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Evaluation Of the Effect of New Generation Quinolone Antibiotics and Immunepotent CRP on The Immune System

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ABSTRACT

Human peripheral blood mononuclear cells (PBMCs) are affected by new generation quinolone antibiotics (Ciprofloxacin & Ofloxacin) as well as Immunepotent CRP (IMP-CRP) when it comes to lymphocyte populations and immune mediator production. The objective of this work is to evaluate the effect of quinolones, Ciprofloxacin and Ofloxacin on the immune system, focusing on the mechanisms of oxidative activity of monocytes, lymphocyte populations and their production of cytokines; contributing to the body of knowledge of these antibiotics; since they are the most used and considered as the new generation. On the other hand, the action of IMP-CRP is evaluated, which has antibacterial and anti-inflammatory activity; on these same parameters, which had not previously been considered. After treating the individuals' blood with quinolones and Immune potent CRP and its various combinations, peripheral blood mononuclear cells were separated by density gradient from the peripheral blood of healthy people. NBT was used to detect monocyte oxidative activity, flow cytometry was used to measure the percentage of B, NK and selected T cells, and ELISA was used to measure the production of the cytokines IL-2, IL-10, and IFN-γ. MTT was used to measure relative cell viability. The results revealed that the medications did not influence the ability of the PBMCs to live. There was no noticeable variation in the oxidative activity of monocytes between treatments and controls, nor was there a discernible difference in the percentages of any of the cell populations studied. IL-2 production was increased in Ciprofloxacin and ofloxacin treatments ($p<0.02$) but unaffected in Immune potent CRP and combination therapies. Treatments with Ciprofloxacin, ofloxacin, and Immune potent CRP were observed to reduce IL-10 production ($p<0.046$). Treatments with Ciprofloxacin had no effect on IFN-γ production; however, treatments with ofloxacin (50 g/mL), Immune potent CRP, and their combinations significantly reduced IFN-γ production (p 0.01). Despite the fact that Ciprofloxacin, ofloxacin, and Immune potent CRP did not affect PBMCs viability, phagocyte activity, or lymphocyte populations, these antibiotics were able to alter cytokine production of IL-2, IL-10, and IFN-γ.

Keywords: *quinolone antibiotics, PBMCs, Ciprofloxacin, ofloxacin, Immune potent CRP*

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INTRODUCTION

In the fight against infection, the immune system is composed of cells and substances with specific roles. Reactions to invading germs can be innate (natural) or exogenous (artificial). In innate responses, a person's body produces the same response no matter how many times they encounter the same infectious pathogen; acquired (adaptive) responses are enhanced by repeated exposure to a given infection (Netea, et al 2020). Both types of immunity work together to protect the body from infection. The lack or defect of one or more components of the immune response leads to susceptibility to infectious diseases whose severity will depend on the affected cell and the type of defect.(Chaplin, 2010)

Quinolones are a group of antimicrobial agents that inhibit bacterial DNA gyrases, inhibiting DNA synthesis (Pham, 2019). These drugs have a broad spectrum of action and great therapeutic use. In addition to their bactericidal properties, there are reports that they are also capable of exerting modulatory effects on the immune system (Hooper, et al 2016). However, studies have focused on the effect of these antibiotics on the activity of transcription factors related to the immune system (eg, NFκB) and the production of proinflammatory cytokines (eg TNF-α, IL- 1 and IL-6) (Iyer, 2012); without finding studies that evaluate its effect on other parameters of the immune response such as the evaluation of lymphocyte populations and the production of other immunological mediators.

IMP-CRP containing transfer factor is a dialysate of substances of heterogeneous mixtures of low molecular weight, obtained after passing the soluble material from the spleen through a membrane with a pore of 12 to 14 kDa. Given this, IMP-CRP can transfer specific immunity to an individual who does not have it, thus constituting a type of passive immunotherapy. Product usage is handled in units (U). Several experiments show the immunomodulatory and immunopotentiating properties of IMP-CRP (Svelander, 2001): it has been observed to protect against endotoxic shock induced by lipopolysaccharide (LPS) in vivo and in vitro, decreasing the production of the cytokines TNF-α and IL-6 and the production of nitric oxide and increasing the production of IL-10 in addition to reducing the production of species in vitro reactive oxygen (Nandi, 2009).

The objective of this work is to evaluate the effect of quinolones, Ciprofloxacin and ofloxacin on the immune system, focusing on the mechanisms of oxidative activity of monocytes, lymphocyte populations and their production of cytokines; contributing to the body of knowledge of these antibiotics; since they are the most used and considered as the new generation. On the other hand, the action of IMP-CRP, produced and marketed by the Laboratory of Immunology and Virology of the Faculty of Biological Sciences, is evaluated, which has antibacterial and anti-inflammatory activity on these same parameters, which had not previously been evaluated.

MATERIALS AND METHODS

Peripheral Blood Mononuclear Cells (Pbmc)

Peripheral blood was collected in tubes with sodium heparin by venipuncture from healthy donors with prior informed consent. The donors were male, aged between 20 and 25 years. PBMC were isolated using a density gradient with Ficoll-Hypaque (Ficoll® Paque Plus, Merck, India) at 2000 rpm for 45 min at 27ºC, according to the manufacturer's instructions. Subsequently, a couple of washes were performed with Hank's buffer solution (HBSS). In the case of the test with monocytes, washings were carried out using cold phosphate buffer solution (PBS) to avoid the adhesion of monocytes to the surface of the tube. PBMCs were suspended in complete RPMI 1640 medium: RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) ((MegaCell™ RPMI-1640 Medium, Merck India), previously inactivated at 60ºC for 45minutes; and supplemented with streptomycin-penicillin antibiotic (Pen-Strep, Thermo Fisher Scientific, USA).

