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PROJECT NAME: "Estudio cuantitativo, cualitativo y funcional del efecto de la solución isotónica Quinton, sobre poblaciones reguladoras del sistema inmunitario (Quantitative, qualitative and functional study of the effect of the isotonic solution Quinton on immune system regulatory populations)"			
C	REPORT ON RESULTS OBTAINED		

OBJECTIVES

The first objective set in the study was to analyse the effects of the Quinton isotonic solution (ISO) on the proliferation of peripheral blood mononuclear cells (PBMCs).

The second objective was to analyse the effects of ISO on the sub-population of T-regulatory lymphocytes (Treg) from peripheral blood samples (PBMC).

The study's third objective was to analyse the effect on the viability and morphology of the hASC treated with different concentrations of the Quinton® isotonic solution (ISO).

MATERIALS AND METHODS. RESULTS

NEGATIVE CONTROL

In all cases, the Quinton isotonic solution was compared with a saline solution of a similar concentration (PBS).

PBMC SAMPLES

All peripheral blood mononuclear cell samples were obtained via density gradient centrifugation (Fycoll-Hypaque), from peripheral blood from healthy volunteers.

VIABILITY OF PBMCs IN CULTURE

Firstly, different tests were conducted to determine the viability of the cells in the presence of the Quinton isotonic solution. The PBMCs were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and were cultured in duplicate at 100,000 cells/well (96-well plates) in pure RPMI culture medium, pure ISO or different proportions of RPMI/ISO (3/1, 1/1). All the media were supplemented with foetal calf serum (10%), penicillin/streptomycin (1%) and glutamine (1%). The cells were stimulated with phytohemagglutinin (PHA) at a concentration of 10 ug/ml. Positive control of the proliferation (maximum theoretical proliferation) was considered at RPMI 100%+PHA. In the same fashion, negative controls were conducted (without stimulus) for each one of the conditions. After 5 days of culturing, cellular proliferation was analysed with flow cytometry. Results showed that both viability and proliferation were measurable in all conditions used, including when nutrients

were absent (ISO 100%) (Fig. 1).

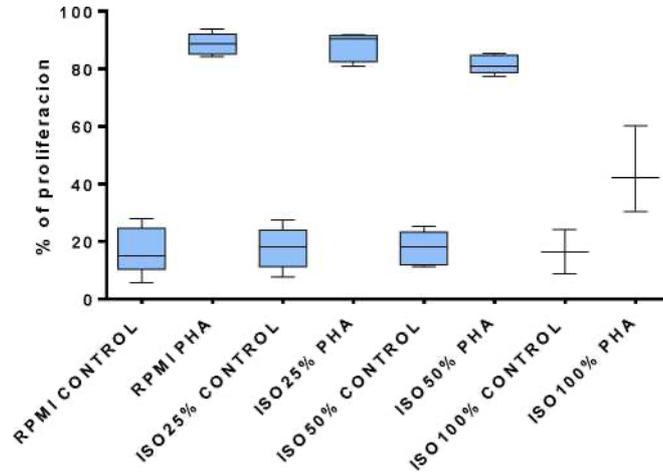


Fig. 1. Proliferation of PBMCs for different RPMI/ISO proportions.

PBMC PROLIFERATION

Once verified that cellular proliferation was measurable for the entire gradient of conditions (from 100% of RPMI medium to 0%), we studied the effect of ISO in and the Quinton hypertonic solution in greater detail, comparing them at the same time with similar solution as far as NaCl concentration is concerned (9 g/l), than in ISO 100%, but that did not contain the rest of its elements; in this case, we used PBS (Phosphate Buffer Saline) 1X. To use the hypertonic solution (33 g/l) to achieve the same final concentration of NaCl as in ISO 100% (9 g/l), said solution was used at a final concentration of 12%. As control for this last condition, PBS 3.5X was used (3.5 times more concentrated than PBS 1X), at a final concentration of 12%, in order to keep the final NaCl concentration of 9 g/l.

