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Immunomodulatory effects of 2-deoxyglucose on peripheral blood mononuclear cells: Implications in COVID-19 treatment

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Abstract
PBMCs play a significant role to produce a wide range of cytokines in non-specific immune response. This might the PBMCs play in important role in the cytokines storm generated in severe case in covid-19. This study examines the relationship of 2-DG in modulating the LPS induce release of proinflammatory cytokines from PBMCs. ELISA was used to estimate the absolute value of cytokines, and statistical analysis was performed. The results showed that LPS treatment significantly increased the levels of both TNF-α, and IL-6 compared to control, while co-treatment with 2-DG prevented the LPS effect on PBMCs. These findings suggest that 2-DG might act as an immunomodulator which explain how it showed some beneficial effect in the treatment of COVID-19.

Keywords: PBMCs. LPS, 2DG, Immunomodulator, TNF- α. IL-6, ELISA

Introduction
In December 2019, a new coronavirus emerged as the cause of a severe acute respiratory disease named (COVID-19). The virus that spilled over to humans in China was classified in the family Coronaviridae, genus Beta coronavirus, and was named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), for its similarity to SARS CoV [1]. The SARS-COV-2 originated from the Hubei province of Central China epicentre being in Wuhan city [2]. This infection has been growing since then and has spread to worldwide [3]. The incubation period of SARS-CoV-2 ranges between 1-14 days with the median incubation period of about 5 days. The primary mode of transmission of SARS-CoV-2 infection is through contact with the infected individuals and by respiratory droplets [4]. The SARS-CoV-2 virus binds the Angiotensin-converting enzyme 2 (ACE2) as receptor for cell entry and the transmembrane protein serine protease TMPRSS2 for S protein priming [5]. One peculiarity of SARS-CoV-2 is represented by its tropism for type I and type II pneumocytes, alveolar macrophages and mucus cells in the nasal cavity [6]. Although beyond the respiratory disease, systemic disorders have also been reported [7]. ACE2 enzyme is widely expressed on the plasma membranes of various cell types in different tissues, including intestinal and endothelial cells [8]. The vascular endothelium is among the tissues affected by the SARS-CoV-2 infection as the normal ACE2 peptidase activity leads, through the G-protein coupled receptor, to the activation of signalling pathways responsible for vasodilation, anti-inflammatory, and anti-fibrotic responses.
The immune system plays a key role in COVID-19. In particular, the immune response, both innate and adaptive, contributes not only to the defence against SARS-CoV-2, but also-if dysregulated and hyperactivated can lead to deleterious effects and the “cytokine storm”, which is the basis of the more severe clinical forms of COVID-19. Cytokine storm syndrome has been described in several immune-based pathologies and its role in COVID-19 has been thoroughly investigated. It is characterized by elevated serum concentrations of pro-inflammatory cytokines (i.e., IL-6; Tumor necrosis factor Alpha-TNF-α) and is associated to fatal multi-organ failure [9]. The immune system is equipped with a sophisticated mechanism that can respond to a variety of infections. The activation of the immune system's inflammatory pathways is required for a normal antiviral immune response; nevertheless, an abnormal or exaggerated immunological response by the host might result in serious illness

if left unchecked. Cytokines have a crucial role in the inflammatory response. Several immune cells, including innate macrophages, dendritic cells, natural killer cells, and adaptive T and B lymphocytes, produce cytokines. Pattern recognition receptors (PRRs) sense diverse molecular structures that are specific to the invading virus during an innate immune response to a viral infection. Pathogen associated molecular patterns are the names given to these molecular structures (PAMPs). The binding of PAMPs to PRRs initiates the inflammatory response against the invading virus, which activates several signalling pathways and, as a result, transcription factors that induce the expression of genes responsible to produce several products involved in the host's immune response to the virus, including genes encoding several pro-inflammatory cytokines [10]. Genes encoding inflammatory cytokines, chemokines, and adhesion molecules are induced by these transcription factors. This series of events causes leukocytes and plasma proteins to be recruited to the infection site, where they conduct numerous effector activities to resist the infection's trigger [11].

