Effect of rose extract treatment on soluble CCR5 and CXCR4 secretion by the endothelial cells in vitro

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ABSTRACT

Background: CC-Chemokine Receptor 5 (CCR5) and Chemokine C-X-C-Motif Receptor 4 (CXCR4) are expressed in various tissues, and they are potential molecules involved in multiple pathways. CCR5 and CXCR4 targets are associated with immune regulation in patients in multiple tissues and numerous clinical conditions. The study was performed searching for a novel therapy for immune regulation on these CCR5 and CXCR4 receptors with rose extract. Methods: The crushed red rose extract was prepared, and it was processed for analysis. The HUVEC cells were obtained for seeding in the cell culture. The cells were tested in normal physiological conditions and varying degrees of hypoxia. The cells were treated with extract for 72 hours, and the resultant secreted supernatants were analyzed for expression of CCR5 and CXCR4 by the Elisa technique. Results: The CCR5 levels were significantly elevated at normoxia compared to untreated controls. The surge of CCR5 was persistent in 12% hypoxia, and at higher degrees of hypoxia, the levels were mildly lower than the untreated levels. The CXCR4 levels were not changed in normoxia, and even with significant hypoxia, the levels were similar or mildly reduced compared to untreated values. Conclusion: The rose extract has the potentials to induce the secretion of soluble CCR5 from the HUVEC cells, and it can prevent the reduction of soluble CXCR4 levels during the hypoxic challenge of the endothelial cells. This in-turn can modulate the receptor levels on the endothelial cells, which has clinical applications.

Key words: CCR5, CXCR4, Immune regulation, Rose extract

INTRODUCTION

Immune-mediated disorders are common in clinical practice. Most clinical disorders — infective, inflammatory, or neoplastic have dys-regulation of immune function in the background. Autoimmune disorders, bacterial and viral infections, and even noncommunicable diseases are regulated to various extents by the immune system. Though many treatment methods are available for immune dys-regulation, there are disadvantages and potential side effects of the existing therapy. This includes steroids and other common immunomodulators, especially when used in long-term or immunocompromised patients.

The study was performed searching for a novel immune regulation agent, which can modulate the immune system without potential side effects. CXCR4 and CCR5 are surface proteins related to prospective heterogeneous multi-functional properties with essential functions in the immune system, including autoimmune disorders and malignancies1-5. The study was performed in search of a novel therapy for immune regulation using rose extract. CCR5 and CXCR4 are associated with immune regulation in human retrovirus-infected patients1-3. Blocking of these receptors prevents internalization of the viruses, and in turn, it is associated with resistance to infection. The genetic polymorphism in the receptors can also be associated with overwhelming infections like West Nile fever. In drug-induced inhibition of the receptor, this is not seen as a side effect6. The secretion of CCR5 further influences the immune system. Hence, in his study, the effect of the extract on the expression of the receptors CCR5 and co-receptor CXCR4 was studied in normal and hypoxic conditions. These receptors are also associated with cardiovascular, tumor development, and nervous system changes. Newer medications are available in the treatment of human retrovirus infections7. However, most of these are associated with significant side effects in the long term. Also, an effective CCR5 and CXCR4 blockage are related to various clinical benefits. Rose extract has minimal stimulatory effects on CD4+ T lymphocytes8. Both CXCR4 and CCR5 are small transmembrane proteins with G-protein coupling, and therefore secretion is not expected in plasma. However, secreted
forms of CXCR4 and CCR5 in plasma have been described in the past in literature, in the setting of human immunodeficiency virus infections.

METHODS

Preparation of red-rose extract
A red rose (Rosaceae) was taken from a plant in a red-rose garden supervised by a botanist. 2091 mg of freshly collected petals were distributed in 6 Precellys CK14 lysing tubes (Bertin Technologies, ref. 03961-1-003) to homogenize soft tissue. One ml of PBS (ThermoFisher, ref. 14190) was added to each tube. The tubes were run on Precellys 24 homogenizer at 5000 rpm for 2 x 30 seconds. Then, the supernatant from all tubes was gathered and spun down at 200g, 4°C, for 5 min. The supernatant was spun down twice at 4°C for 5 min, the first at 2000 g and the last at 10000 g. The final supernatant was 0.22 μm filtered (Sartorius, ref. 16534-K) and stored until use at -20°C.