The culture used for each volunteer in the study and that acted to evaluate the comparative effect of the different solutions was:

	RPMI 100%	ISO 12.5 %	PBS 12.5 %	ISO 25%	PBS 25%	ISO 50%	PBS 50%	ISO 75%	PBS 75%	ISO 100%	PBS 100%	HYPHER 12%	PBS 3.5X 12%
CONTROL													
CONTROL													
PHA													
PHA													

The proliferation was measured similarly to the previous description. The results showed that the ISO solution in practically all concentrations used, provides an advantage over PBS as far as cellular proliferation is concerned. In all cases (except for absent nutrient conditions), statistically significant differences are observed, which suggest that adding the Quinton isotonic solution, regarding PBS, leads to an increase in lymphocyte proliferation. On the other hand, it was observed that by only adding 12.5% of 25% of the Quinton isotonic solution to the RPMI medium (classically considered the optimum medium for PBMC culturing), in every case, led to a large increase in proliferation, as opposed to the maximum theoretical proliferation (RPMI 100%), which suggests that the Quinton isotonic solution provides the RPMI with a protective or synergy factor (Fig.2) for cellular proliferation.

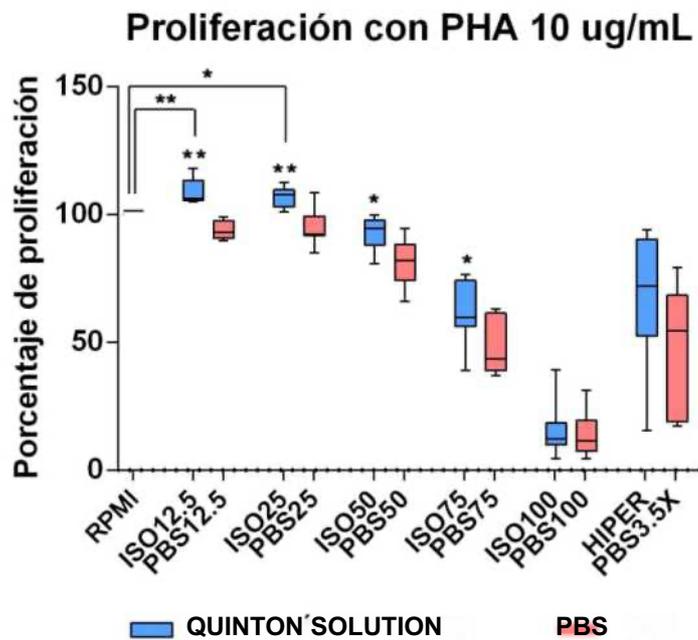


Fig.2 . Percentage of PBMC proliferation with PHA at 10 ug/mL

After these observations, we wanted to study the effect of the ISO solution on PBMC proliferation, using different stimulus concentrations (PHA).

The results obtained were (Fig.3):

