heating pad underneath the animal. The pulse-oxymeter was attached to the ear of the animals in order to monitor saturated oxygen level of arterial blood (Fig 5) (Govoni *et al*., 2012; Krimsky *et al.,* 2009).

vi. Electrocardiography (ECG)

Adhesive electrocardiogram (ECG) patches or button electrodes were placed on right arm (red), left arm (yellow) and left leg (green) (Mirabella *et al*., 2014). Rabbits were kept on right lateral recumbency after applying gel on the corresponding site. The ECG tracing consisted of three primary complexes: P wave, QRS complex and T wave, which represent atrial depolarization and ventricular depolarization, respectively. ECG was used to detect abnormal rhythms associated with these conditions, including atrial fibrillation, ventricular premature complexes, ventricular tachycardia and supraventricular tachycardia. Bradycardia was noticed after induction of anaesthesia, whereas atrial fibrillation, ventricular premature complexes, ventricular tachycardia and supraventricular tachycardia were not observed (Fig 6).

Paper speed of ECG	: 25 mm/sec
Sensitivity	: 4/mv
One of the ECG calculated values	
P wave amplitude	: 0.05 mv
Duration	: 0.04 sec
R wave amplitude	: 0.25 mv

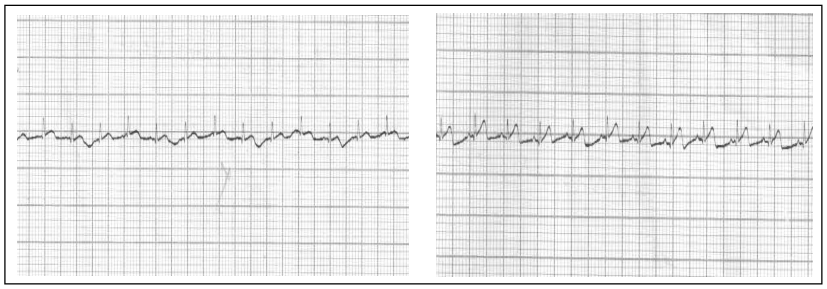


Fig 6: Electrocardiography (ECG).

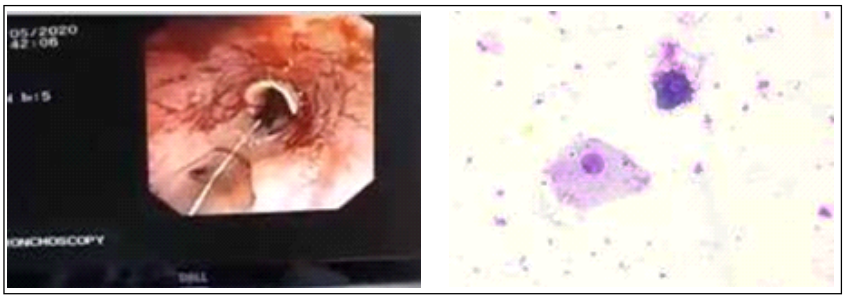


Fig 7: Broncho-alveolar lavage fluid (BALF) collection aided with endoscopy and staining of epithelial cells (40x).

vii. Cell analysis in the broncho-alveolar lavage fluid (BALF)

The lung was lavaged with saline (0.9% NaCl, 37°C, 3 × 10 ml/kg b. wt.) (Fig 7). BALF was collected and centrifuged at 1500 rpm for 3 min. One drop of BALF sediment was used for smear preparation and microscopically examined for inflammatory cells/parasites after staining by Leishman, Giemsa and cocktail stain.

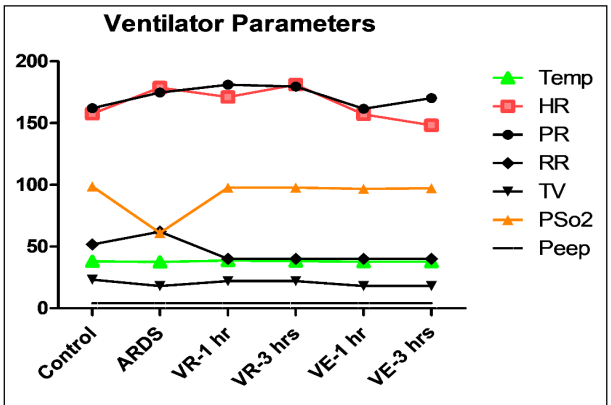
Statistical analysis

Results were analyzed by complete randomized design using one-way ANOVA test and comparison of the means was done by Duncan's Post-hoc test (multiple comparison test).