Looking into various biological applications of 2 deoxyglucose (2-DG), the previous study is designed to the immunomodulatory effects of 2-DG on peripheral blood mononuclear cells to analyze the immune responses. In the previous study done by Uehara *et al*., was found that the glycolytic inhibitor 2-deoxy-D-glucose (2-DG), a simple monosaccharide, attenuated cellular responses to IL-6 by inhibiting N-linked glycosylation of the IL-6 receptor gp130 [12]. 2-DG also blocked TNF-α, IL-1β, and IFN-γ signals, and efficiently alleviated a mouse model of inflammatory bowel disease and human rheumatoid arthritis and prevented death following lipopolysaccharide (LPS) shock, a mouse model of a cytokine storm [13]. And attenuated LPS-induced pulmonary inflammatory responses, a mouse model of ARDS [14]. Moreover, the study has been assessing the Immunomodulatory effects of 2-DG in healthy donors via subsequent production of pro-inflammatory cytokines. Experimental validation of the effectiveness of 2-DG in triggering immune responses may be beneficial for adjunct treatment of SARS-COVID- 19.

Aims and Objectives

1. To isolate PBMCs from blood of healthy donors.
2. To study the *in vitro* activation characteristics of PBMCs upon stimulation by LPS.
3. To examine if 2 deoxyglucose has any immuno-modulatory role on the activation of PBMC.

Materials and Methods

Lab wares: All common laboratory glass wares were purchased from Borosil, India sterile tubes vacutainer syringe, vacutainer EDTA tubes, falcon tubes, microcentrifuge tubes, microtips and all other plasticwares (96 well plate, 12 well plate, 6 well plate, T-25 flask, t-75 flask) were purchased from Genaxy, New Delhi, India. Adjustable variable volume micropipettes use in this study were purchased from Eppendorf, India.

Chemicals and reagent: Ficoll-hypaque, phosphate buffer saline, Cell culture media RPMI-1640 media, and Fetal bovine serum (FBS), were purchased from Gibco, USA Penicillin, streptomycin, trypan blue dye, trypsin, 2- deoxy glucose, LPS, BCA assay kit, IL-6, TNF-α and IL-β1 ELISA kit, (Invitrogen & Sigma respectively) Glycerol, ethanol, and methanol were purchased from HiMedia, Mumbai India.

Instruments

- **CO_2 Incubator:** Thermo scientific, India
- **Weighing balance:** Wensar
- **Digital pH meter:** Eutech instruments
- **Centrifuge:** Remi, India
- **Multi-mode plate reader (Spectramax Id3):** Molecular devices, USA
- Hemocytometer
- **Automated Cell Counter:** Thermofisher, USA
- **Inverted microscope:** Nikon, India

The present study was undertaken in the department of biochemistry, M.M Institute of Medical science and Research Mullana Ambala.

Human volunteers

Blood was collected from healthy human donors in compliance with the ethical committee guidelines of MMDU Mullana Ambala, India.

Specimen collection

5 ml of venous blood sample was collected from an anti-cubital vein of the subjects in a disposable vacutainer syringe under aseptic condition and transferred to a sterile, dry EDTA vial for isolation of PBMC. The blood was processed within 2 hours for the better isolation.

Isolation of Human Peripheral blood mononuclear cells (PBMC)

Blood was diluted with the equal volume of PBS (1: 1) and then layered on lymphocytes separation media (HiMedia Laboratories Pvt. Ltd). The mixture was centrifuged at 2000 rpm for 20 min. after centrifugation formed Three-layer first layer is plasma, second layer PBMC, third layer red blood cell (RBC). The undisturbed PBMC layer was isolated, washed twice with PBS twice and re-suspended in RPMI-1640 media supplemented with 10% Fetal bovine serum (FBS), and antibiotics (100 µg/ml penicillin and streptomycin). To analyze cell viability and cell number, cells was counted with a hemocytometer, and viability was tested by trypan blue dye exclusion assay.

After that $1X10^6$ cells will be seeded on 25 cm^2 flask or 0.2 X 10^6 cells on 6 well plates. Cells (70% confluent) was incubated for 48 h without (control) or with 5 Mm. concentrations of 2- deoxyglucose and LPS concentration 10µg/ml

Enzyme linked immunosorbent assay (ELISA) of TNF-α and IL-6: TNF-α and IL-6 was analyzed by Enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Invitrogen & Sigma respectively). The supernatant of different samples was collected and used for the estimation of the TNF-α and IL-6 according to the manufacturers protocol ELISA as published earlier.