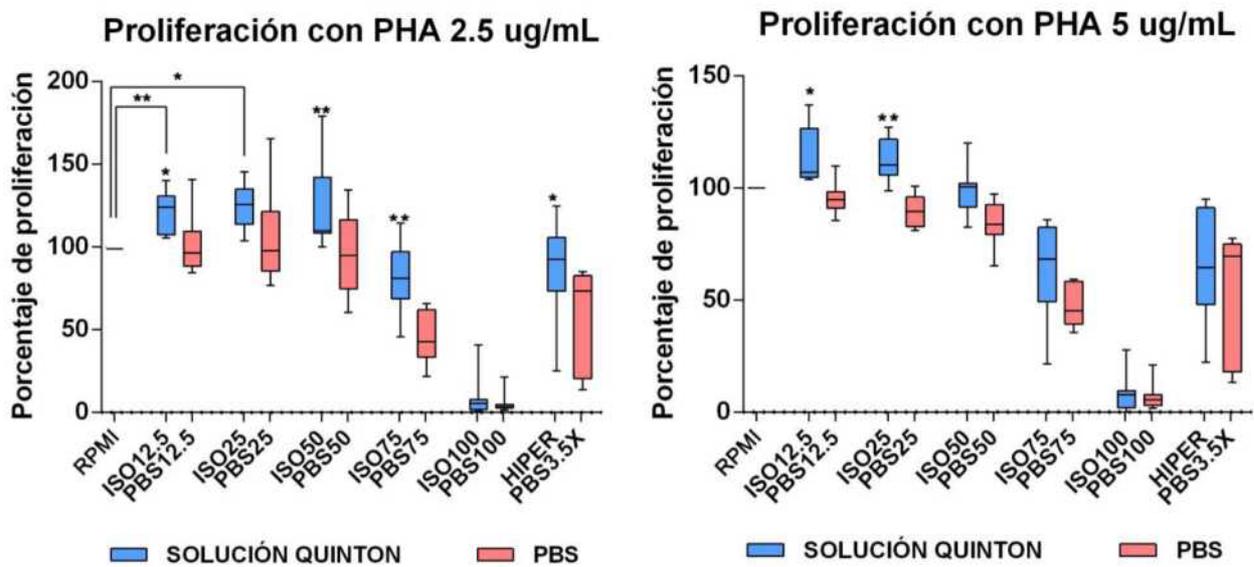


Fig.3 . Porcentaje de proliferación de PBMCs con PHA a 2,5 ug/mL y 5 ug/mL

- QUINTON SOLUTION -PBS

Fig. 3. Percentage of PBMC proliferation with PHA at 2.5 ug/mL and 5 ug/mL

In all cases, basically the same results were obtained, but it was observed that a lesser concentration of stimulus (PHA) caused a greater difference between the proliferation data obtained with the ISO solution, as opposed to those obtained with PBS, for each one of the conditions analysed. Said effect is also observed in greater detail in the figure below (fig. 4), showing the net difference (in percentage) of proliferation between the Quinton isotonic solution and the PBS for different conditions.

Efecto de la concentración de PHA

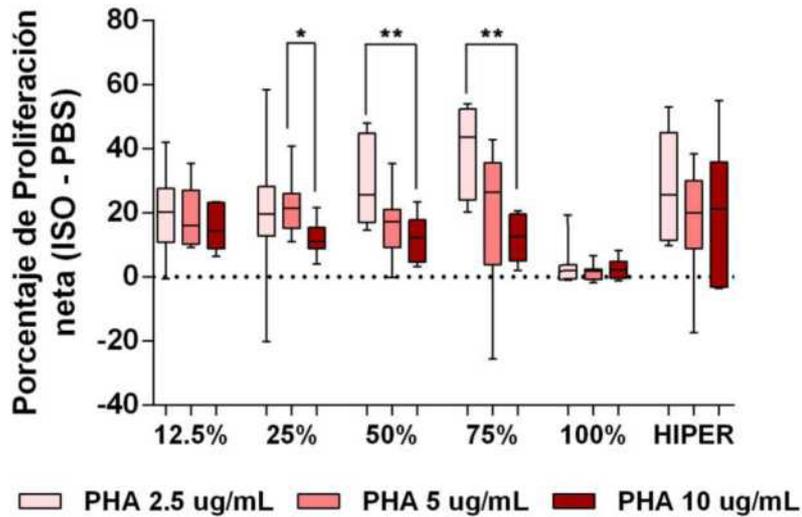


Fig.4. Effect of PHA concentration in net proliferation (ISO - PBS) for each one of the conditions.

MODULATION OF REGULATORY T CELLS

The PBMCs were cultured on 96-well dishes in duplicate, with 100,000 cells/well in pure RPMI culture medium, pure ISO or with different proportions of RPMI/ISO (12.5, 25 and 50% ISO). All the media were supplemented with foetal calf serum (10%), penicillin/streptomycin (1%) and glutamine (1%). The cells were stimulated with phytohemagglutinin (PHA) at a concentration of 10 ug/ml. In the same fashion, negative controls were conducted (without stimulus) for each one of the conditions. After 3 days of culturing, CD4 and CD25 membrane markers were analysed, as well as the FoxP3 intranuclear transcription factor, by labelling with monoclonal antibodies, permeation and then analysis with flow cytometry. All results were compared with a PBS solution (Phosphate Buffer Saline) 1X, with a NaCl concentration similar to the ISO 100% (9 g/L), but that did not contain the rest of its elements.

The culture used for each volunteer in the study and that acted to evaluate the comparative effect of the different solutions was:

	RPMI 100%	ISO 12.5 %	PBS 12.5 %	ISO 25%	PBS 25%	ISO 50%	PBS 50%	ISO 100%	PBS 100%
CONTROL									
CONTROL									
PHA									
PHA									

The results were analysed thus:

Firstly, lymphocytes expressing CD4 were selected (Fig. 5.A) and the percentages of the different populations expressing CD25 and/or FoxP3 were quantified in them (Fig.5.B). Moreover, the median fluorescent intensity of CD25 and FoxP3 markers was quantified, both in the total population of CD4 lymphocytes and in the sub-population CD4+CD25+FoxP3+.