RESULTS AND DISCUSSION

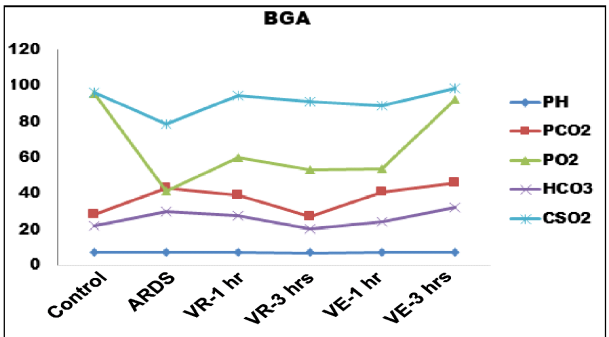
Ventilator parameter

The parameters like temperature, pulse rate and PEEP did not show any significant differences in all four groups (Groups: I-Control, II-ARDS, III-VR and IV-VE). Significant difference ($p<0.05$) was observed in heart rate within VR group with 1 hr ventilation treatment (130.25±17.14) and 3 hrs ventilation treatment (181.00±4.30). Whereas, non significant difference was observed in VE groups where a constant heart rate (157±4.71) was maintained. A highly significant difference was observed in the initial respiratory rates in the ARDS induced groups (II-ARDS, III-VR and IV-VE) when compared with control and recorded as 62±2.58 in ARDS groups and 51.75±1.70 in control group. The normal respiratory rate of rabbits ranged between 40 to 60 breaths per minute and the obtained results confirm that the ARDS was successfully induced in ARDS, VR and VE groups, where the respiratory rate was increased due to acute stress. Simultaneously, control group was observed with 23.25±1.97 of tidal volume and 98.75±0.25 of SpO_2, which significantly differ ($p < 0.01$) from other ARDS induced groups (II-ARDS, III-VR and IV-VE) as they recorded 18.00±1.15 of initial tidal volume and 61±7.69 of initial SpO_2. After 1 hr ventilation treatment, both the RV and EV significantly reduced the respiratory rate (40.00±0.00), increased the tidal volume (RV: 22.00±0.15; VE: 18.00±1.15) and SpO_2 (VR: 97.75±0.63; VE: 96.75±1.11) of each groups. No significant difference was observed between and within



VR: Referral ventilator; VE: Experimental Ventilator; ARDS: Acute Respiratory Distress Induced rabbit; Heart rate and respiratory rate were marginally elevated and significantly elevated in ARDS group respectively when compared with other three groups. SpO_2 levels of 95% to 100% are considered to be clinically normal. ARDS group showed a significant decline in SpO_2 levels whereas no significant difference was noticed between control, VR and VE groups. Tidal volume of ARDS VE-1hrs, VE-3 hrs showed significant decrease ($p<0.05$). Whereas, tidal volume of VR - 1 hr and VR - 3 hrs was not significant when compared to control group and ARDS.

Fig 8: Line chart for comparing ventilator parameters before and after treatment (after 1 hr and 3 hrs).



VR: Referral ventilator; VE: Experimental Ventilator; ARDS- Acute Respiratory Distress Induced rabbit;
Blood gas analysis was done and found no significant deviation in blood pH levels between the groups. PO2 levels in ARDS group showed significant decrease when compared to other groups

Fig 9: Line chart for BGA.

Table 1: Ventilator parameter.