Treatment of HUVEC cells
HUVEC, which was commercially available (Human Umbilical Vein Endothelial Cells - Cell Applications, ref. 200-05n, lot 3054) was procured, and subcultures were performed. HUVEC cells were seeded at passage 8 in 48-well plates at 9.500 cells/well (10000 cells/cm²) in 250 μL of endothelial cell growth medium (Cell Applications, ref. 211-500). 24 h after plating, the cells were treated in duplicate with 250 μL of red rose extract diluted at 0.5%, 0.05%, and 0.005% (v/v) in endothelial cell growth medium (Cell Applications, ref. 211-500) or with growth medium only as the control. Our previous study showed cytotoxicity of the rose extract at different condition was collected and pooled after 72 h. The cell viability was evaluated under a microscope. The collected medium samples were stored at -80°C for the profiling of secreted cytokines.

CCR5 evaluation
For the evaluation, the standard Abbkine Human CC-Chemokine Receptor 5 (CCR5) ELISA Kit (Ref. KTE62546) was used. The samples tested were diluted at 1/5 in monoplicate. This Human CC-Chemokine Receptor 5 (CCR5) ELISA Kit employs a two-site sandwich ELISA to quantitative CCR5 in samples. An antibody specific for CCR5 has been pre-coated onto a microplate. Standards and samples were pipetted into the wells, and the immobilized antibody binds any CCR5 present. After removing any unbound substances, a biotin-conjugated antibody specific for CCR5 was added to the wells. After washing, Streptavidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells, and color develops in proportion to the amount of CCR5 bound in the initial step. The color development was stopped, and the intensity of the color was measured.

RESULTS

CCR5
All samples were tested diluted at 1/5 in monoplicate (one well per sample). Using diluted human CCR5 standard at 320; 160; 80; 40; 20, and 10 pg/ml, a standard curve was drawn by calculating a 4 parameters logistic regression (Figure 1). The coefficient of determination R² is over 0.999. The minimum detectable dose of Human CCR5 is typically less than 1 pg/ml.
The regression shows the calculation of the concentrations of CCR5 in samples (Table 1 and Figures 2 and 3). The CCR5 concentrations were elevated with rose extract treatment. The rise in the CCR5 levels was preserved in normoxia and treatment at 12% $O_2$. The increase in CCR5 was seen at all the rose extract 0.005%, 0.05%, and at a level of 0.5%. Predominantly the increase was observed at treatment with 21% and 12% oxygen. At lower oxygen saturation of 5% and 1%, the secretion was reduced at all concentrations of the rose extract (Panels A and B, Figure 3). Compared to treatment at 21% oxygen saturation, 12% had higher values of CCR5 at all concentrations of the rose extract (Panel C, Figure 3). Table 1 shows the highest values of changes with treatment at 0.005% rose extract concentration. There was no change or reduction in CCR5 values when treated with severe hypoxia (5% and 1% $O_2$) irrespective of the rose extract concentration. In severe hypoxia-treated samples, the levels of CCR5 were reduced compared to untreated levels.

**CXCR4**

All samples were tested undiluted in monolopicate (one well per sample). Using diluted human CXCR4 standard at 5000; 2500; 1250; 625; 312.5; 156.25, and 78.13 pg/mL, a standard curve was drawn by calculating a four parameters logistic regression (Table 2, Figures 4 and 5). The coefficient of determination $R^2$ is over 0.999. The CXCR4 levels were similar to the untreated levels at normoxia and 12% oxygen levels. However, unlike CCR5 values, in severe hypoxia, the levels of CXCR4 were preserved compared to normoxia levels. There was a minimal increase in the levels of CXCR4 at 12% $O_2$ 0.005 percent rose extract concentration and at 5% $O_2$ concentration — 0.5% rose extract treatment. The minimal increase in the CXCR4 was also observed at 5% $O_2$ 0.5% rose extract (Panels A and B, Figure 5). There was a decrease in the levels of CCR5 compared to values at 21% $O_2$ when treated at 12, 5 and 1% $O_2$ levels (Panel C, Figure 5). Table 2 shows only minimal elevations in CXCR4 levels at 0.005% + 12% $O_2$ treatment and 0.5% + 5 and 1% $O_2$.