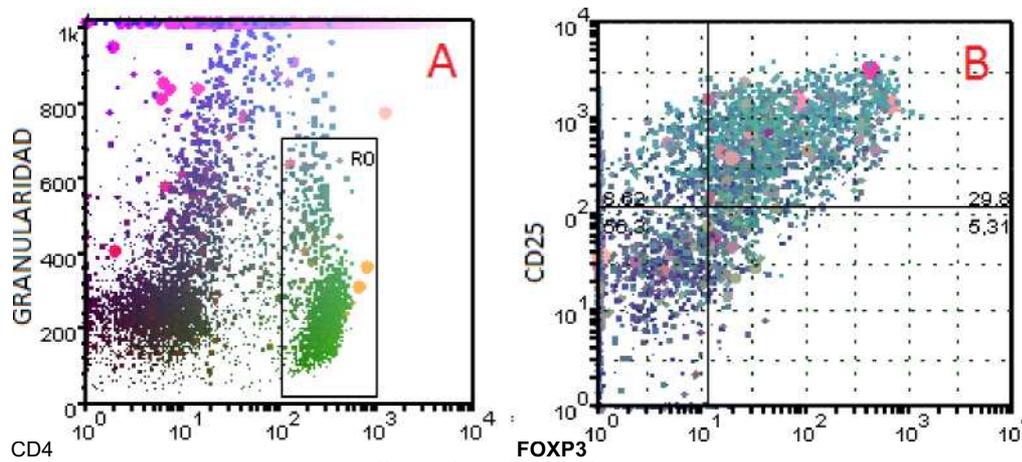


Fig. 5. Cells analysed with flow cytometry.

Results obtained were homogenised regarding the 100% RPMI condition of each one of the volunteers, this being considered the optimum culture condition standard for the PBMCs. Said results showed that the cells cultured with a Quinton isotonic solution, in comparison with PBS, expressed a higher percentage of CD4+CD25+FoxP3+ cells in all cases (Fig. 6), these differences being significant with absent nutrients (100% isotonic solution). With the rest of conditions, the aforementioned trend continues, obtaining values close to significant with 12.5% isotonic solution ($p = 0.09$). When the ISO solution is present, it is striking that the percentage of CD4+CD25+FoxP3+ cells remains practically similar to the percentage observed with 100% RPMI, even under conditions with 50% nutrient absence, unlike what was observed with cells cultured with PBS, as would be expected.

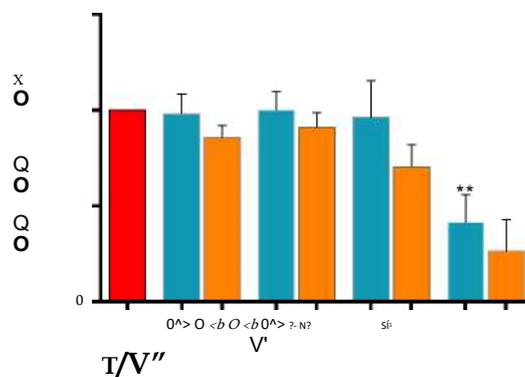


Fig. 6. Percentage of CD4+CD25+FOXP3+ populations.

Currently, most authors identify authentic regulatory T cells (Treg) as those that express with CD25 activation marker with a high intensity (CD25^{high}), that simultaneously express the FoxP3 transcription factor. This population was also analysed in this study, showing results very similar to previous results (Fig. 7), but with even greater differences than those observed in the previous case, specifically for the 12.5 and 50% ISO concentrations. Just like in the previous analysis, significant results were obtained for the 100% isotonic solution condition. Once again, the percentage of CD4+CD25^{high}FOXP3+ cells (Treg) remains practically constant, even under conditions with 50% nutrient absence, the opposite of what occurs with PBS. It is surprising that the percentage of Treg cells under some culture conditions (ISO 12.5%) was even greater than the percentage obtained for the 100% RPMI condition.

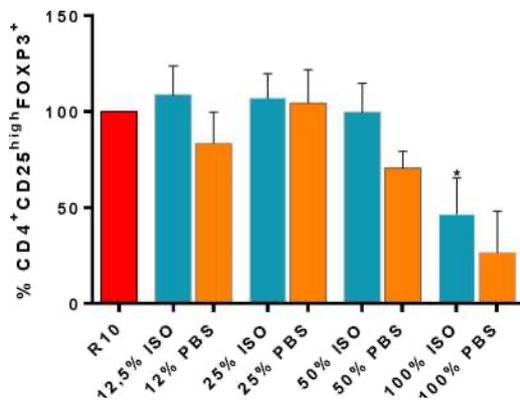


Fig. 7. Percentage of CD4+CD25highFOXP3+ populations.