Parameter	Group-I (Control)	Group-II (ARDS)	Group-III (VR-1hr)	Group-III (VR-3 hrs)	Group-IV (VE-1 hr)	Group-IV (VE-3 hrs)	F value	Sig
Temp	38.13±0.14^{ab}	37.53±0.35^{a}	38.68±0.27^{b}	38.33± 0.32^{ab}	37.70± 0.30^{a}	37.80±0.15^{a}	2.630	0.059
Heart rate	157.50±14.55^{ab}	178.75±4.78^{b}	130.25±17.74^{a}	181.00± 4.30^{b}	157.00±4.71^{ab}	148.25±8.97^{ab}	3.260	0.029
Pulse rate	162.00±3.19^{a}	174.74±4.21^{a}	181.00±7.87^{a}	179.50±8.1^{a}	161.50±6.89^{a}	170.25±5.36^{a}	1.840	0.155
Respiratory rate	51.75±1.70^{b}	62.00±2.58^{c}	40.00±0.00^{a}	40.00± 0.00^{a}	40.00±0.00^{a}	40.00±0.00 ^{a}	113.68	0.000
Tidal Volume	23.25±1.97^{b}	18.00±1.15 ^{a}	22.00±1.15^{ab}	22.00±0.15^{ab}	18.00±1.15^{a}	18.00±1.15^{a}	3.443	0.023
SpO_2	98.75±0.25^{b}	61.00±7.69^{a}	97.75±0.63^{b}	97.75±0.95^{b}	96.75±1.11^{b}	97.25±0.85^{b}	-	-
PEEP	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	-	-

Note: Data are presented as mean±S.E. of four animals in each group.

Data with similar superscripts in a row or column do not differ significantly (p>0.05).

Data with different superscripts in a row or column showed significant (p<0.05) or highly significant (p<0.01) difference.

Table 2: Blood gas analysis (BGA).

Parameter	Group-I (Control)	Group-II (ARDS)	Group-III (VR-1hr)	Group-III (VR-3 hrs)	Group-IV (VE-1 hr)	Group-IV (VE-3 hrs)	F value	Sig
PH	7.48±0.042^{a}	7.45±0.019^{a}	7.44±0.026^{a}	7.37±0.095^{a}	7.44±0.147^{a}	7.45±0.029^{a}	0.262	0.928
PCO_2	28.75±1.46 ^{ab}	43.35±3.30^{c}	39.33±4.02^{bc}	27.33±5.06^{a}	40.90±2.43^{c}	46.03±4.02^{c}	4.706	0.006
PO_2	95.70±1.82^{c}	41.80±1.72^{a}	60.18±11.39 ^{b}	53.58±6.42^{ab}	53.90±3.77^{ab}	92.20±1.37^{c}	15.440	0.000
HCO_3	22.22±2.11^{ab}	30.45±1.29^{bc}	27.85±1.27^{abc}	20.70±2.47^{a}	24.38±4.48^{abc}	32.55±3.76^{c}	2.758	0.051
CSO_2	96.33±0.70^{cd}	79.00±2.64 ^{a}	94.63±2.022^{bcd}	91.43±3.41^{bc}	88.88±1.38^{b}	98.78±0.26^{d}	11.886	0.000

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1 hr and 3 hrs of ventilation in both the VR and VE groups (Table 1 and Fig 8).

Blood gas analysis (BGA)

There was non significant difference in the pH values of control and other groups (p>0.05). But parameters such as initial PCO_2, PO_2, HCO_3 and SO_2 showed high significant difference between the control and other ARDS induced groups (II-ARDS, III-VR and IV-VE). After 1 hr ventilation treatment both the RV and EV significantly reduced the PCO_2, PO_2, HCO_3 and SO_2 to the normal level. No significant difference was observed between and within 1 hr and 3 hrs of ventilation in both the VR and VE groups. The obtained results revealed that there were no functional differences between referral and experimental ventilators in the treatment of ARDS (Table 2 and Fig 9).

Electrocardiography (ECG)

The ranges of different parameters of VR and VE group was given in the Table 3. Non-significant differences were found in the ECG analysis between the two treated groups.