Drugs

The quinolone antibiotics Ciprofloxacin (Cipro I.V. 200mg/100ml) (Vidharbha Pharma LLP, Mumbai, Maharashtra, India) and Ofloxacin (DIGIFLOX-IV. 0.2% W/V) (DIGIFLOX-IV Senen Biotech, Haryana, India) were used. For use in cell culture, the antibiotics were sterilized using syringe filters of 0.22 microns (Sree Aadya Scientifics, Hyderabad, India).

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Immunepotent Crp (Imp-Crp)

IMP-CRP was used, a dialyzable extract of leukocytes of bovine origin, containing transfer factor; elaborated in the Biological Production Plant of the Immunology and Virology Laboratory of the Faculty of Biological Sciences of the Baghdad University of Medical sciences. One unit (U) is the dialysate of 1.5 x 109 bovine leukocytes. For use in cell culture, 10 U of lyophilisate were suspended in 2 mL of PBS (5 U/mL) and sterilized using 0.22-micron syringe filters.

Treatment Of Pbmc

PBMCs were seeded in a complete RPMI 1640 medium and treated using Ciprofloxacin, ofloxacin, and IMP-CRP.

Cell Viability

To evaluate the viability of the treated cells, the assay was performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole (MTT) Bromide (Sigma Chemical CO, St. Louis, MO, USA) as described below. The PBMCs were seeded in complete RPMI 1640 medium at a concentration of 104 cells per well in 96-well culture plates, the treatments were applied and incubated at 37ºC with 5% Carbon dioxide for 72 h. Once the incubation period was over, 20 µL of MTT (5 mg/mL) in PBS were added to each well and the plate was incubated again at 37ºC with 5% CO2 for 2 h. Then, the plate was centrifuged at 1600 rpm for 10 min, the supernatant was discarded and 100 µL of dimethyl sulfoxide (DMSO) was added to each well; it was gently shaken and the absorbance at 540 nm was determined using a Synergy HT plate reader. (BioTek Instruments, Inc.USA). The absorbance values obtained for the treatments were compared with those of the control to obtain the percentage of relative cell viability.

Superoxide Production

PBMCS were seeded in complete RPMI 1640 medium in 24-well plates at a concentration of 3.5 x 106 cells/well. Subsequently, the treatments mentioned above were added and the cells were incubated at 37ºC in an atmosphere of 5% CO2 for 3 h. Non-adherent cells were discarded by decanting the medium from the plates and washing twice with Hank's buffer solution (HBSS). Once the monocytes adhered to the plate were obtained, 0.1% nitroblue tetrazolium chloride (NBT) (Merck, India) diluted in HBSS plus 600 ng/mL of phorbol-myristate was added to each well. acetate (PMA) (Merck, India). The monocytes were incubated for 1 h at 37°C in a 5% CO2 atmosphere and subsequently, the NBT solution was discarded and washed with PBS at 37°C; then methanol was added to fix the cells and allowed to dry. Then 120 µL of potassium hydroxide plus 140 µL of DMSO were added and gently stirred (100-200 rpm) for 10 min at room temperature. Finally, 200 µL of the solution from each treatment were transferred to 96-well plates and the absorbance was read on a Synergy HT plate reader at 620 nm. Absorbance values are directly proportional to the amount of superoxide anion production.

Determination Of Lymphocyte Populations

PBMCs were seeded in complete RPMI 1640 medium in 12-well plates at a concentration of 2 x 106 cells/well. Treatments were added and then stimulated by adding phytohemagglutinin (PHA) (Merck, India), a polyclonal T-cell activator, diluted in complete RPMI 1640 medium at a final concentration of 5 µg/mL per well. Subsequently, they were incubated at 37ºC in an atmosphere of 5% CO2 for 72 h in order to stimulate cell proliferation. At the end of the incubation, the cells of each well were collected and adjusted to 2 x 105 in 50 µL of PBS + 5% FBS + 0.01% sodium azide solution and placed in 5 mL Falcon tubes (BD Biosciences, USA). 5 µL of the corresponding cocktail of antibodies labeled with the fluorochromes fluorescein isothiocyanate (FITC) and phycoerythrin (PE) from the kit. Becton Dickinson Simultest™ IMK-Lymphocyte the mixture was homogenized and incubated at room temperature and in the dark for 20 min. Subsequently, a solution of PBS + 5% FBS + 0.01% sodium azide was washed to eliminate the excess of labeled antibodies, and finally, the samples were fixed by adding 500 µL of 1% paraformaldehyde in PBS. Counting was performed in Beckman Coulter Epics XL Flow Cytometer.

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Cytokine Production
PBMCs were plated in complete RPMI 1640 medium in 12-well plates at a concentration of 1 x 106 cells/well. The treatments and the phytohemagglutinin stimulator were added at a final concentration of 5 μg/mL per well and subsequently incubated at 37ºC in an atmosphere of 5% CO2 for 24 h. The cells were centrifuged at 1600 rpm for 10 min and the supernatants of each treatment were collected, which were stored at -20 °C until used for evaluation of the production of the cytokines IL-2, IL-10 and IFN-γ. The determination of cytokine production was performed using the following commercial ELISA kits: Human IL-2 Immunoassay Kit (Thermo Fisher Scientific, USA), Human IL-10 Immunoassay Kit (Thermo Fisher Scientific, USA) and Quantikine Human IFN-γ Immunoassay (Thermo Fisher Scientific, USA), respectively, according to the protocol described by the manufacturer. Plates were read at 450 nm on a Synergy HT plate reader.

