IMMUNOMODULATORY ACTIVITIES OF QUINTON'S ISOTONIC AND HYPERTONIC SOLUTION. Part I

A study aimed at testing out the immunomodulatory activities of Quinton's isotonic and hypertonic solution, both *in vitro* and *in vivo* included the possible effects of both products *in vitro*, on human mononuclear cells of peripheral blood (PBMNc) in cultures, taken from healthy individuals. This involved assessing the following general aspects during the culture:

- Microscopic study of the possible effects on the cell viability, morphology, number and size of aggregated cells.
- Study of the possible effects on specific and general cellular proliferation.
- Study the possible effects on cellular proliferation in different lymphocyte populations.
- Effect on haemoglobin released into the medium, in order to discover the possible effect of protecting or preserving the red blood cells.

In this study 10 ml of anticoagulant blood (EDTA) was taken from ten healthy volunteers.

The method used is as follows:

- Cell cultures: The PBMNc under study were obtained by means of a Ficoll-Hypaque density-gradient centrifugation, adjusted to 1x106/ml and grown in Costar 96 well cell culture in the ratio of 200,000 cells/well.

The cell culture conditions were as follows:

- RPMI, supplemented with 1% of antibiotic, 1% of glutamine and 10% of fetal calf serum (FCS); pH = 7.3
- Quinton isotonic solution alone (ISO-); pH = 7.3
- Quinton hypertonic solution alone; pH = 7.3
- Quinton Isotonic solution (ISO+) supplemented with 1% of antibiotic, 1% of glutamine and 10% of FCS; pH = 7.3
- Quinton hypertonic solution supplemented with 1% of antibiotic, 1% of glutamine and 10% of FCS; pH = 7.3
- Physiological saline solution supplemented (SS) with 1% of antibiotic, 1% of glutamine and10% of FCS; pH = 7.3

As stimulants for the different conditions, phytagglutinin (PHA), phorbol esters and ionomycin (PMA+Io), anti-CD3+anti-CD28 (CD3+CD28) were used together with unstimulated cells (negative controls). The culture plates were cultivated for 4 weeks in a CO2 incubator, at 37ºC, 95% humidity and 5% CO_2.

- Inverted/optical microscope: this was used to calculate the cell viability parameters, morphological cell changes, number and size of aggregated cells. Cell viability is analysed by using vital dyes (trypan blue).
- Flow cytometry: to analyse cell proliferation, the [5(6)] carboxyfluorescein diacetate succinimidyl ester or the CFSE (Sigma-Aldrich Co) technique was applied.

At the same time, by means of the Direct Immunoflurescence technique, the PBMNc were incubated with different combinations of monoclonal antibodies aimed at the membrane antigens CD3, CD4, CD8 and CD25, and combined with different fluorophores (FITC, PE, PE-Cy5).

Results

- With regard to ISO+, cell viability after 4 days of being cultivated was similar to that of the RPMI and that of the SS. This dropped slightly when Quinton isotonic solution was used alone. No cellular morphological changes were detected with regard to the RPMI or the SS. As for Quinton hypertonic solutions, alone or supplemented, viability was already minimum in the first 12 hours of the culture (unproven data), which is why its use in later experiments was ruled out.
- With regard to the number and size of the aggregated cells (Picture 1), cell cultures with RPMI were seen to have aggregated cells in all of the stimulated wells. In the case of PMA+Io they were small and numerous, and then bigger but fewer in the case of the PHA and CD3+CD28. In the latter case they were considerably bigger. When ISO- was used, no aggregated cells were detected, apart from a few isolated cases in that of PHA and antiCD3+antiCD28. Nevertheless, when the ISO+ was used, the same thing happened as in the case of the RPMI for the different stimulus, although the aggregates were not as significant. The fact that unstimulated aggregates were detected in the cell controls is significant. With regard to the SS, similar aggregates to those concerning ISO+ were obtained, although no aggregates were detected in the unstimulated cells.