Regarding the intensity with which the CD4+CD25+FOXP3+ cell population expresses its phenotype markers (Medium Fluorescence Intensity - MFI), it is observed that the CD25 activation marker, with stimulus and absent nutrients, has a significantly greater intensity ($p = 0.026$) than obtained with the same cells cultured with PBS (Fig. 8B), keeping similar expression levels or even slightly greater than those observed for the different proportions of the RPMI and ISO solution. Something similar occurs with the CD25 marker on the entire Th lymphocyte population (CD4+) (Fig. 5F). The expression level remained the same for all conditions studied, except for the PBS 100% condition, where MFI falls.

With the FoxP3 FMI, with absent nutrients (ISO 100%), it should be noted that the non-stimulated cells tend to decrease expression (Fig. 9G), while those stimulated with PHA can maintain it, reaching statistically significant differences vs. PBS (Fig 5G and 5H).

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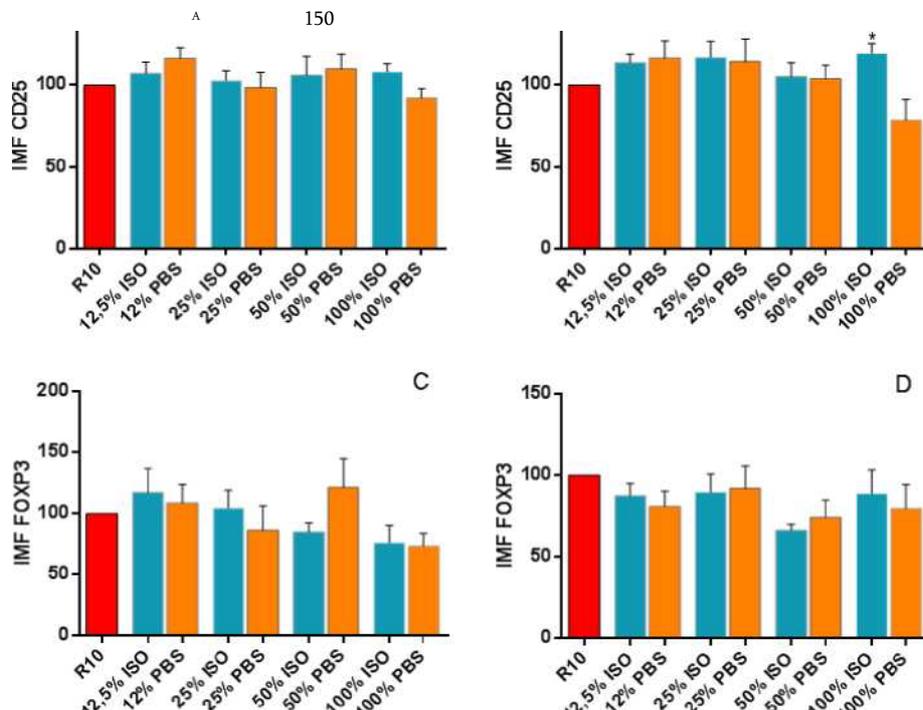


Fig. 8. Medium fluorescence intensity for the CD4+CD25+FoxP3 population. MFI of CD25 (A, B) and MFI of FoxP3 (C, D). Control (A, C). PHA (BD).