Statistic Analysis
Each experiment was performed twice in triplicate. Analysis of variance (ANOVA) and Dunnett's test was used to determine if there was a difference between treatments, taking values of $p < 0.05$ as significant.

RESULTS

Cellular Viability Of Treated Pbmcs
To determine if the treatments used in this study have cytotoxic effects against PBMCs that could alter the results of subsequent tests, the cell viability study was carried out using the MTT technique, finding that treatments with Ciprofloxacin, ofloxacin and IMP-CRP did not affect the relative cell viability of PBMCs after 72 h of exposure (Figure 1).

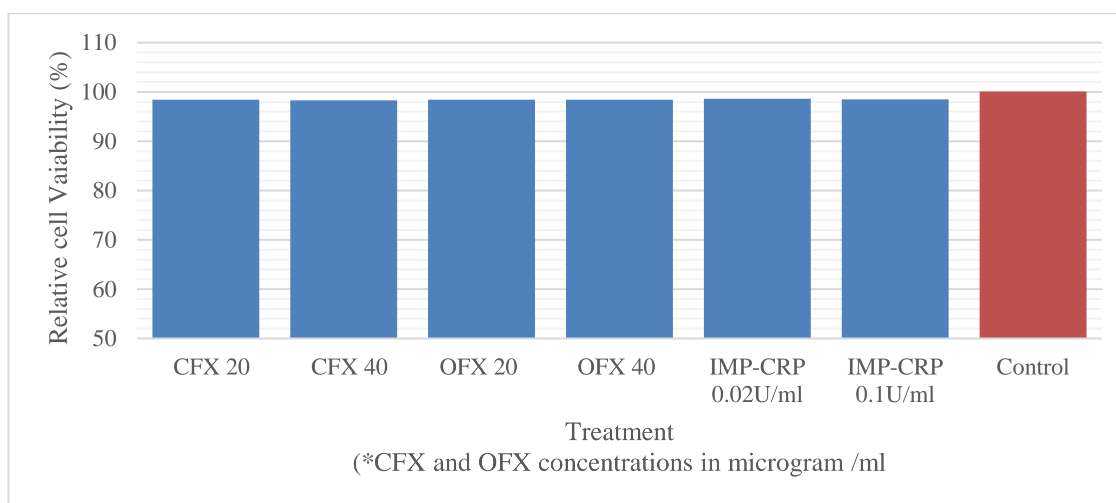

FIGURE 1. Relative cell viability of PBMCs 104 PBMCs were cultured and treated with Ciprofloxacin 20 and 40 μg/mL, ofloxacin 20 μg/mL and 40 μg/mL and IMP-CRP 0.02 and 0.1 U/mL, and incubated at 37ºC, 5% CO2 for 72 h. Subsequently, 20 μL of MTT were added to each well and incubated for 2 h, then the plate was centrifuged, the supernatant was discarded and 100 μL of DMSO was added and the samples were read at a wavelength of 540 nm. A value of p<0.046 (*) was considered significant. CFX: Ciprofloxacin; OFX: ofloxacin; IMP-CRP

Oxidative Activity Of Monocytes
The oxidative activity of monocytes obtained from PBMCs was evaluated by the intracellular reduction of NBT. The absorbance value obtained is directly proportional to the amount of NBT oxidized, indirectly indicating the amount of superoxide anion produced intracellularly.

The results show that the treatments did not affect the production of superoxide anion compared to the control (Figure 2).

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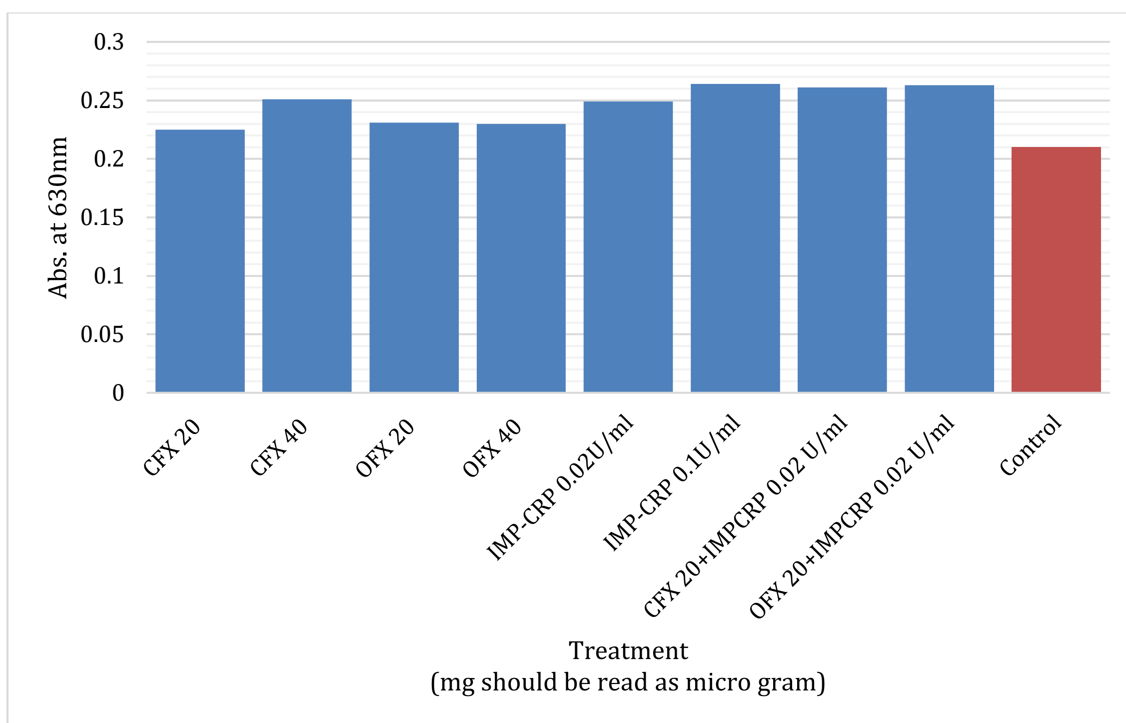