Broncho-alveolar lavage fluid (BALF)

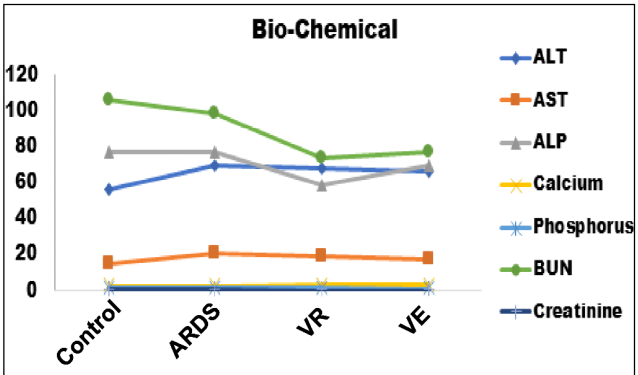
The highest number of cells retrieved from both the VR and VE treated groups by BAL were the inflammatory cells namely macrophages, neutrophils which line the alveolar space. The alveolar macrophages were the most common cells in the BALF. Bacterial organisms (cocci) were also present in the BALF. This could possibly be due to the acute damage caused to the alveoli of lung as a result of ARDS (Bohlin *et al.,* 2005; Fan *et al.,* 2018).

Biochemical and Haematological profile

No significant difference was observed in the biochemical profile of ARDS induced groups (II-ARDS, III-VR and IV-VE) when compared with control (Table 4). There were no significant differences between the initial and final values of haemoglobin, PCV, RBC, WBC, platelet, monocyte and eosinophil count in the ARDS induced groups (VR and VE) before and after treatment (Table 5). Whereas, significant variations (p<0.05) were observed in other blood parameters like neutrophil (N) and lymphocytes (L) count between VR (N=33.75±2.17; L=62.00±2.12) and VE (N=60.75±2.06; L=36.25±1.93) groups and they were also significantly different from control (N=45.50±3.85; L=50.00±4.06) and ARDS (N=39.75±1.11; L=58.50±1.66) groups. These all were indicating that the ARDS induction may cause wide range of inflammatory response in the respiratory track which ultimately triggers the different range inflammatory cells counts in all ARDS, VR and VE groups. These different range of inflammatory cell counts shows non-significant difference with ARDS recovery rate in both ventilators treated group of VR and VE (Fig 10 and 11).

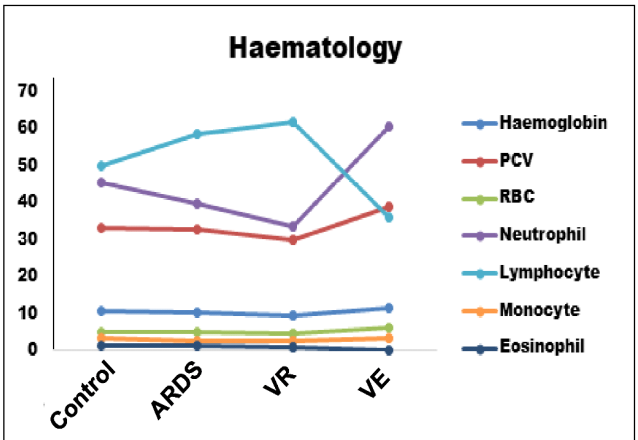
Table 3: Electrocardiography (ECG).

Parameters	Range
P wave amplitude	0.04 to 0.05 mV
P duration	0.03 to 0.04 sec
PR interval	0.06 to 0.08 sec
R wave amplitude	0.08 to 0.25 mV
QRS complex	0.03 to 0.04 sec
QT interval	0.08 to 0.12 sec
T wave amplitude	0.05 to 0.075
Heartrate	160 to 240 beats/minute



VR: Referral ventilator; VE: Experimental Ventilator; ARDS: Acute Respiratory Distress Induced rabbit; Biochemical profile revealed no significant difference between the groups.

Fig 10: Line chart for Bio-chemical parameter.



VR: Referral ventilator; VE: Experimental Ventilator; ARDS- Acute Respiratory Distress Induced rabbit; VE - group revealed highly significant increase in Neutrophils (p<0.01) when compared to other groups. Similarly, lymphocyte count of ARDS and VR groups did not differ significantly (p>0.05) from each other. However, lymphocyte counts of the VE group showed highly significant difference (p<0.01) when compared to other groups. Eosinophil counts of VE ventilator revealed significant difference (p<0.05) when compared to control and ARDS group.