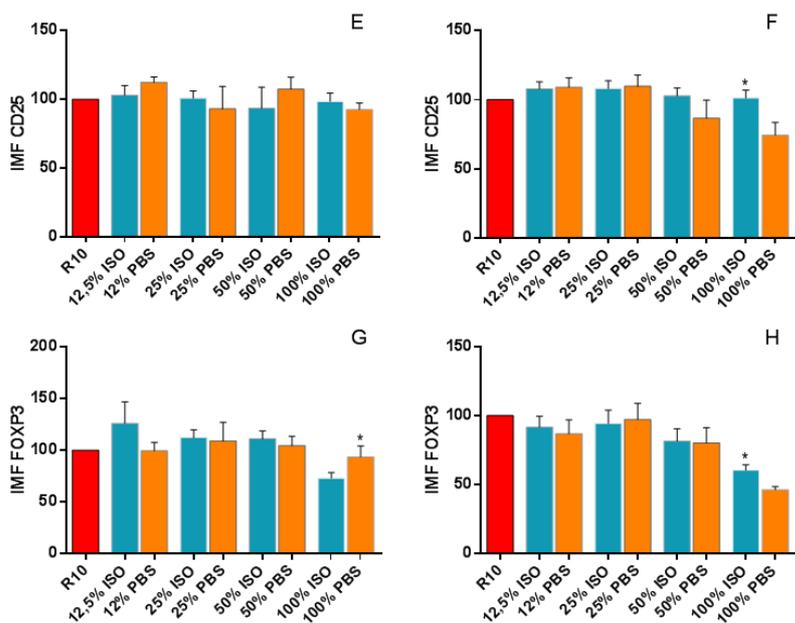


Fig. 9. Medium fluorescence intensity for the CD4⁺ population. MFI of CD25 (E, F) and MFI of FoxP3 (G, H) Control (E, G). PHA (F, H).

EFFECT ON THE VIABILITY AND MORPHOLOGY OF MESENCHYMAL STEM CELLS

Mesenchymal stem cells are a kind of stem cell found in numerous adult body tissues. These cells can be divided into adipocytes, chondrocytes and osteocytes, and also bear a property that makes them a great tool to treat immunological diseases, since these cells have great immunomodulatory potential. In fact, they are able to decrease activation of most of the immune system's cells, all while increasing Treg cell proportion. In addition to the effects described on lymphocyte proliferation, the Quinton solutions appears to be having a positive effect on the Treg cell proportion. Since this is a primary culture, its growth is limited, and any factor contributing to increased proliferation and viability after cryopreservation would be welcome, since it would provide for greater performance in possible cell therapy. Additionally, optical microscopy was used to analyse the effect of these same proportions of culture medium and Quinton isotonic solution on different mesenchymal stem cells from adipose tissue, or stromal cells (ASC). hASC clones were obtained from different donors, isolated and cultured in Costar plates with 96 wells with 10,000 cells/well, with different culture medium and saline solution proportions (Fig. 10). The clones not used were cryopreserved at -80°C in standard freezing medium. In all cases, the final concentration of foetal calf serum, antibiotic and glutamine was 10%, 1% and 1% respectively, and the final volume was 200 µL/well. In this case, cells were cultured in DMEM culture medium, an ideal substrate for this kind of cell, over the course of 3 days. Results are shown in Fig. 10, where once again, we clearly observe a protective effect of the Quinton isotonic solution in comparison with PBS under nutrient-absent conditions, with more round ASC cells separated from the culture flask (dead), suggesting a possible protective effect of the ISO solution, which would be able to somehow delay the death of these cells. These effects can be better observed in Fig. 11, showing the ratio of dead cells in each condition for 100% RPMI, with the following formula:

$$\text{RATIO} = \frac{\text{DEAD CELLS}}{\text{RPMI DEAD CELLS}}$$

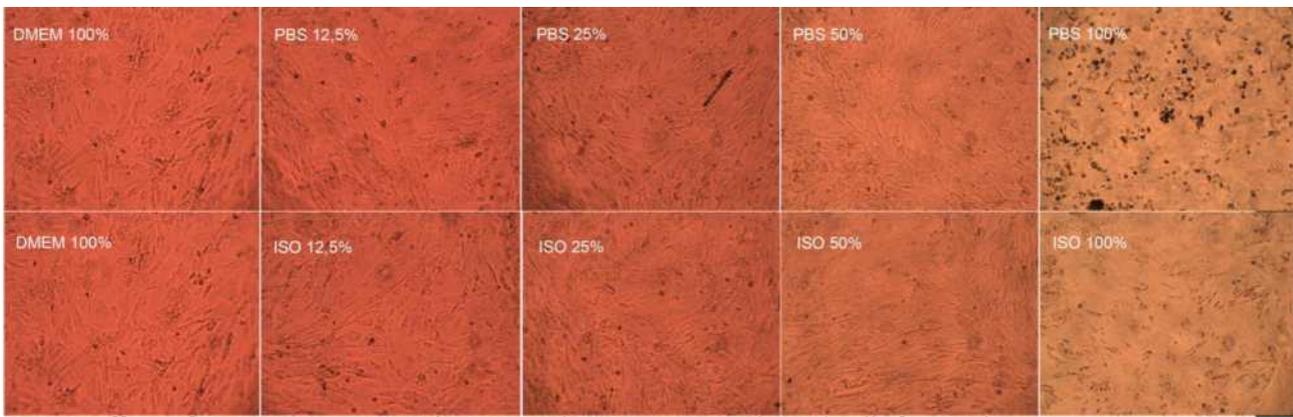


Fig. 10. Effect of Quinton isotonic solutions and PBS on mesenchymal cells from adipose tissue.