FIGURE 2. Superoxide anion production in monocytes. 3.5 x 106 PBMCs were cultured and treated with 20 µg/mL and 40 µg/mL Ciprofloxacin, 20 µg/mL and 40 µg/mL ofloxacin, and 0.02 and 0.1 U/mL IMP-CRP, and 20 µg/mL Ciprofloxacin combinations. + IMP-CRP 0.02 U/mL and ofloxacin 20 µg/mL + IMP-CRP 0.02 U/mL and incubated at 37ºC, 5% CO2 for 3 h. The protocol described in the materials and methods was followed to determine the production of superoxide anion. A value of $p<0.046$ (*) was considered significant. CFX: Ciprofloxacin; OFX: ofloxacin

Evaluation Of Treatments On Lymphocyte Populations

To determine whether treatments with Ciprofloxacin, ofloxacin, IMP-CRP, and their combinations caused a change in the proportions of lymphocyte populations, PBMCs cultured with the treatments were labeled with fluorochrome-labeled antibodies and subsequently read in a flow cytometer, finding some changes in the proportions of B and NK cell populations between treatments and control, which however were not statistically significant (Figure 3 a and b). In addition, the populations stimulated with phytohemagglutinin show an increase in the percentages of both cell types (B and NK) with respect to the non-stimulated ones, which, however, was not statistically significant (Figure 3 a and b).

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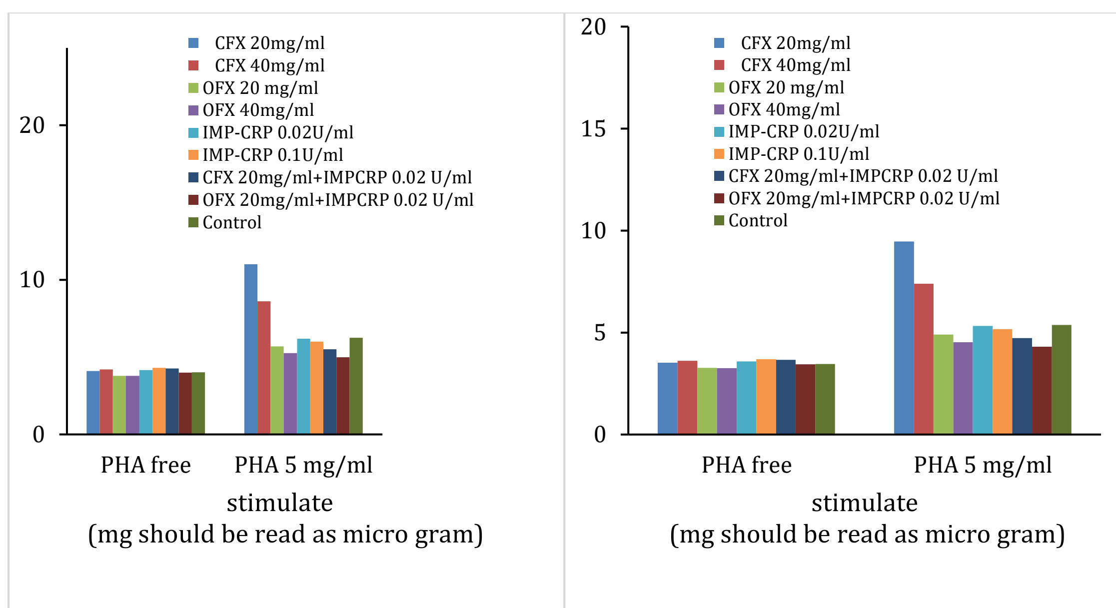

(b)

FIGURE 3. Evaluation of the percentage of B and NK cells in PBMCs. 2 x 106 PBMCs were cultured and treated with Ciprofloxacin 20 µg/mL and 40 µg/mL, ofloxacin 20 µg/mL and 40 µg/mL and IMP-CRP 0.02 and 0.1 U/mL, and the combinations of Ciprofloxacin 20 µg/mL + IMP-CRP 0.02 U/mL.
mL and ofloxacin 20 µg/mL + IMP-CRP 0.02U/mL; they were stimulated or not with 5 µg/mL of phytohemagglutinin and incubated at 37ºC, 5% CO2 for 72 h. The cells were then incubated with fluorochrome-labeled antibodies, fixed, and subsequently, read in a flow cytometer to obtain the percentage of B (a) and NK (b) cells. A value of $p<0.046$ (*) was considered significant. CFX: Ciprofloxacin; OFX: ofloxacin; phytohemagglutinin: phytohemagglutinin.

