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Short Communication

Lack of Effect of Human Chorionic Gonadotropin in Mixed Lymphocyte Reaction in Xenotransplantation

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Abstract

It has been speculated that the immunomodulation associated with pregnancy, e.g., decreasing pro-inflammatory cytokines, increasing anti-inflammatory cytokines, upregulation of T regulatory cells (Tregs), is in part due to the effect of human chorionic gonadotropin (hCG). In this study, we tested the effect of hCG on proliferation of human peripheral blood mononuclear cells (PBMCs) stimulated by irradiated pig PBMCs. Mixed lymphocyte reaction (MLR) was carried out with human PBMCs as responders and irradiated wild-type pig PBMCs as stimulators, with or without hCG. The spontaneous mean proliferation of $CD3^+$T cells was 7% and, when stimulated by phytohemagglutinin (PHA) was 43%. When stimulated with irradiated wild-type pig PBMCs, $CD3^+$T cell proliferation was 18%. When hCG (at concentrations of 100 IU/ml, 500 IU/ml, and 1,000 IU/ml) was added to the MLR, the proliferation of $CD3^+$T lymphocytes was 20%, 20%, and 18%, respectively. hCG also had no effect on the proliferation of $CD4^+$T and $CD8^+$T cells. hCG does not suppress human lymphocyte proliferation stimulated by wild-type pig PBMCs in MLR (unless this is related to an increased number of Tregs, which was not tested in this study).

Keywords
Chorionic gonadotropin; human; mixed lymphocyte reaction; pig; T cells; Xenotransplantation

1. Introduction

Pregnancy is the single example of foreign tissue avoiding rejection by the host's immune system, without requiring immunosuppressive therapy (Table 1) [1-7]. Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein primarily produced by syncytiotrophoblasts, often known as the 'pregnancy hormone [8]. The primary functions of hCG in pregnancy are to stimulate progesterone secretion to maintain uterine decidua, and modulate the maternal immune system [8]. In a mouse skin transplantation model, multiparous mice induced tolerance to syngeneic male skin grafts [9], whereas following a single pregnancy there was no significant protective effect on paternal skin allografts [10].

Table 1 Mechanisms active in fetal tolerance during pregnancy and associated observations.

Reference	Suggested mechanism
[1, 2, 4]	Anatomical barriers (uterus and placenta). Immature antigen expression by the fetus. Selective immunosuppressive state of the maternal immune system.
[3, 5]	Immunomodulation by expression of the HLA-G at feto-maternal surface.
[5]	HLA-G Influences T cells and Natural Killer cells towards tolerant phenotypes.
[4, 6]	Hormones of pregnancy play a role in 'reprogramming' immune cells to a more immunotolerant phenotype.
[7]	Modulation of innate and adaptive immunity by hCG (increased Tregs, Bregs, and anti-inflammatory cytokines).

The postulate of hCG as an immunomodulatory molecule capable of influencing conversion to a tolerogenic immunological environment has been explored in many studies (Table 2). It is associated with a decrease in cells producing pro-inflammatory cytokines and an increase in those producing anti-inflammatory cytokines. Transfection of various cell types with hCG plasmid DNA attracts T regulatory cells (Tregs), and in pregnancy there is a greater number of Tregs attracted to the placenta [11-13]. These results suggest that hCG might be used as an immunosuppressive agent in transplantation [14] because of its effect on the regulation of immune reactivity. There are several studies that demonstrate a beneficial effect of hCG on skin allograft survival in mice, although the prolongation of allograft survival was minimal (less than a week) [15-17]. These effects of hCG make the hormone an appealing subject for study in xenotransplantation.

Table 2 Evidence of effects of hCG during pregnancy.

Reference	Suggested mechanism
[8, 18]	Crucial role of hCG in zygotic implantation, Improvement of implantation rates with pretreatment of intrauterine hCG during in vitro fertilization.
[19, 20]	In vivo, exogenous hCG influences conversion to a tolerogenic immune environment by increasing Tregs and anti-inflammatory cytokines.
[13]	Recruitment of Tregs to fetomaternal interface. Lower levels of hCG are associated with spontaneous abortion.
[21]	Promotes a regulatory phenotype of B cells (Bregs).
[17, 22-24]	hCG promotes reduction of inflammation by blunting the effects of TNF-α pathway (in vitro), increasing anti-inflammatory cytokines, e.g., IL-10, IL-27, and decreasing pro-inflammatory cytokines, e.g., IL-17
[25, 26]	Promotion of dendritic cell profile associated with secretion of increased indoleamine-2,3-dioxygenase and expression of adhesion/costimulatory molecules.