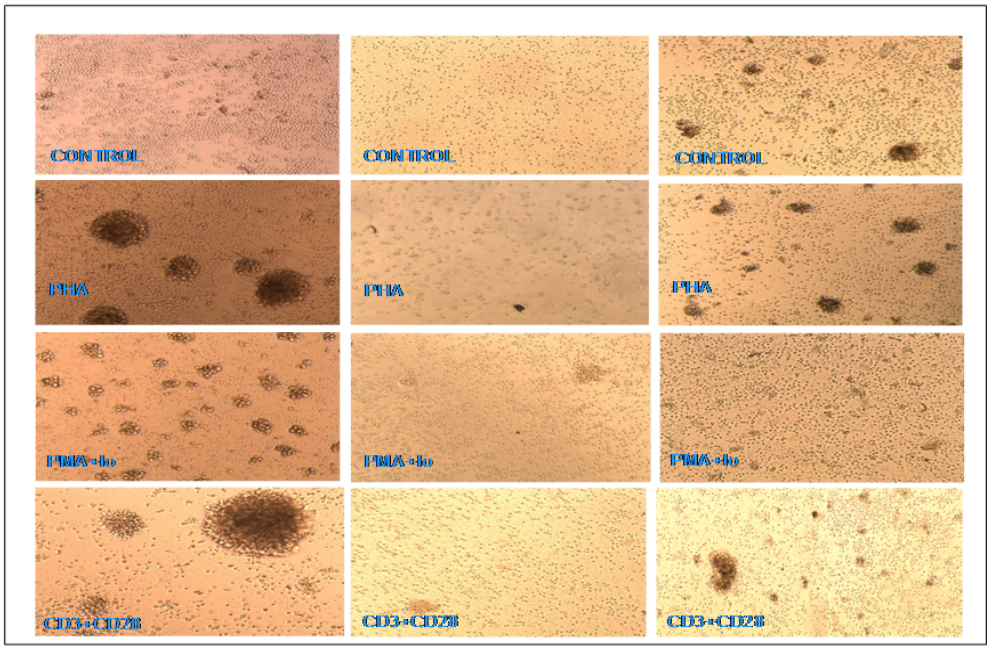

Figure 1: PBMNC Cultures

- With regard to analysing cellular proliferation, the CFSE technique was applied so that tritium labelled thymidine didn't have to be used. Figure 2

shows the total (CD3+) T lymphocyte gates analysed both unstimulated and stimulated

- with anti-CD3+anti-CD28 (CD3+CD28+). The *dot-plot* shown in figures 3 and 5 respectively show proliferation (measurement in % of CFSE+ cells) and the lymphocyte activation (% of CD3+CD25+ cells) from the unstimulated and stimulated cells (CD3+CD28) in the different culture mediums used. The maximum proliferation and/or activation occurred, as was expected, in the stimulated cells in RPMI; proliferation in the other two mediums was however limited and there was no noticeable difference between them. Nevertheless, when histograms were used to measure proliferation (figure 4), differences were indeed observed. The proliferation (left fluorescence deviation) of stimulated cells in the ISO+ medium was greater than proliferation in the ISO-; in the latter case proliferation was similar to that observed in control cells. This data also tallies with the greater cell activation found in cells stimulated in ISO+ (figure 5), which could justify the existence of aggregated cells in the cultures with this medium.