On the other hand, the results show that there was no difference in the proportion of total T cells between the treatments and the control. However, a difference ($p<0.046$) was found between phytohemagglutinin-stimulated and non-stimulated cell populations (Figure 4). When evaluating the proportion of T CD4+ and T CD8+ cells, these were not affected by the treatments either, but as in the case of total T lymphocytes, there was a decrease in T CD4+ and T CD8+ lymphocytes in the cell population that was stimulated with 5 µg/mL of phytohemagglutinin (Figure 4).

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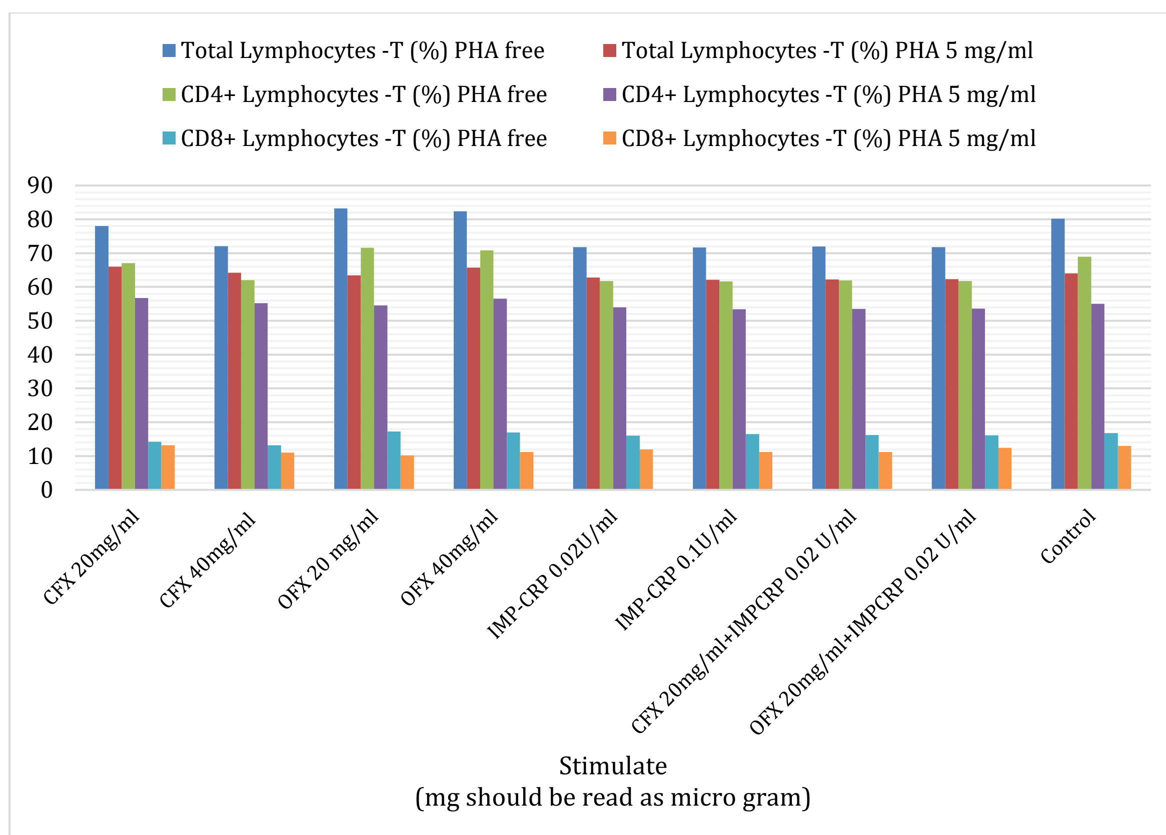

FIGURE 4. Evaluation of the percentage of total T, CD4+ T and CD8+ T lymphocytes in PBMCs. 2 x 106 PBMCs were cultured and treated with Ciprofloxacin 20 µg/mL and 40 µg/mL, ofloxacin 20 µg/mL and 40 µg/mL and IMP-CRP 0.02U/mL and 0.1 U/mL, and the combinations of Ciprofloxacin 20 µg/mL + IMP-CRP 0.02U/mL U/mL. mL and ofloxacin 20 µg/mL + IMP-CRP 0.02U/mL U/mL; they were stimulated or not with 5 µg/mL of phytohemagglutinin and incubated at 37ºC, 5% CO2 for 72 h. The cells were then incubated with fluorochrome-labeled antibodies, fixed, and subsequently read in a flow cytometer to obtain the percentage of total T cells (a), CD4+ T cells, and CD8+ T cells. A value of p<0.046 (*) was considered significant. CFX: Ciprofloxacin; OFX: ofloxacin; IMP-CRP; phytohemagglutinin: phytohemagglutinin.

Cytokine Production

After exposing the PBMCs to the treatments for 24 h in the presence or absence of phytohemagglutinin stimulation, the production of the cytokines IL-2, IL-10 and IFN-γ were evaluated by ELISA (Beatriz, 2000). The statistical analysis showed that the treatments did not affect the production of IL-2 in the cells not stimulated with phytohemagglutinin. Still, a significant difference was observed between the treatments in the cells stimulated with phytohemagglutinin, finding that Ciprofloxacin and ofloxacin, at doses of 20 µg/mL and 40 µg/mL, increased the production of IL-2 by about 29.9%(p<0.02) and that IMP-CRP alone and combined in its different doses did not affect the production of this cytokine (Figure 5).