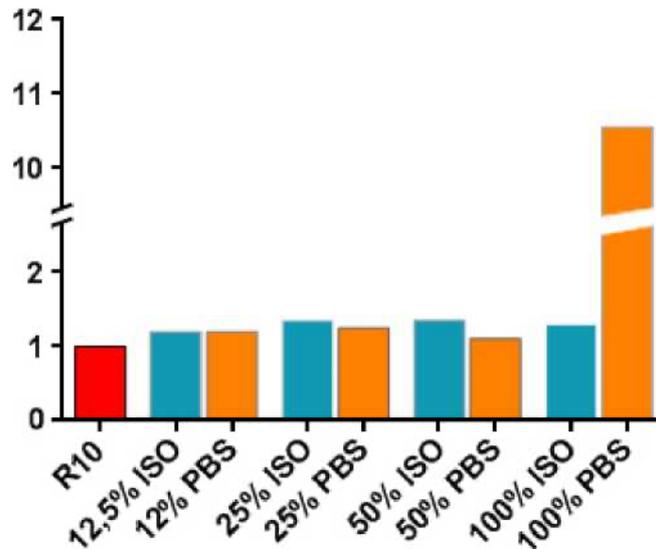


Fig.11. Ratio of dead cells for 100% RPMI

EFFECT ON VIABILITY

Once the effects of the morphology and its possible protective nature based thereupon, the quantitative analysis of ASC viability was studied at different times and with different concentrations of the Quinton isotonic solution. Several hASC clones were unfrozen. They were cultured on 12-well Costar plates. They expanded until reaching confluence and were trypsinized and cultured again at 50000 cells per well. They were cultured with different isotonic solution/DMEM proportions. After 1, 3 and 5 days, cell viability was analysed via phase microscopy and intracellular labelling with Tripan. A total cell recount was conducted with a haemocytometer. They were then cryopreserved in Quinton isotonic solution to again analyse viability after unfreezing after a few months. In all concentrations, the Quinton® isotonic solution increased the number of hASC over the control condition with a saline solution with a similar concentration (Fig. 12). These numbers were higher when the Quinton® solution was administered at a concentration of 12.5%, doubling the figure over a positive control with 100% DMEM (Fig.13). Under conditions when the culture medium with 100% PBS and ISO solutions were absent, cell values were null.

DMEM 100%	ISO 12,5%	PBS 12,5%	ISO 25%	PBS 25%	ISO 50%	PBS 50%	ISO 100%	PBS 100%

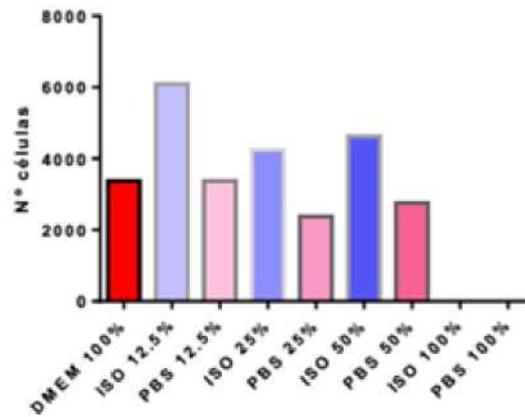


Figure 12. Total number of cells obtained after trypsinization.

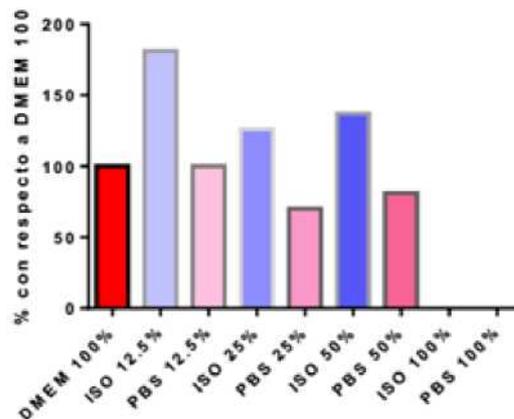


Figure 13. Percentage of cells obtained from 100% DMEM condition after trypsinization

The photographs of cultures show how, as the DMEM concentration decreased, so did the number of cells (Fig. 14), even showing large areas with absent hASC under 100% PBS condition. And not this alone: the fusiform morphology characteristic of hASC is better conserved when the Quinton® isotonic solution is present (for PBS).