The standard curve was generated by using synthetic TNF-α and IL-6 peptide supplied with the kit.

Determination of TNF-α
Reagent preparation
Substrate solution
Freshly prepared Substrate solution A and B was mixed in equal volume then need to use.

TNF-α standard
A series of standard was prepared by dilution in deionized water cover in a range for 15.6 pg/ml to 1000 pg/ml

Assay protocol
In the first step, 50 µL Assay Diluent 1F is added to each well. Then, 200 µL of standard or sample is added to each well. After this, the plate is sealed and are incubated at room temperature for 2 hours. The contents of the plate are then discarded, and it is washed three times with washing buffer. Then, 200 µL conjugate antibody is added to each well. This plate is covered with adhesive strip and is incubate 1 hour at room temperature. The contents of the plate are again discarded, followed by washing with wash buffer thrice. Then 200 µL of substrate solution is added to each well. The plate is again incubated for 20 minutes at room temperature. After that, 50 µL of stop solution is added to each well. Finally, the optical density is taken at 450 nm and results were calculated.

Determination of IL-6
Assay protocol
A series of standard was prepared by dilution in assay buffer - A provides in the kits cover in a range for 7.8 pg/ml to 500 pg/ml.
The plates are first washed 4 times with 300 µL of 1X wash buffer and then 50 µL of assay buffer is added each well. The plate are sealed and are incubated foe 2 hours at room temperature with shaking at 200 rpm. The contents of the plate are discarded and again the plates are washed 4 times with 1X buffer. After that, 100 µL of human IL-6 antibody solution is added to each well. The plate is then sealed and is incubated at room temperature for 1 hour. Followed by this, contents are again discarded and washing using 1X wash buffer is done for four times. In the next step, 100 µL of avidin-HRP A solution is added to each well, the plates are sealed and are incubated at room temperature for 30 minutes. Contents are poured down and the plates are washed five times 1X wash buffer. Now, 100 µL of substrate solution F is added to each well. And the plate is incubated in the dark 15 minutes. After that, 100 µL of stop solution added to each well. Then finally, the optical density is recorded at 450 nm and results are calculated. The optical density took 30 minutes. Concentration of IL-6 was calculated by comparing the OD of sample to standard curve after the appearance of colour.