When evaluating the production of IL-10, it was observed that the treatments did not affect the production of this cytokine in the cells not stimulated with phytohemagglutinin, (Kallio, 2003) but its production was affected when the cells were stimulated with phytohemagglutinin, in which a decrease was observed. of the production of IL-10 with respect to the control in a dose-dependent manner: 15% (p<0.046) and 25% (p<0.02) for Ciprofloxacin 20µg/mL and 40 µg/mL; 50% (p<0.02) and 69% (p<0.02) for ofloxacin 20 µg/mL and 40 µg/mL; 75% (p<0.02) and 82% (p<0.02) for IMP-CRP 0.02 U/mL and 0.1U/mL; and 79% (p<0.02) and 83% (p<0.02) for the combinations of Ciprofloxacin 20 µg/mL + IMP-CRP 0.02 U/mL and ofloxacin 20 µg/mL + IMP-CRP 0.02 U/mL, respectively (Figure 5).

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IFN-γ production was not altered under any treatment in cells not stimulated with phytohemagglutinin. Regarding the cells that were stimulated with phytohemagglutinin, the production of IFN-γ was not affected by the Ciprofloxacin treatments (Holland, 1998) nor by the 20 µg/mL dose of ofloxacin. In the rest of the treatments, a decrease in IFN-γ production was observed: 34% ($p<0.02$) for ofloxacin 40 µg/mL, 40% ($p<0.02$) and 67% ($p<0.02$) for IMP-CRP 0.02 and 0.1U/mL, respectively; 49% ($p<0.02$) for the combination of Ciprofloxacin 20 µg/mL + IMP-CRP 0.02 U/mL and 43% ($p<0.02$) for the combination of ofloxacin 20µg/mL + IMP-CRP 0.02 U/mL (Figure 5).

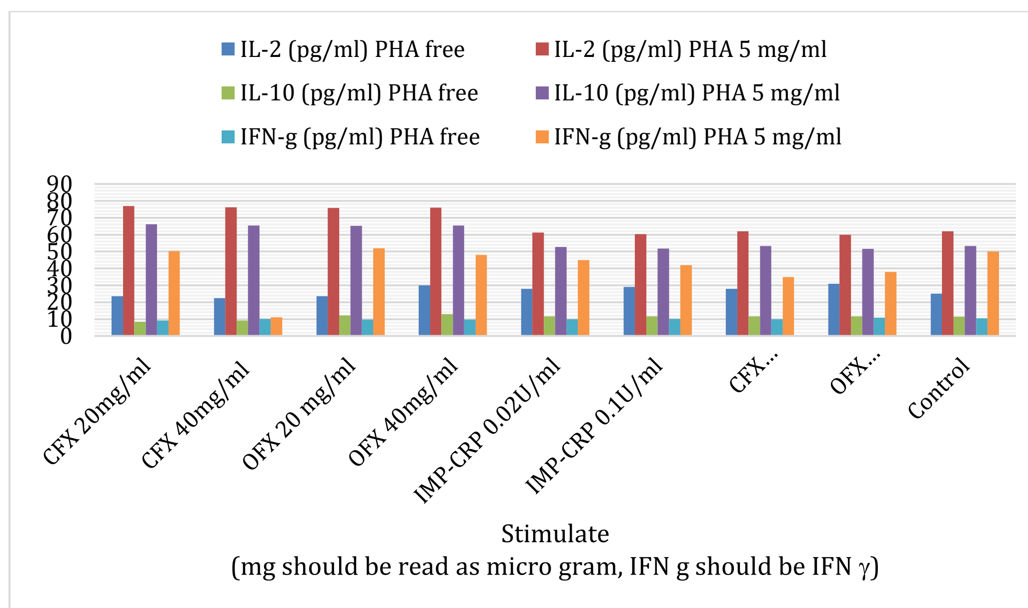

FIGURE 5. Evaluation of IL-2, IL-10 and IFN-γ production in PBMCs. 10^6 PBMCs were cultured and treated with Ciprofloxacin 20 and 40 µg/mL, ofloxacin 20 and 40 µg/mL and IMP-CRP 0.02 and 0.1 U/mL, and the combinations of Ciprofloxacin 20 µg/mL + IMP-CRP 0.02 U/mL and ofloxacin 20 µg/mL + IMP-CRP 0.02 U/mL; they were stimulated or not with 5 µg/mL of phytohemagglutinin and incubated at 37ºC, 5% CO_2 for 24 h. Subsequently, culture supernatants were obtained and ELISA was performed for IL-2, IL-10, and IFN-γ. Values of $p<0.02$ (*) and $p<0.02$ (**) were considered significant. CFX: Ciprofloxacin; OFX: ofloxacin; IMP-CRP: IMMUNEPOTENT CRP; PHA: phytohemagglutinin; pg: picograms.

DISCUSSION

Quinolones are broad-spectrum synthetic antibiotics that can modulate the immune system (Young, Lowell. 2014). Two quinolones currently used on a large scale are Ciprofloxacin and ofloxacin (Seral, 2005). IMP-CRP also has immunomodulatory effects. Given the therapeutic use of the compounds mentioned earlier, defining their role in the modulation of the immune system is crucial since it can be of great clinical importance, especially in immunodeficient patients or intensive care units.

As part of this study, the effect of Ciprofloxacin, ofloxacin, and IMP-CRP on PBMCs viability was evaluated. The results obtained show that there were no significant variations in the relative viability of PBMCs at any of the doses used, previous corroborating research by Germann, et al. (2013), Chaudhry, (1990), for Ciprofloxacin, ofloxacin, and IMP-CRP, respectively. This indicates that these compounds are safe for administration by any route and also gives us the guideline to attribute any immunomodulatory effect found to the action of the compound independent of cell toxicity.

