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HOW SAFE IS THE USE OF OVER-THE-COUNTER MEDICATIONS BY PREGNANT WOMEN?

QUÃO SEGURA É A UTILIZAÇÃO DE MEDICAMENTOS ISENTOS DE PRESCRIÇÃO POR MULHERES GRÁVIDAS?

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Abstract

Although it is strongly recommended that pregnant women avoid taking medication during pregnancy, some drugs are often indicated by physicians to treat their ailments. Recent studies describe the *in vitro* and *in vivo* effects of these medications, but their effects on trophoblasts cytotoxicity and maintenance/conversion of Tregs has not been studied in detail. We tested the cytotoxic effects in a mouse trophoblast cell line (SM9.1 cells), and the Treg maintenance/conversion by these cells, under the influence of 3 medications: Paracetamol, Ibuprofen, and Cephalexin. Cytotoxic effects in SM9.1 cells and changes in Treg cell maintenance/conversion were observed only at relatively high medication doses. The direct correlation between medication doses and SM9.1 cytotoxicity can contribute to deleterious effects of these medications during pregnancy, while the Treg maintenance/conversion for some medications at tested concentrations were increased. Therefore, a better understanding of how these drugs affect the physiology of different cell types is needed.

Resumo

Embora seja fortemente recomendado que mulheres grávidas evitem tomar medicamentos durante a gravidez, alguns medicamentos são frequentemente indicados por médicos para tratar seus problemas de saúde. Estudos recentes descrevem os efeitos *in vitro* e *in vivo* desses medicamentos, mas seus efeitos na citotoxicidade dos trofoblastos e na manutenção/conversão de células T reguladoras (Tregs), que são essenciais para manutenção da gravidez, não foram estudados em detalhes. Testamos os efeitos citotóxicos em uma linha de células de trofoblasto de camundongo (células SM9.1) e a manutenção/conversão de Tregs por essas células, sob a influência de 3 medicamentos: Paracetamol, Ibuprofeno e Cefalexina. Efeitos citotóxicos nas células SM9.1 e alterações na manutenção/conversão das células Treg foram observados apenas em doses relativamente altas de medicamento. A correlação direta entre as doses de medicamento e a citotoxicidade em SM9.1 pode contribuir para efeitos prejudiciais desses medicamentos durante a gravidez, enquanto a manutenção/conversão de Tregs para alguns medicamentos nas concentrações testadas foi aumentada. Portanto, é necessário um melhor entendimento de como esses medicamentos afetam a fisiologia de diferentes tipos de células.

Keywords: Ibuprofen; Tylenol; Cephalexin; trophoblast; regulatory T cell

Palavras-chave: Ibuprofeno; Tylenol; Cefalexina; trofoblasto; célula T reguladora

Introduction

Pregnancy is a fascinating process with respect to immunology because it allows fetal development without rejection. During gestation, the placenta expresses paternal antigens, but an antagonistic maternal T cell response is not observed. Therefore, some form of immunosuppression is clearly associated with pregnancy, and is the subject of many studies aimed at understanding the role of different cells and possible mechanisms.

Since regulatory T cells (Tregs) are one of the major players in tolerance (SAKAGUCHI et al., 2006), many results describe both their roles and those of dendritic cells (DCs) in tolerance induction during pregnancy (LEBER; TELES; ZENCLUSSEN, Ana C, 2010; PLAKS et al., 2008; SAMSTEIN et al., 2012). While the precise mechanisms that trigger this tolerance are still unknown, observations show that Tregs and DCs aggregate within the uterus (KALLIKOURDIS; BETZ, 2007; KAUSHIC et al., 1998; ZARNANI et al., 2006), perhaps to inhibit the stable contacts between DCs and conventional CD4 T cells, which are essential for activating the immune system (MEMPEL; HENRICKSON; ANDRIAN, VON, 2004; MILLER et al., 2004).

Despite this immunological tolerance, a controlled and local inflammation in the endometrium could be important for implantation (MOR et al., 2011). Nevertheless, if inflammatory reactions occur just after conception or during pregnancy, implantation and fetal development could be compromised, leading to abortion (HVIID et al., 2010; WOUDWYK et al., 2012). Thus, there appears to be a delicate balance between inflammation levels; small responses would allow implantation and fetal development, while strong responses would cause abortion (TADOKORO, 2012). Taken together, studies of the role of medications during pregnancy would be particularly relevant, especially for those commonly used medications for which experimental data in placental cytotoxicity and Treg biology are absent.

Many medications are often prescribed to pregnant women, some “without any restrictions” (such as Paracetamol and Cephalexin), and some after MD approval only (like Ibuprofen). Although the best scenario for the pregnant woman is to avoid taking all types of medications, they are often recommended in practice to counteract various conditions triggered by the pregnancy. As such, Paracetamol is considered to be safe for pregnant

women and has become the preferred pain reliever during pregnancy. Nonetheless, recent studies have correlated its use to disorders from autism spectrum (ANDRADE, 2016), attention deficit by hyperactivity disorder (ADHD) (FAYS, DE et al., 2015), and other neurological disorders (LIEW et al., 2016). Apart from these studies, only one examined the effects of these medications in trophoblasts, where paracetamol was shown to inhibit the placental barrier by functional alteration of ABC pumps, promoting the transport of maternal-to-fetal billi acids (BLAZQUEZ et al., 2014). It is still unknown whether this mother-to-fetus transport of billi acid is deleterious.