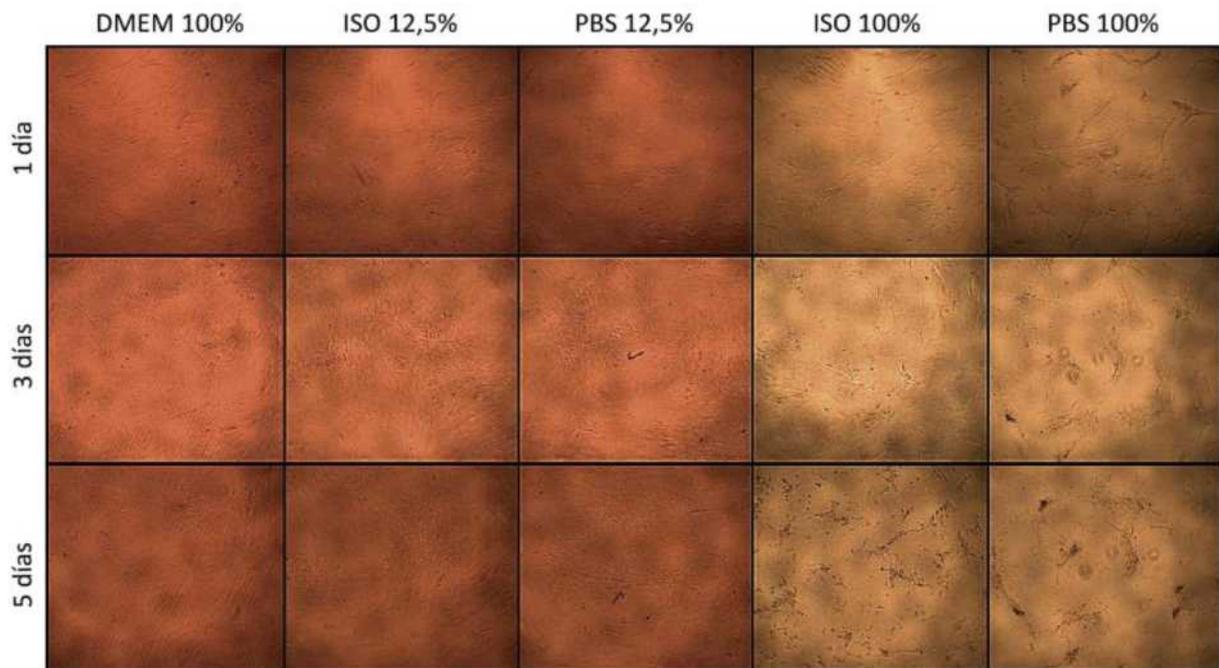


Figure 14. Photographs taken 1, 3 and 5 days of hASC cultured with different ISO and PBS concentrations.

CONCLUSIONS

1. Although preliminary, the data obtained to date suggest that there would appear to be a clear synergy in the isotonic and hypertonic Quinton solutions, with the PHA, which is intensified at low concentrations in the latter. This complements previous results obtained to date with both solutions, which describe both of their potential to increase lymphocyte activation.
2. The data obtained on the effect of the Treg cells indicate that the Quinton isotonic solution seems able to modify (maintain and/or raise) the percentages of Treg cells, both if we use the CD4+CD25+FoxP3+ cell population and the CD4+CD25^{high}FoxP3+ population as a reference.
3. Moreover, the intensity with which the CD25 marker is expressed is more homogeneous when the isotonic solution is present, regardless of the culture medium proportions used, and even when absent. Regarding FoxP3 intensity, clear effects of the ISO solution in the absence of nutrients are observed, both with and without stimulus, which indicate a possible regulatory effect for the solution on the expression of said transcription factor.
4. Results obtained on the effect that the ISO solution could have on mesenchymal stem cells from adipose tissue indicate a possible protective cellular effect, which we have previously described in prior studies (with red blood cells), that could be delaying or preventing apoptosis and later cellular death.
5. Finally, regarding the effect of the Quinton isotonic solution on the viability of mesenchymal cells, we can affirm that the presence of different proportions of the Quinton isotonic solution in culture helps to maintain the integrity and viability of hASC *ex vivo*.

In San Vicente del Raspeig, 9 June 2017

Head researcher

Signature: José Miguel Sempere Ortells

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