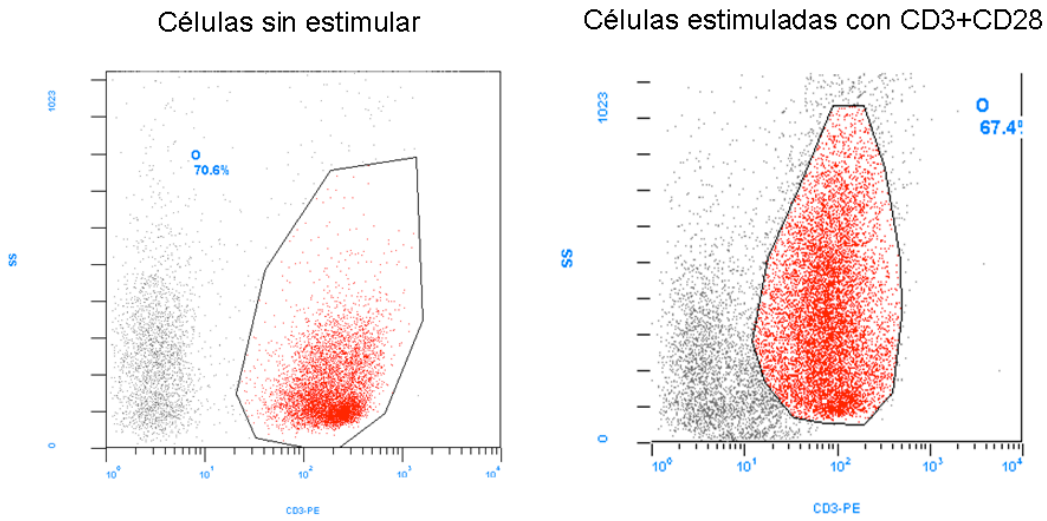

Figure 2: Selecting the population

- When proliferation and/or activation in CD4+ y CD8+ T lymphocyte subpopulations was analysed, the results were similar to those described for the total T lymphocytes (CD3+). Therefore activation and proliferation was detected in both lymphocyte populations, although the parameters were greater in the CD4+ T lymphocyte population than in that of the CD8+. The results on the proliferation of the B lymphocytes and NK cells were not conclusive.
- To gauge the effect of Quinton's hypertonic and isotonic solutions on the red blood cell lysis, the spectrophotometric method used by the Drabkin reagent was modified. This method is commonly used to measure total haemoglobin levels (free haemoglobin + intraeritrocitary haemoglobin) in blood. We adapt the method to measure only the free haemoglobin at different times during the culture (8, 12, 24, 48, 72 and 96 hours) and with different pure culture

mediums without adding (RPMI, SS, Quinton isotonic and hypertonic solutions). The suitable calibrated curve was established beforehand with bovine haemoglobin. This was later used to incorporate the different absorbance obtained in the sample and in this way obtain approximate concentrations of free haemoglobin in each sample. The results obtained were as follows:

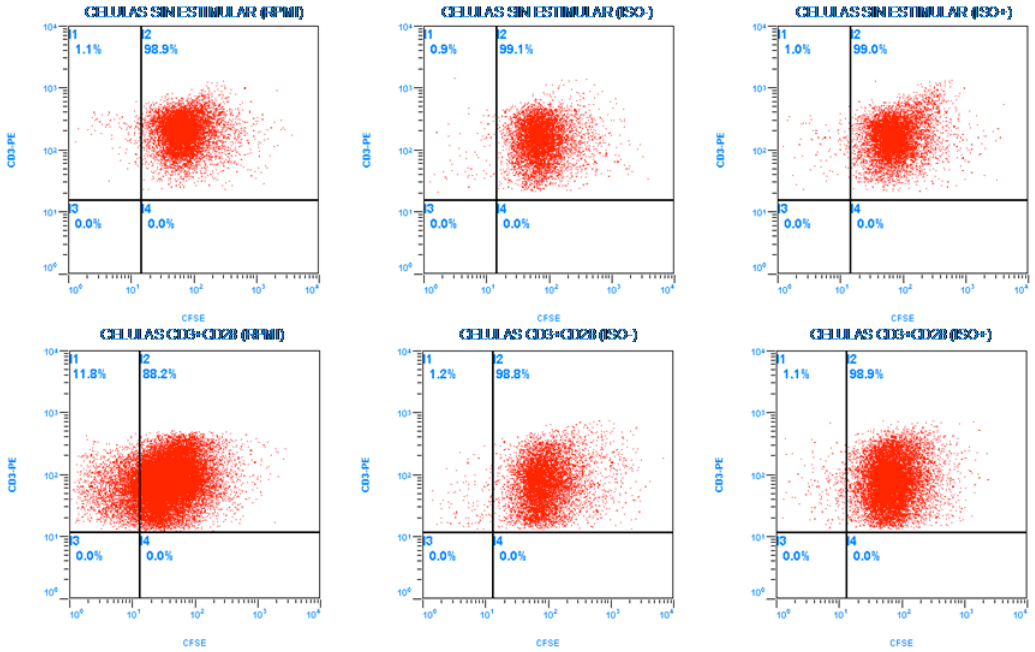

Figure 3: Cell proliferation (CFSE)

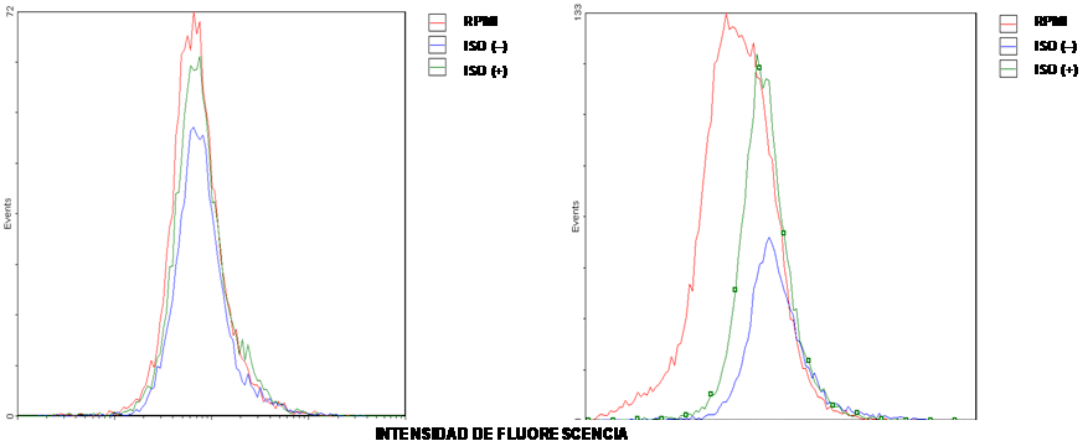

Figure 4: Cell proliferation

- For the first 8 hours all the culture mediums seemed to keep the red blood cells in good condition, the absorbance was similar in all the samples analysed.
- After 8 hours of culture, the blood cell lysis started to increase considerably and progressively in Quinton's hypertonic solution. Maximum absorbance figures were obtained at 96 hours.