**DISCUSSION**

The study shows high levels of CCR5 after rose treatment, whereas the CXCR4 levels were maintained in normoxia conditions, and the CXCR4 levels were elevated in hypoxic conditions after rose treatment. CCR5 and CXCR4 act as co-receptor for the entry of the virus inside the endothelial and immune regulatory cells. Blocking of these receptors is known to delay the manifestations of the disease. Therefore, CCR5 or CXCR4 genetic mutations are associated with resistance or delay in clinical disease manifestation at the population level after retrovirus infection. Hence, this observation can help treat immunodeficiency disorder or reduce the manifestations of the disease. The secretion by endothelial cells could result from cell lysis based as it is a transmembrane protein. CXCR4 is 45 kDaltons and the molecular weight of CCR5 is 40 kDaltons. The predicted molecular weight of these would fall in the category of middle molecules comparable by size with Beta2 microglobulins, cystatins, complement factors, etc. In our previous study, we have shown that cell lysis does not happen with rose extract concentrations < 0.1 percent. Our observations in this study have shown that the secretion was observed at much lesser concentrations. Hence, the observations cannot be attributed to cell damage or lysis as the mechanism. It has been demonstrated that the CCR5 and CXCR4 receptors could be blocked by CCR5 or CXCR4 molecules by the phenomenon of competitive antagonism. Many selective agonists, both peptide and non-peptide molecules, have consistently shown internalization of the receptors after binding to the CCR5 receptors. CCL5 or RANTES is a close molecule associated with the binding of the CCR5 receptors, and non CCL5 mechanisms are also involved in activating the CCR5 axis. In blocking the receptors, the agonist binds and internalizes the receptors and acts similar to the antagonist. This mechanism is also demonstrated distinctively in CXCR4 receptors. It has been consistently shown that the retrovirus entry into the target cells is mediated through CXCR4 and CCR5 co-receptors. It has also been shown that the CCR5 inhibitor maraviroc to have clinical benefits by delaying the progression of the disease and its complications in achieving this effect by its inhibition. The results need to be tested and validated in animal and human studies in the future.

CCR5 is consistently shown to stimulate angiogenesis in the cancer tissues. There is a close crosstalk between CCR5 and immune system defense. In our previous study, the rose extract has shown an inhibitory action on IL2, IL1, TNF beta, and certain other vascular inflammatory markers. RANTES is associated with stimulation of angiogenesis by CCR5 mediated response. The CCR5 is actively involved in mobilizing the polymorphonuclear myeloid cells into the blood and directing them to the tumors. Hence, inhibition of the receptors could effectively result in the delay of tumor growth.

CCR5 is involved in cardiac fibrosis processes, especially in Chagas disease, and also antibodies to CCR5
Figure 1: The figure shows the workflow evaluating CCR5 and CXCR4 levels by Elisa.
Table 1: Results of human CCR5 ELISA testing which shows CCR5 concentration, absorbance observed at various oxygen concentrations, and the percentage change observed