The purpose of the present study was to determine whether hCG would suppress the human T cell proliferative response to wild-type (WT) pig peripheral blood mononuclear cells (PBMCs) in vitro.

2. Materials and Methods

2.1 Sources of Human PBMCs

Institutional Review Board (IRB) approval was obtained for withdrawal of blood from healthy, reproductive age female volunteers who are not on hormonal contraceptive pills (n = 3). The samples were obtained in accordance with the Declaration of Helsinki, with the informed consent of the subjects. PBMCs were isolated, as previously described [27].

2.2 Human Chorionic Gonadotropin (hCG)

Recombinant hCG (Ovidrel, Merck KGaA, Darmstadt, Germany) (250µg in 0.5ml) has been documented to be equivalent to 5000 IU urinary gonadotropin [28]. The concentration of hCG selected was 100 IU/ml, as it is the average concentration of hCG in maternal blood during second and third trimesters [29]. We also used high concentrations, i.e. 500 IU/ml and 1000 IU/ml.

2.3 CFSE Mixed Lymphocyte Reaction (MLR)

Freshly harvested human PBMCs were labeled with CFSE, and co-cultured for 6 days with irradiated WT PBMCs with or without hCG, as previously described [30]. Following staining of the cells with the Live/Dead fixable stain kit, flow cytometry was performed using antibodies to CD3 (Pacific Blue, clone SP34-2, BD Pharmingen, San Jose, CA), CD4 (PE-Cy7, clone SK3; BD Pharmingen), and CD8 (PE, clone RPA-T8; BD Pharmingen). Proliferation of subpopulations of human PBMCs was quantified by CFSE dilution (% CFSE cells). Spontaneous (responders alone) and phytohemagglutinin

(PHA, Roche, Basel, Switzerland) (final concentration 5µg/ml)-treated cells were used as negative and positive controls, respectively [30].

2.4 Statistical Analyses

Data are presented as mean and standard deviation (SD) for all variables. The statistical significance of differences was determined by non-parametric Kruskal-Wallis tests followed by Dunn's multiple comparisons test, as appropriate, using GraphPad Prism version 7 (GraphPad Software, San Diego, CA). A p value of <0.05 was considered to be statistically significant.

3. Results

Summary data (Table 3) are reported as the means of % CFSE cells ± SD (n = 3 for each group). There was no significant difference in proliferation of the $CD3^+T$, $CD4^+T$, or $CD8^+T$ cells when stimulated with irradiated wild-type pig PBMCs with or without hCG (Figure 1).

Table 3 Percentage proliferation of $CD3^+$, $CD4^+$, $CD8^+$ T lymphocytes stimulated by irradiated wild-type pig PBMCs with or without hCG (at 3 different concentrations).

Cells	Spontaneous	pPBMC+ hPBMC	pPBMC+ hPBMC+ hCG 100 IU	pPBMC+ hPBMC+ hCG 500 IU	pPBMC+ hPBMC+ hCG 1000 IU	PHA
$CD3^+T$	6.929 ± 1.018	18.25 ± 4.621	19.50 ± 6.525	20.40 ± 4.775	18.00 ± 3.928	43.29 ± 16.23
$CD4^+T$	4.929 ± 1.018	13.63 ± 2.504	15.00 ± 2.976	15.40 ± 1.949	13.88 ± 2.900	28.57 ± 17.35
$CD8^+T$	8.786 ± 0.6986	19.38 ± 4.779	17.88 ± 3.271	21.20 ± 3.962	18.88 ± 4.190	36.71 ± 15.85

hCG = human chorionic gonadotropin; hPBMC = human peripheral blood mononuclear cells; PHA = phytohemagglutinin; pPBMC = WT pig peripheral blood mononuclear cells.