- After 48 hours, the blood cell lysis also started to increase slowly and progressively with RPMI and SS; maximum absorbance figures were obtained at 96 hours, more so for SS than for the RPMI. In any case, both absorbance figures were less than those detected for the Quinton hypertonic solution.
- With Quinton isotonic solution, similar absorbance figures were constant during the 96 hours of culture and no increase was shown in any of the samples analysed. ***This might be due to the fact that Quinton Isotonic solution protects the red blood cells***.

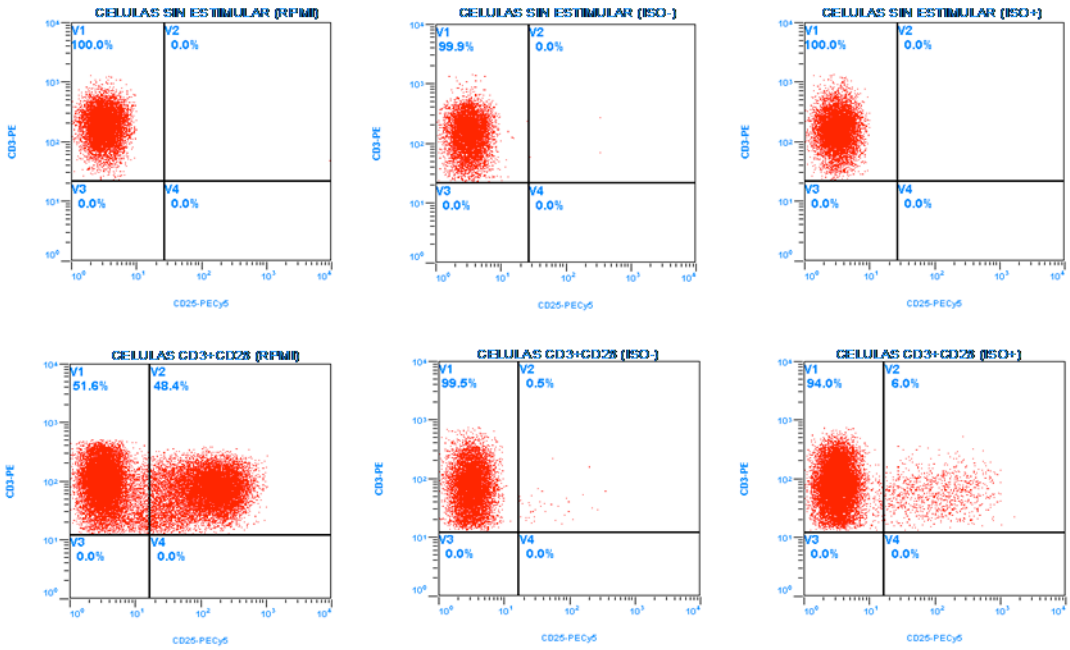

Figure 5: Cell activation

Conclusions

1.-The PBMNs cultivated in vitro with Quinton isotonic solution, maintain their morphology and viability throughout the four days of the culture, especially with ISO+. solution.

2. - ***The ISO+ solution*** acts like the RPMI culture medium in terms of the aggregation, proliferation and/or cell activation. Moreover, to a certain degree, ***it seems to be able to activate cells by itself, which is proved by the existence of cell aggregates in the unstimulated medium.***

3.- According to the aforementioned, the fact that there is hardly any aggregation, proliferation and/or cellular activation in the ISO- could be due to the fact that there aren't any nutrients in the medium.

4. - Quinton isotonic solution clearly protects/preserves red blood cells, which is proved by the fact that hardly any haemoglobin is freed during the 96 hours of culture.
5. - The high tolerance of the PBMNc *in vitro* with Quinton isotonic solution is evident, as is its potential to activate cells, which suggests that in ideal conditions it could replace conventional culture mediums. Likewise apart from this potential to protect red blood cells, the results obtained could be used as the basis for future *in vitro e in vivo* studies with the product that might even reveal that it can help preserve organs.