Often, if Tylenol™ (paracetamol) is either ineffective or causes secondary effects, the use of Ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), may be recommended by the MD, after weighing its risks. Among the collateral effects observed in pregnant women that used this medication are fetal weight loss (NEZVALOVÁ-HENRIKSEN et al., 2016), spontaneous abortion (DANIEL et al., 2014; EDWARDS et al., 2012), and congenital malformation (DANIEL et al., 2012). Apart from these findings concerning the deleterious effects to the fetus, there are only two studies about the effects of Ibuprofen in trophoblasts: that Ibuprofen affects latic acid absorption (EMOTO et al., 2002) and it inhibits leptin absorption (DUTTAROY et al., 2002). However, these studies do not provide any indication about how such alterations could affect pregnancy.

Another medication that is considered safe for pregnancy is Cephalexin, often indicated for infections in the urinary tract. The effects of this antibiotic in trophoblasts is still not reported, but it is known that Cephalexin can cross the placenta (CREATSAS et al., 1980) and it is believed to act without fetal compromise (CAMPBELL-BROWN; MCFADYEN, 1983; JEPSEN et al., 2003). However, the lack of data does not imply that it is safe, since it is possible that latent collateral effects are still to be discovered.

Given the lack of data and detailed understanding about the effect of these medications in pregnancy, this study uses *in vitro* protocols to investigate whether these three medications (Ibuprofen, Paracetamol, and Cephalexin) could compromise trophoblast survival, as well as Treg maintenance by these cells.

Materials and methods

1. Mice

All procedures agreed with the rules established by the National Council for Control on Animal Experimentation (Concea), which supervises animal use by educational/research institutions through Institutional Ethics Committees for Animal Uses (CEUA). Therefore, our project was submitted and approved by the "*Universidade Vila Velha*" CEUA (protocol number 387/2016). The experiments used primary cell cultures from the thymuses and spleens of 4-week-old female Swiss mice. These mice were euthanized with 20 μL ketamine (10 mg/mL) and separated into different experimental groups (n=5/experiment). All mice were previously kept in SPF conditions in ventilated racks (Alesco Ltd., Brazil).

2. Cell cultures

In this study, the trophoblast cell line SM9.1 and the primary cell cultures, obtained from the thymuses and spleens of the Swiss female mice described above, were used. Each cell was cultivated/maintained as follows.

The SM9.1 cells (the trophoblast cell line established from pregnant Swiss mice at gestational day 9) were cultured in 25 or 75 cm^2 culture flasks (Greiner Bio-One, Germany), with complete RPMI-1640 medium (HEPES at 25 mM, L-glutamine 2 at mM, penicillin at 100 U/mL, streptomycin at 0,1 mg/mL, sodium pyruvate at 0,11 mg/mL, sodium bicarbonate at 2 mg/mL, 2-mercaptoethanol at 5 mM, and fetal calf serum at 10%). All reagents were acquired from Sigma-Aldrich Inc. USA. These cells were kept at 37º C, in a humidified chamber with 5% CO_2, for 2 to 3 days, when they reached 80 – 90 % confluence. At this time, these cultures were split in new culture flasks by removal with a trypsin incubation (0.25%, up to 5 min of incubation at 37 ^oC; gentle tap, flask wall wash for 2 to 3 times with a complete RPMI medium, centrifugation at 390 G). For the cytotoxicity tests, each well of a 96 well-plate received 2 x 10^4 cells, with 100 μl of complete RPMI medium.

For the thymus and spleen primary cell cultures, each female Swiss mouse was euthanized by intravenous injection of ketamine (20 μL/mice; stock at 10 mg/mL). The Thymus and spleen were removed, ground in PBS, and each cell suspension transferred to 15 ml tubes (Greiner One Inc., Germany), for centrifugation (390 G, 10 min, 4^oC). After that, the supernatants were removed, the cells were suspended in complete RPMI medium, and counted in a Neubauer chamber. After counting, cell suspension concentrations were adjusted to 3x10^6 and seeded over or not SM9.1 cells. Although this basic protocol was used for both the thymus and spleen cell suspensions, the spleen cell suspension was submitted

to a red blood cell lysis protocol just after the first centrifugation. For this, each spleen cell pellet was suspended in a 0.5 ml solution of red cell lysis buffer (8,26g NH_4Cl; 1,19g $NaHCO_3$; 200µL EDTA 0,5M; 100mL distillated water, pH 7,3), incubated at room temperature for 1 to 5 min, and centrifuged at 390 G, 10 min, 4°C. After this centrifugation, the protocol was identical to the protocol described for the thymus cell suspensions.