Results

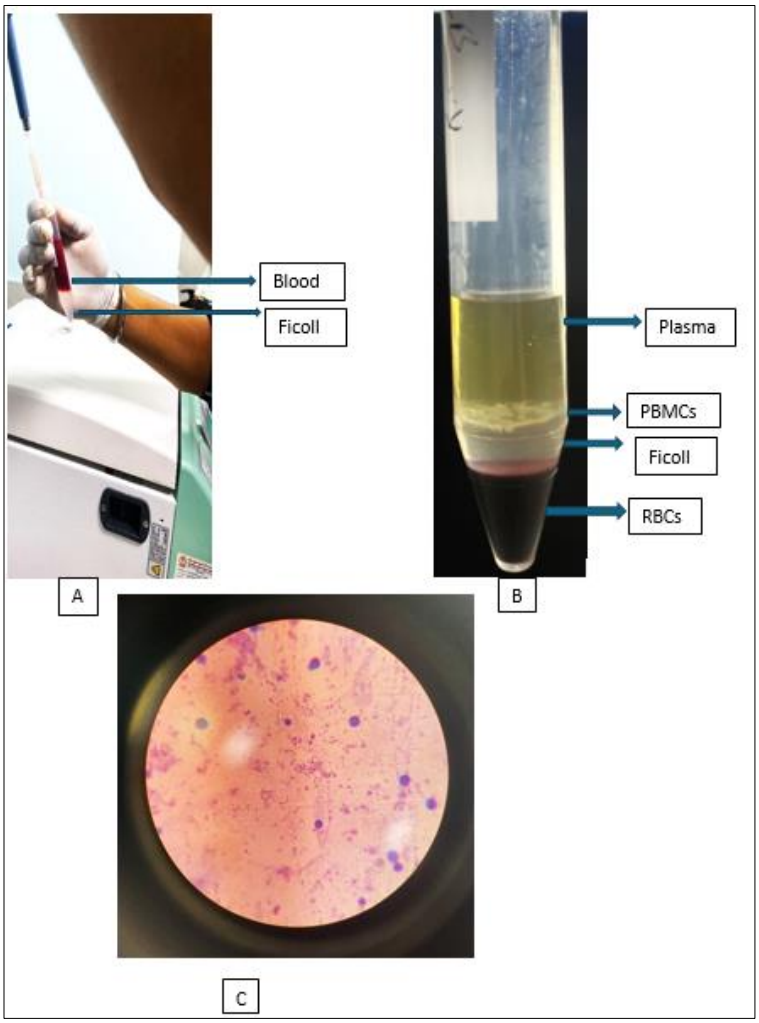

Fig 1: Separation of PBMCs from peripheral venous blood by density gradient centrifugation. a) Layers before ficoll spin b) Layers after ficoll spin and c) Peripheral blood mononuclear cells

Estimation of proinflammatory cytokines TNF-α and IL-6 after administration of LPS, 2-DG results

Estimation of TNF-α

Table 1: Absolute value of control, LPS, AND LPS+2DG

S. No	Controls	LPS (pg/ml)	LPS+2-DG (pg/ml)
1.	2.15	3.05	2.17
2.	1.96	3.17	2.25
3.	1.92	3.20	2.31
4.	2.12	3.11	1.98
5.	1.98	3.27	2.14

Table 2: Effect of LPS treatment on TNF-α

Treatment	Mean	Median	Standard deviation	P value
Control	2.026	1.980	0.1024	<0.0001***
LPS	3.160	3.170	0.08426	

Mean ± SD of controls and LPS were 2.026 ± 0.1024 and 3.160 ± 0.08426 respectively and median of controls and LPS were 1.980 and 3.170 respectively and the difference was statistically highly significant. (***= Highly Significant)

Table 3: Effect of LPS+2-DG treatment on TNF-α

Treatment	Mean	Median	Standard deviation	P value
Control	2.026	1.980	0.1024	0.1192
LPS+2DG	2.170	2.170	0.1255	

Mean ± SD of controls and LPS +2-DG were 2.026 ± 0.1024 and 2.170 ± 0.1255 respectively and median of controls and LPS were 1.980 and 2.170 respectively and the difference was statistically highly significant.

Table 4: Effect of LPS+ LPS+2DG treatment on TNF-α:

Treatment	Mean	Median	Standard deviation	P value
LPS	3.160	3.170	0.08426	0.0001***
LPS+2DG	2.170	2.170	0.1255	

Mean ± SD of LPS and LPS +2DG were 3.160 ± 0.08426 and 2.170 ± 0.1255 respectively and median of controls and LPS were 3.1710 and 2.170 respectively and the difference was statistically highly significant. (***= Highly significant)

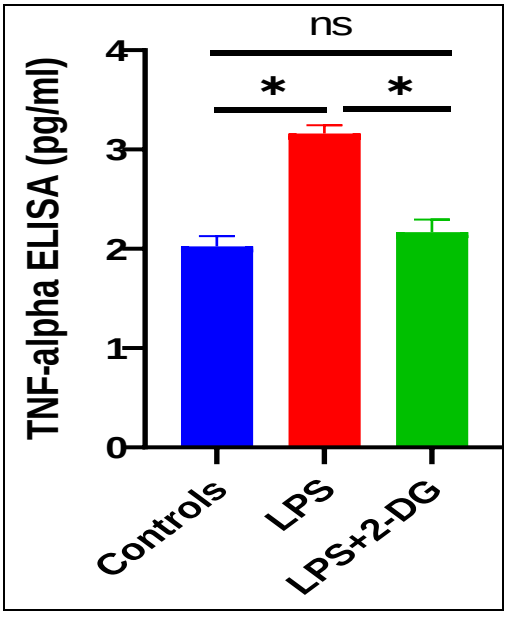

Fig 2: TNF-α was estimated by ELISA for the supernatant of control +LPS +2-DG from PBMCs and described in this figure