| Samples | Oxygen level (%) | Rose extract concentration (%) | Absorbance without blank (450nm) | CCR5 concentration in diluted samples (pg/ml) | Dilution | CCR5 concentration (pg/mL) | Treated vs. untreated (%) | Percentage Change |
|---------|------------------|-------------------------------|----------------------------------|-----------------------------------------------|----------|----------------------------|--------------------------|---------------------|
| 21      | 0                | 0.214                         | 16.02                            | 5                                             | 80.12    | 100%                       | 100%                    | 100%                |
| 12      | 0                | 0.314                         | 21.86                            | 5                                             | 109.31   | 100                        | 136.4                   |                    |
| 21      | 0.5              | 0.281                         | 19.96                            | 5                                             | 99.82    | 124.6                      | 100%                    | 100%                |
| 12      | 0.5              | 0.457                         | 29.77                            | 5                                             | 148.87   | 136.2                      | 149.1                   |                    |
| 21      | 0.05             | 0.358                         | 24.36                            | 5                                             | 121.78   | 152                        | 100%                    |                    |
| 12      | 0.05             | 0.507                         | 32.48                            | 5                                             | 162.42   | 148.6                      | 133.4                   |                    |
| 21      | 0.005            | 0.585                         | 36.7                             | 5                                             | 183.5    | 229                        | 100%                    |                    |
| 12      | 0.005            | 0.65                          | 40.14                            | 5                                             | 200.71   | 183.6                      | 109.4                   |                    |
| 5       | 0                | 0.379                         | 25.5                             | 5                                             | 127.49   | 100                        | 159.1                   |                    |
| 1       | 0                | 0.509                         | 32.6                             | 5                                             | 162.99   | 100                        | 203.4                   |                    |
| 5       | 0.5              | 0.269                         | 19.27                            | 5                                             | 96.35    | 75.6                       | 96.5                    |                    |
| 1       | 0.5              | 0.26                          | 18.76                            | 5                                             | 93.79    | 57.5                       | 94                      |                    |
| 5       | 0.05             | 0.225                         | 16.69                            | 5                                             | 83.43    | 65.4                       | 68.5                    |                    |
| 1       | 0.05             | 0.235                         | 17.28                            | 5                                             | 86.41    | 53                         | 71                      |                    |
| 5       | 0.005            | 0.282                         | 20.02                            | 5                                             | 100.1    | 78.5                       | 54.6                    |                    |
| 1       | 0.005            | 0.362                         | 24.55                            | 5                                             | 122.75   | 75.3                       | 66.9                    |                    |

are associated with the reversal of fibrosis. It is also implicated in neointimal proliferation and vascular injuries. Inhibition of the CCR5 is associated with the reversal of autoimmune myocarditis in bench studies. Blocking the CXCR4 was associated with increased contractility and reduced inflammation-mediated injuries. CCR5 blockade reduces atherosclerosis by reducing arterial injury. CCR5 and CXCR4 inhibitions are involved in the positive remodeling of the myocardium after myocardial infarctions. CCR5 inhibition or polymorphisms is associated with reduced mortality in dialysis patient and reduced mucosal inflammation in murine colitis. In the previous study, rose extract has been shown to inhibit inflammation markers significantly, including RANTES. CCR5 inhibitors block heat shock protein associated with HIV and inflammatory bowel diseases and reduce IL13 induced injuries. CCR5 is an active target receptor for inhibition in the therapy of pulmonary arterial hypertension. CXCR4 inhibition also was independently shown to have an attenuation of pulmonary arterial hypertension. Downregulation of CXCR4 is associated with alleviating pain ischemia-reperfusion-induced spinal cord injuries due to TLR4 inhibition. CCR5 receptors are also expressed in the central nervous system by the neuronal, endothelial, and immune regulatory cells. CCR5 axis is involved in Alzheimer’s disease, CNS infections, and blood-brain barrier maintenance during stroke recovery. They are associated with neuronal survival in stroke therapy. Recent SARS-CoV-2 infections are associated with high morbidity and mortality. The spike protein of SARS-CoV-2 has been associated with genomic sequences with overlaps in HIV-gp 120 protein, which could be a mutation by the natural evolution of viruses. This could also be a random association of genomic sequences. Nevertheless, the pathophysiology of the SARS CoV-2 is still not well understood. If
this utilizes a CCR5 pathway with versatile functions, blocking the receptors could be helpful, though this is speculative. CCR5 and CXCR4 are transmembrane receptors, and the mechanism of secretion into the plasma is not very clear. In the past, soluble CCR5 and CXCR4 receptors are observed in the plasma\textsuperscript{58,59}. Also, in the past recombinant soluble mimetics of CCR5 have been synthesized and evaluated as a treatment method in acquired immunodeficiency syndrome\textsuperscript{60,61}. The inhibition of the CCR5 and CXCR4 could also have been by a cross-reacting antibody mechanism that could have existed, which needs to be evaluated. This could be a mechanism of shedding the receptors into the plasma from the endothelial cells either by synthesis or the existing receptors after the rose extract treatment. The results are preliminary, with results involving minimal samples only. Further large in\textit{vitro} tests and animal studies need to be performed to study the immune actions, and the side effects profile of the extract also needs to be evaluated. Nevertheless, this is the first study showing the benefits of rose extract treatment at the molecular level. Further studies need to be performed to assess the help of the extract at the cellular and clinical levels.