Another parameter considered was the oxidative activity of monocytes, evaluated through the intracellular production of superoxide anion. Said activity was not affected after administering the different treatments of Ciprofloxacin, ofloxacin, IMP-CRP and the combinations (see materials and methods for clarifications).

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These results differ from those previously reported, in which quinolone treatments were found to increase superoxide production in PMA-stimulated rat macrophages. The difference in the results is probably due to the difference in the model since studies carried out in human cells show that ofloxacin does not affect the oxidative activity of neutrophils after activation with Candida albicans and Staphylococcus aureus (Miramón, 2012). Regarding IMP-CRP, previous studies report that it has the ability to inhibit the production of reactive oxygen species in macrophages. Although, it should be noted that the stimulant was LPS, which has been reported to induce a strong production of nitric oxide, but not superoxide. In our study, PMA is used, a stimulator that, contrary to LPS, induces the production of superoxide but not nitric oxide; Therefore, our results do not overlap or contradict those of Vadiveloo, (2001), but rather complement them. Given the previous reports, it isn't easy to define the role of Ciprofloxacin, ofloxacin and IMP-CRP on the activities of phagocytes.

Various modulatory effects have been reported for the compounds of interest in this study on essential transcription factors involved with the immune system, such as NF-κB, AP-1 and MAP-kinase. Given the roles of these transcription factors as signal integrators in lymphocyte activation, we were interested in whether Ciprofloxacin, ofloxacin, and IMP-CRP promoted or inhibited the proliferation of any lymphocyte subpopulation, which could be reflected in an increase or decrease in its proportion. The analysis was performed by flow cytometry, giving as a result that the different doses of the compounds and their combinations did not vary the percentages of B, NK, total T, T CD4+ and T CD8+ lymphocytes compared to the control. The absence of significant difference between the treatments and the control occurred both for cells without stimulation and for cells stimulated with phytohemagglutinin. These experiments seem to be the first carried out in this regard since no bibliography was found in which the in vitro effect of treatment with quinolones or IMP-CRP on the percentages of lymphocyte subpopulations was reported. Furthermore, our results showed that when cells were stimulated with phytohemagglutinin, the percentage of T-lymphocyte subpopulations was decreased (Figure 4), which occurred regardless of treatment. This finding is not new since Fock, et al. (2010) had already reported this phenomenon, which was later confirmed by Gilicze, et al. (2019), who attributed it to the loss of expression of the surface marker CD3 due to activation of the Apoptotic mechanisms of lymphocytes caused by stimulation with phytohemagglutinin.

The phytohemagglutinin stimulant was also used to assess cytokine production by PBMCs treated with Ciprofloxacin, ofloxacin, IMP-CRP, and combinations. The cytokines evaluated were IL-2, IL-10, and IFN-γ, which are known to be secreted by activated T lymphocytes, except IL-10, which is also secreted by monocytes. The results show that the production of the cytokines as mentioned earlier did not vary between treatments and control in PBMCs that were not stimulated. However, when the PBMCs were stimulated with phytohemagglutinin, it was possible to observe how the treatments had significant effects on the production of cytokines (Figure 5). In this regard, it is prudent to mention that the stimulatory or inhibitory effects of quinolones in terms of cytokine synthesis can only be observed when cells are exposed to stimulants such as lectins (eg phytohemagglutinin, Concanavalin A), cytokines (eg. TNF-α, IL-1), LPS or PMA; or to stressors such as radiation or cytotoxic drugs (Sodhi, 2007). IMP-CRP seems to behave similarly to quinolones since, at least regarding the production of the cytokines evaluated here, it did not exert any notable effect in the absence of phytohemagglutinin (Figure 5).

Regarding the specific effects of each treatment, we have that Ciprofloxacin increased the production of IL-2 at doses of 20 µg/mL and 40 µg/mL, an effect that agrees with that reported by Riesbeck, et al. (1998) for this quinolone. Ofloxacin also increased IL-2 production at doses of 20 µg/mL and 40 µg/mL, a finding that differs from that reported by Li, et al (2009), who found no significant difference in IL-2 production in cells treated with ofloxacin 10 µg/mL and stimulated with phytohemagglutinin, which could indicate that a higher dose of quinolone is required for it to exert an effect on IL-2 production. The effect on IL-2 overproduction does not appear to correlate with cytometry results in which the proportion of T lymphocytes does not show a significant increase over control for any dose of Ciprofloxacin or ofloxacin.

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A possible response could be an inhibition in the surface expression of the IL-2 receptor, as reported by by Riesbeck, et al. (1998) for Ciprofloxacin, which would cause an accumulation of IL-2 in the culture supernatant without considerably affecting the number of T lymphocytes. The clinical effect is difficult to define, but given the functions of IL-2 as the leading promoter of the development of regulatory T cells, the overproduction of IL-2 could indirectly promote a beneficial anti-inflammatory environment to control acute inflammation caused by bacterial infections, although it could, in turn, be harmful in the case of cancer patients.

IMP-CRP did not show any effect on IL-2 production at any dose, in agreement with previous reports for transfer factor, as well as its combinations with quinolones, which indicates that IMP-CRP somehow modulates the effects of these antibiotics, at least with respect to this parameter. This is likely related to the protective properties of IMP-CRP against side effects of cancer chemotherapy drugs reported by Li, et al (2009). However, studies are still needed to elucidate the mechanism of this process.