Estimation of IL-6

Table 5: Absolute value of control, LPS, AND LPS+2DG

S. No	Controls	LPS (pg/ml)	LPS+2-D (pg/ml)
1.	200.26	250.24	212.85
2.	207.35	257.23	205.17
3.	202.21	261.90	210.35
4.	198.70	242.70	217.17
5.	191.35	264.52	202.30

Table 6: Effect of LPS treatment on IL-6:

Treatment	Mean	Median	Standard deviation	P value
Control	200.0	200.3	5.820	<0.0001***
LPS	255.3	257.2	8.897	

Mean ± SD of controls and LPS were 200.0 ± 5.820 and 255.3 ± 8.897 respectively and median of controls and LPS were 1.980 and 3.170 respectively and the difference was statistically highly significant (***= Highly Significant)

Table 7: Effect of LPS+2DG treatment on IL-6:

Treatment	Mean	Median	Standard deviation	P Value
Control	200.0	200.3	5.820	0.1192
LPS+2-DG	209.6	210.4	5.944	

Mean ± SD of controls and LPS+2-DG were 200.0 ± 5.820 and 209.6 ± 5.944 respectively and median of controls and LPS were 200.3 and 210.4 respectively and the difference was statistically highly significant.

Table 8: Effect of LPS+LPS+2DG treatment on IL-6:

Treatment	Mean	Median	Standard deviation	P VALUE
LPS	255.3	257.2	8.897	<0.0001
LPS+2DG	209.6	210.4	5.944	

Mean ± SD of LPS and LPS +2DG were 255.3 ± 8.897 and 209.6 ± 5.944 respectively and median of controls and LPS were 257.2 and 210.4 respectively and the difference was statistically highly significant.

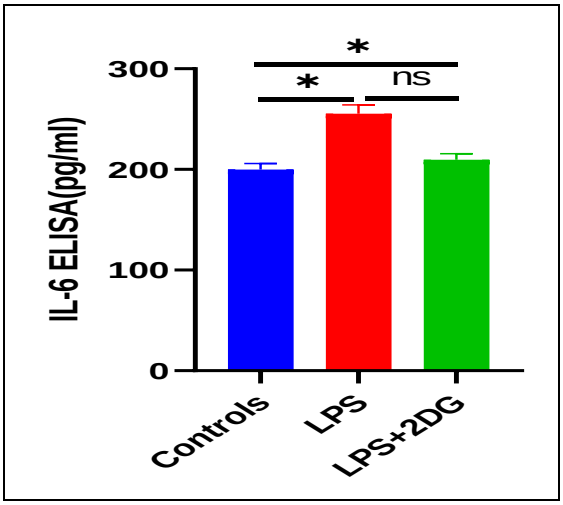

Fig 3: IL-6 was estimated by ELISA for the supernatant of control +LPS +2-DG from PBMCs and described in this figure.

Discussion

The autopsy of the lungs SARS-COV-2 infected patient suggested that the radiological findings of patchy ground-glass opacities, and the histology of the lung parenchyma showed a variety of findings from exudative hyaline

membrane disease to organized pneumonia, At the gross examination, the lungs were frequently described as heavy, congested, and edematous, frequently with a focal two-sided consolidation [15]. Massive pulmonary embolism was cited as the primary cause of death in a number of instances [16]. Lobar infarction was also frequently reported to be brought on by sub-segmental pulmonary emboli [17]. Patients with COVID-19 may experience the fibrosing pattern as a result of DAD [18]. Analysing the vascular modification reveals that thrombotic microangiopathy coexists with other illnesses including endothelialitis and pulmonary angiogenesis in SARS-COV-2, where vascular injury is a distinguishing clinical hallmark. The development of type II pneumocytes with nucleomegaly and conspicuous nucleoli, together with an accumulation of macrophages, lymphocytes, and multinucleated giant cells, were distinctive findings of the COVID-19 injury at the level of the lung [19]. The presence of numerous megakaryocytes was accompanied by the discovery of several infiltrating lymphocytes [20]. Neutrophil extracellular traps (NETs) can also cause thrombosis, fibrosis, and lung damage linked with inflammation [21]. A variety of cytokines are produced by PBMCs in a non-specific inflammatory response to SARS-COV-2 infection [22]. A number of proinflammatory cytokines, including IL-1β SARS-CoV-2, appear to effect on the development and activation of IL-1β, which in turn stimulates TNF-α and other proinflammatory [22]. IL-1β thus contributes to the cytokine storm brought on by coronavirus infections [23]. Epithelial and endothelial cells undergo apoptosis, as well as vascular leakage and death, as a result of a cytokine storm that is brought on by the unchecked inflammatory responses brought on by the SARS-CoV-2 infection [24].