CONCLUSIONS

The rose extract increases the secretion of CCR5 by the endothelial cells, and CCR4 levels were not changed, but it prevents the reduction in the levels of CXCR4 with hypoxia treatment. Further large in\textit{vitro} and in\textit{vivo} studies are required for the evaluation of immune modulation by the rose extract.

ABBREVIATIONS

CCR: CC-Chemokine Receptor

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None.

AUTHOR’S CONTRIBUTIONS

MCA conceived the idea and method, designed the study, interpreted results and wrote the paper. EM
Figure 3: Quantification of human CCR5 in the samples. Panel A shows the results of concentrations changes in CCR5 levels after treatment of various concentrations of rose extract (0.005, 0.05 and 0.5 percent v/v of rose extract) and various degrees of hypoxia. Panel B shows the changes in percentage of CCR5 compared to baseline (Zero percent rose concentrations). Panel C shows changes CCR5 under various degrees of hypoxia and rose extract concentrations. * indicates an increase in value from baseline, O indicates a decrease in value from baseline.
prepared the methods’ protocol, performed the tests and derived the results. All authors read and approved the final manuscript.

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None.

**AVAILABILITY OF DATA AND MATERIALS**

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

Not applicable.

**CONSENT FOR PUBLICATION**

Not applicable.

**COMPETING INTERESTS**

The authors declare that they have no competing interests.

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### Table 2: Results of human CCR5 ELISA testing which shows CXCR4 concentration, absorbance observed at various oxygen concentrations, and the percentage change observed

| Samples | Oxygen level (%) | Rose extract concentration (%) | Absorbance without blank (450 nm) | CXCR4 concentration (pg/mL) |
|---------|-----------------|-------------------------------|---------------------------------|----------------------------|
|         |                 |                               | 2180.1                          |                            |
|         | 21              | 0                             | 2190.1                          |                            |
|         | 12              | 0                             | 2021.2                          |                            |
|         | 21              | 0.5                           | 1905.7                          |                            |
|         | 12              | 0.5                           | 2034.8                          |                            |
|         | 21              | 0.05                          | 2275.9                          |                            |
|         | 12              | 0.05                          | 1984.3                          |                            |
|         | 21              | 0.005                         | 2082.9                          |                            |
|         | 12              | 0.005                         | 2142.4                          |                            |
|         | 5               | 0                             | 1940.6                          |                            |
|         | 1               | 0                             | 1781.8                          |                            |
|         | 5               | 0.5                           | 2253.2                          |                            |
|         | 1               | 0.5                           | 1880.3                          |                            |
|         | 5               | 0.05                          | 1668.8                          |                            |
|         | 1               | 0.05                          | 1784.6                          |                            |
|         | 5               | 0.005                         | 1852.7                          |                            |
|         | 1               | 0.005                         | 1776.7                          |                            |

### Change Percentage

| Treated vs. untreated (%) | Hypoxia vs. 21%O₂ (%) |
|---------------------------|-----------------------|
| 100                       | 100                   |
| 100                       | 87.2                  |
| 87                        | 100                   |
| 106.8                     | 100                   |
| 118.2                     | 118.2                 |
| 98.7                      | 98.7                  |
| 102.9                     | 102.9                 |
| 88.6                      | 88.6                  |
| 81.4                      | 81.4                  |
| 86.8                      | 86.8                  |
| 73.3                      | 73.3                  |
| 78.4                      | 78.4                  |
| 88.9                      | 88.9                  |
| 85.3                      | 85.3                  |
Figure 4: Standard curve of human CXCR4 ELISA assay which shows the standard CXCR4 concentration values against absorbance observed at 21% O₂.

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Figure 5: Quantification of human CXCR4 in the samples. Panel A shows the results of concentrations changes in CXCR4 levels after treatment of various concentrations of rose extract (0.005, 0.05 and 0.5 percent v/v of rose extract) and various degrees of hypoxia. Panel B shows the changes in percentage of CXCR4 compared to baseline (Zero percent rose concentrations). Panel C shows changes CXCR4 under various degrees of hypoxia and rose extract concentrations. * indicates an increase in value from baseline, ○ indicates a decrease in value from baseline.
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