Another of the cytokines whose production was evaluated in this study is IL-10. The results indicate that all the treatments evaluated cause, to a greater or lesser extent, the inhibition of IL-10 production (Figure 5). Our results differ from those of Riesbeck, et al. (1998) for ofloxacin, where there was no statistically significant inhibition at a dose of 10 µg/mL and under LPS stimulation. However, they mention that a dose-dependent pattern was noted in its inhibition, so if the doses of 20 µg/mL and 40 µg/mL were used, it would be possible to find inhibition. The difference between our results and those of Riesbeck, et al. (1998) could also be due to the stimulus used (his LPS against our phytohemagglutinin), since different stimuli can cause differential effects of quinolones on cytokine production, probably due to the different pathways of signalling that stimulant activate.

On the other hand, our results agree with those of Shaw, et al. (2009) for trovafloxacin since a dose-dependent inhibition of IL-10 production is shown, although it is essential to mention that trovafloxacin was discarded from the market due to its hepatotoxic effects. They also agree with the reports by Mold, et al. (2003) for IMP-CRP, in which the production of IL-10 and other blood cell cytokines is inhibited after stimulation with LPS. Given the role of IL-10 as a suppressor of the inflammatory response, its inhibition could enhance inflammatory reactions during infections. However, it is difficult to discern the clinical implications of our results since in vivo studies of LPS-induced septic shock have shown that IL-10 production is increased after administration of the quinolone ciprofloxacin. Still, it decreases after the administration of IMP-CRP in both cases attenuating the severity of the symptoms and favoring survival. This indicates that the modulation of the production of IL-10 alone is insufficient for attributing pro- or anti-inflammatory properties to the compounds.

Regarding the effect of the treatments on the production of IFN-γ, it was found that the dose of ofloxacin 40 µg/mL and all the treatments that contained IMP-CRP significantly decreased the production of this cytokine. In contrast, the rest of the treatments did not affect it, although it should be noted that the 20 µg/mL dose of ofloxacin also showed inhibition, but without becoming significant. No reports on the influence of Ciprofloxacin on this parameter were found. Regarding ofloxacin, some reports agree with our results, such as the work of Gürbay, (2001), in which no effect was found on the production of IFN-γ at a dose of 10 µg/mL, an effect that, according to our results, it only appears under higher doses (above 20 µg/mL). Riesbeck, et al. (1998) report that ofloxacin decreases the percentage of CD4+ T cells that express intracellular IFN-γ in a dose-dependent manner from a dose of 2 µg/mL of quinolone. It should be noted that in the study as mentioned earlier, they used a different stimulator (PMA + Ionomycin) than the phytohemagglutinin that we used, which is known to cause different results. In addition, the effect on a specific population may differ from the global effect since there are other populations of IFN-γ-secreting cells, such as CD8+ T cells and NK cells, which could act in a compensatory manner.

There are no previous reports of the effect of IMP-CRP on IFN-γ production so this result will serve as a precedent for future research in this regard. As with the IL-2 results, the effect of IMP-CRP on IFN-γ production overlaps with the effect of quinolones.

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The inhibition of IFN-γ production observed for ofloxacin and IMP-CRP could be related to the previously observed anti-inflammatory effects of these compounds, indicating that these effects not only act immediately on innate mechanisms but also the acquired response.

The reason for combining quinolone treatments with IMP-CRP was to see if there was an interaction between these drugs and their possible relationship with their concomitant use or non-use. As mentioned above, for IL-2 and IFN-γ, the effects of IMP-CRP outweigh those of the quinolones (Figure 5). The mechanism of this event is unknown, but as mentioned above, it could be related to the mechanism of protection against side effects of cancer chemotherapy drugs. Furthermore, it is possible that such interactions also exist with stimulating agents (eg, LPS, phytohemagglutinin), in which case their beneficial effects would be due to blockade of the toxin rather than modulation of the functions of the affected cells. It is, therefore, necessary to carry out studies to elucidate such mechanisms.

The effects found in this study for Ciprofloxacin, ofloxacin and IMP-CRP contribute to the body of knowledge of the immunomodulatory properties of these compounds. However, it is necessary to continue conducting in vitro and in vivo studies in animal and human models concerning the effect of these drugs on the immune system to elucidate their function on the components of this system.

CONCLUSIONS

- Ciprofloxacin, ofloxacin and IMP-CRP do not affect the cell viability of PBMCs after 72 h of culture.
- Ciprofloxacin, ofloxacin, and IMP-CRP do not affect intracellular superoxide anion production levels in stimulated monocytes.
- Ciprofloxacin, ofloxacin, and IMP-CRP do not affect the proportions of lymphocyte subpopulations in PBMCs after culture for 72 h with or without the stimulator phytohemagglutinin.
- Ciprofloxacin increases IL-2 production, decreases IL-10 production, and does not affect IFN-γ production in PBMCs stimulated with phytohemagglutinin.
- Ofloxacin increases IL-2 production, decreases IL-10 production, and decreases IFN-γ production in PBMCs stimulated with phytohemagglutinin.
- IMP-CRP does not affect IL-2 production and decreases IL-10 and IFN-γ production in PBMCs stimulated with phytohemagglutinin.
- Combinations of IMP-CRP + Ciprofloxacin and IMP-CRP + ofloxacin do not affect IL-2 production, decrease IL-10 production to a greater extent than either compound alone, and decrease IFN-γ production.

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