The results of our study indicate that the administration of LPS significantly increases the levels of proinflammatory cytokines TNF-α and IL-6 in PBMCs, the increase in TNF-α levels with LPS treatment is consistent with previous studies that have shown LPS to be a potent inducer of TNF-α in immune cells (Kapasi *et al*., 2007). TNF-α is a key mediator of the inflammatory response and plays a crucial role in the pathogenesis of various inflammatory diseases (Saghazadeh *et al*., 2014). The attenuated increase in TNF-α levels with 2-DG treatment suggests that glycolysis may play a role in the induction of TNF-α by LPS. This is consistent with previous studies that have shown a role for glycolysis in the induction of inflammatory cytokines in immune cells (O'Neill *et al*., 2016).

The increase in IL-6 levels with LPS treatment is also consistent with previous studies that have shown LPS to be a potent inducer of IL-6 in immune cells (Kapasi *et al*., 2007). IL-6 is a pleiotropic cytokine that plays a role in the regulation of immune responses, inflammation, and haematopoiesis (Hunter and Jones, 2015). The lack of attenuation of IL-6 levels with 2-DG treatment suggests that glycolysis may not play a major role in the induction of IL-6 by LPS.

The co-administration of 2-DG with LPS reduces the levels of TNF-α and IL-6 compared to LPS alone. The decrease in the levels of these proinflammatory cytokines may suggest that 2-DG has anti-inflammatory properties.

Similar results have been reported in other studies where 2-DG was shown to have anti-inflammatory effects. For example, a study by Lee *et al*., (2015) found that 2-DG reduced the production of proinflammatory cytokines TNF-α, IL-1β, and IL-6 in macrophages stimulated with LPS. Another study by Cheng *et al*., (2016) showed that 2-DG reduced the expression of proinflammatory cytokines TNF-α and IL-6 in microglia stimulated with LPS. These studies suggest that 2-DG may have potential as an anti-inflammatory agent.

Furthermore, other studies have also reported the role of proinflammatory cytokines TNF-α and IL-6 in various diseases. For example, TNF-α has been implicated in the pathogenesis of rheumatoid arthritis (RA) (Feldmann *et al*., 1996), while IL-6 has been associated with the development and progression of various cancers (Heikkila *et al*., 2019). Therefore, the reduction in the levels of these cytokines by 2-DG may have therapeutic implications in these diseases.

In addition to its anti-inflammatory properties, 2-DG has also been shown to have anticancer effects. A study by Kurtoglu *et al*. (2007) demonstrated that 2-DG induced apoptosis in cancer cells by inhibiting glycolysis. Another study by Zhang *et al*., (2018) showed that 2-DG sensitized cancer cells to chemotherapy by inhibiting the repair of DNA damage. These findings suggest that 2-DG may have potential as an anticancer agent.

Overall, the results of the study indicate that 2-DG may have anti-inflammatory properties, and its ability to reduce the levels of proinflammatory cytokines TNF-α and IL-6 may have therapeutic implications in various diseases. However, further studies are needed to explore the full potential of 2-DG as an anti-inflammatory and anticancer agent.

Conclusion

There is no effective medicine for SARS-COV-2 so far and attempts are being made to use different immunomodulator to modify the course of the disease inhibition by generation of cytokines storm in covid-19.2-Deoxy glucose was suggested as a possible drug covid-19 and was given rapid approval by Government of India in the covid-19 to combat covid-19 pandemic; however, the mechanism is not clearly known. Our results show that 2-Deoxy glucose probably prevents the development of cytokine storm during covid-19 by inhibiting the release of pro- inflammatory cytokines from PBMCs. However, this is a preliminary study, and more work is necessary in this area.

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