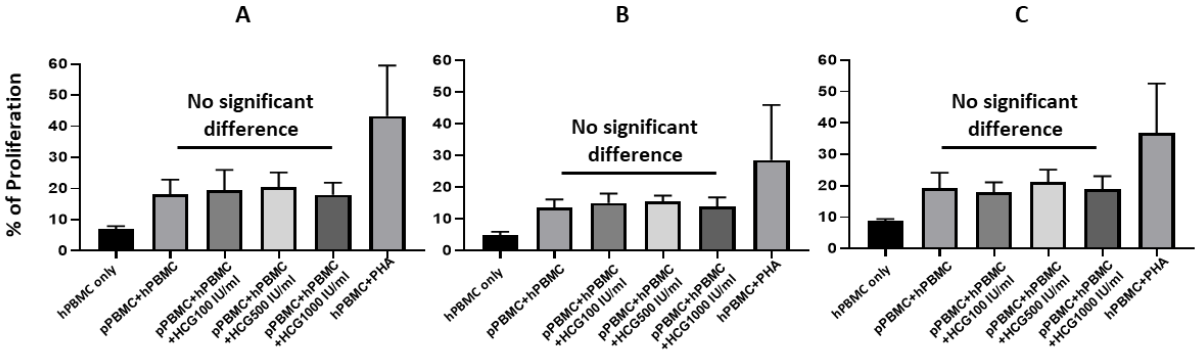

Figure 1 Percentage proliferation of (A) $CD3^+$, (B) $CD4^+$, and (C) $CD8^+T$ lymphocytes. From left to right, spontaneous (negative control), when stimulated with irradiated wild-type pig PBMC without (experimental control) or with hCG at three different concentrations or by PHA (positive control).

4. Discussion

The desirable properties of hCG, e.g., upregulation of Tregs and chemoattraction, could be useful in xenotransplantation (Table 2). A number of studies have shown a suppressive effect of crude hCG on lymphocyte proliferation [31-34], but the suppressive effect was not reproducible with purified hCG [35]. One study demonstrated that, when cultured with hCG, there was conversion of pregnancy-primed female $CD4^+FoxP3^-$T cells to $CD4^+FoxP3^+$T cells (from 4% to 7-8%), and subsequent suppression of autologous and allogeneic lymphocyte proliferation when cultured in the presence of anti-$CD3^+$, anti-$CD28^+$ and IL-2 [36].

In the present study, we were unable to detect any suppressive effect of hCG (even at the highest concentration of 1,000 IU/ml) on xenogeneic MLR. The absence of suppression of lymphocyte proliferation could be associated with an absence (or minimal number) of Tregs expansion during culture with pig PBMCs [30], even in the presence of hCG. We did not investigate Treg phenotype ($CD4^+FoxP3^+$). To increase the frequency of Tregs, PBMCs would possibly need to be incubated with hCG before setting up the MLR. However, we would have anticipated that, during the 6 days of the MLR, some suppressive effect of hCG on T cell proliferation would have been observed.

Limitations of our study include that we tested hCG from only one source, though we have no reason to believe that hCG from other sources may have been more effective. Neither did we test the effect of hCG on human antibody binding to pig PBMCs, nor on human serum cytotoxicity of pig PBMCs, but there is no evidence to suggest that hCG would have any impact in this respect.

Although this small study had a negative result, we believe that, in the light of previous studies of hCG in allotransplantation, it is important to report our data, which we believe are the first reported in a pig-to-human xenotransplantation model.

5. Conclusions

Our limited study suggests that hCG does not suppress human lymphocyte proliferation stimulated by pig PBMCs (unless this results from an increase in the number of Tregs, which we did not investigate).

Abbreviations

hCG = human chorionic gonadotropin
MLR = mixed lymphocyte reaction
PBMCs = peripheral blood mononuclear cells
Tregs = T regulatory cells

Author Contributions

AJ participated in the performance of the research, data collection, statistical analysis and in the writing of the paper. CAB and HI participated in the performance of the research and in the review of the paper. DKCC participated in research design and in writing the paper. HH participated in research design, the performance of the research, and writing the paper. HH is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Competing Interests

The authors have declared that no competing interests exist.

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