Fig 11: Line chart for Haematological parameter.

Table 4: Biochemical parameter.

Parameter	Group-I (Control)	Group-II (ARDS)	Group-III (VR)	Group-IV (VE)	F value	Sig
ALT	56.00±3.85[a]	70.00±7.79[a]	67.75±5.64[a]	66.25±5.20[a]	1.142	0.372
AST	15.00±1.22[a]	20.50±2.47[a]	19.00±1.96[a]	17.00±1.08[a]	1.821	0.197
ALP	77.50±7.47[a]	77.38±13.15[a]	59.00±12.44[a]	69.75±4.94[a]	0.747	0.545
Calcium	2.98±0.15[a]	2.98±0.32[a]	3.08±0.14[a]	3.55±0.17[a]	1.717	0,216
Phosphorus	1.50±0.07[a]	1.55±0.19[a]	1.58±0.06[a]	1.43±0.09[a]	0.326	0.807
BUN	106.33±17.99[a]	98.93±16.92[a]	73.83±6.64[a]	76.75±6.34[a]	1.496	0.266
Creatinine	0.58±0.63[a]	0.60±0.12[a]	0.55±0.96[a]	0.55±0.96[a]	0.064	0.978

Note: Data are presented as mean±S.E. of four animals in each group.

Data with similar superscripts in a row or column do not differ significantly (p>0.05).

Data with different superscripts in a row or column showed significant (p<0.05) or highly significant (p<0.01) difference.

Table 5: Haematological parameter.

Parameter	Group-I (Control)	Group-II (ARDS)	Group-III (VR)	Group-IV(VE)	F value	Sig
Haemoglobin	10.63±0.58[a]	10.35±o.63[a]	9.55±1.04[a]	11.65±1.53[a]	0.722	0.558
PCV	33.20±1.79[a]	32.88±1.58[a]	30.00±2.98[a]	38.98±4.09[a]	1.809	0.199
RBC	4.85±0.17[a]	4.83±0.28[a]	4.59±0.41[a]	6.18±0.51[b]	3.955	0.036
WBC	8175±1423.83[a]	6725±694.47[a]	6250±490.75[a]	6250±492.44[a]	1.110	0.383
Platelet	461000±51768.72[a]	351500±83080.78[a]	319250±35959.18[a]	399500±31237[a]	1.284	0.324
Neutrophil	45.50±3.86[b]	39.75±1.11[ab]	33.75±2.17[a]	60.75±2.06[c]	21.373	0.000
Lymphocyte	50.00±4.06[b]	58.50±1.66[c]	62.00±2.12[c]	36.25±1.93[a]	19.114	0.000
Monocyte	3.25±0.25[a]	2.75±0.48[a]	2.75±0.25[a]	3.25±0.25[a]	0.517	0.517
Eosinophil	1.25±0.48[b]	1.50±0.29[b]	1.00±0.41[ab]	00.00±0.00 [a]	0.046	0.046

Note: Data are presented as mean±S.E. of four animals in each group.

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CONCLUSION

Our findings are supported by the SpO_2 values of the experimental ventilator (VE) which is 96.75±1.11 and 97.25±0.85 at one hour and three hours, respectively. The present study reveals that the VE (model: Bravo ICU ventilator) performance was at least as protective as VR (Hamilton-C2). We conclude that VE is equal to that of VR. Furtherance, it is promulgated to carry out human clinical trials in respiratory distress like COVID-19. The VE was found to be alleviating the respiratory crisis induced by ARDS in rabbits and there were no adverse effects observed. After experiment, animals were kept under observation, no morbidity and mortality were observed. All the rabbits were recovered from the ARDS condition and it is suggested that after post treatment care, these animals may be used for similar kind of studies. This research suggests that for reducing rabbits use in ventilator assessment during COVID-19 pandemic situation this non-invasive method of ventilator assessment study may be the best choice which strongly supports the 3R's principle. This research may support successful evaluation of newly made ventilators during this COVID-19 crisis.

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Conflicts of interest

The investigators declare that there is no conflict of interest in undertaking this study.

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