3. MTT Viability test

To evaluate the medication cytotoxicity of the SM9.1 cells, different drug doses were applied to the SM9.1 cell cultures. All medications were administered at 50 mg/ml with a 1:2 dilution in the following cell culture wells. DMSO at 0.5 % was used as a solvent since it has no cytotoxic effect on SM9.1 cells at this concentration (data not shown). All medications, as well as DMSO, were acquired from Sigma-Aldrich Inc., USA.

An MTT viability test (MOSMANN, 1983) was used, where 100 µL of a MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Dyphenyltetrazolium Bromide) solution (5 mg/mL) was seeded on top of $2x10^4$ SM9.1 cells/well. These cells were plated 48 h prior to MTT incubation and, 24 h before MTT, they received all medication doses. All cell cultures were incubated with MTT for 4 h, when supernatants were harvested and 100 µl DMSO was added to each well to solubilize formazan crystals. Absorbance were read at 595 nm in a spectrophotometer (Celer Ltd., Brazil). As a positive control for 100% cell death, incubation with 50 µl DMSO was used and, as a negative control for cell death, 50 µl complete RPMI medium was added to each well.

4. Analysis of Treg maintenance/conversion

To evaluate the medication effects on Treg maintenance/conversion by SM9.1 cells, 10^6 SM9.1 cells/well were plated in 24-well plates and, 24 h later, $3x10^6$ thymus or spleen cells were added to each well. According to our MTT viability assays, we determined two medication doses: Paracetamol, 6.2 (low) and 50.0 mg/ml (high) (<[Para] and >[Para], respectively); Ibuprofen, 3.1 and 6.2 mg/ml (<[Ibup] and >[Ibup], respectively); and Cephalexin, 6.2 and 12,5 mg/ml (<[Ceph] and >[Ceph], respectively). The low medication doses correspond to the first dose where no cytotoxic effect was observed, and the high medication doses corresponds to approximately 50% of SM9.1 cells were dying.

Phytohemaglutinin (PHA; Sigma-Aldrich Inc., USA) 5 μg/ml was used to stimulate thymus and spleen cell cultures. Unstimulated cell cultures received complete RPMI only. Each plate was incubated for 24, 48, and 72 h, at 5% CO_2, 37 °C, humidified chamber.

5. Flow cytometry

To evaluate the amount of Treg cells, each sample of the thymus/spleen cells co-cultured with SM9.1 cells was incubated with fluorophore labeled monoclonal antibodies (mAb) and read in a flow cytometer. Therefore, each sample was harvested and initially incubated with Fc block for 20 min, 4ºC. After that, each sample received the following fluorophore-labelled mAb from BD Biosciences Inc., USA: anti-CD4 (cat. # 12-0441-83), anti-CD8 (cat. # 12-0081-83), e anti-CD25 (cat. # 12-0251-82), for 30 min, 4ºC, in a dark chamber. After that, each sample was centrifuged for 5 min, 390 G, 10 °C, and 100 µL/sample of *cytofix/cytoperm* buffer (BD Bioscience Inc., USA) was added for 30 min, RT, in a dark chamber. A new centrifugation round was performed and 100 µL/sample of an permeabilization buffer (PBS, 1% paraformaldehyde (PFA), 0,5% Tween-20) was added, for 30 min, RT, in a dark chamber. After a new centrifugation round, 100 µL/sample of anti-Foxp3 mAb (eBioscience Inc., USA, cat. # 12-5773-82) was added for 1h, RT, dark chamber. A final centrifugation round was performed, and each sample was resuspended in FACS buffer containing 1% PFA and stored at 4 ºC until reading in a cytometer (FACS Calibur, BD Biosciences Inc., USA).

6. Statistical analysis

To compare the cytotoxic effects of different medications in SM9.1 cells, as well as medication effects on the Treg maintenance/conversion, analysis of variance (ANOVA) with Tukey correction was performed in Treg indexes. These indexes were calculated by conversion of Treg percentages found during flow cytometry analysis and they include values between 0 to 1, which correspond to 0 to 100% of Tregs, respectively. Values were considered statistically different when $p < 0.05$. All graphics and statistical analysis were performed in a Prism Graphics 5.0 software (GraphPad Software Inc., USA).

Results and Discussion

1. Cytotoxic effect of Paracetamol, Ibuprofen, and Cephalexin on SM9.1 cells

Although the use of medications during pregnancy should be avoided, there are cases where their use is recommended. However, few data are available about their effect on trophoblast cell lines (BLAZQUEZ et al., 2014; DUTTAROY et al., 2002; EMOTO et al., 2002). Therefore, we first evaluated the medication effects on SM9.1 cell death. For this, these cell cultures were incubated with different medication concentrations and an MTT viability test was performed 24 h after these stimulations (Figure 1).

When Paracetamol was added to these cell cultures (Figure 1a), doses from 50.0 to 12.5 mg/ml were toxic for these cells, while doses lower than 6.2 mg/ml had no effect. It was already described that Paracetamol can alter trophoblast physiology, (BLAZQUEZ et al., 2014), although nobody knows if this negatively affects the fetus. Nevertheless, it is assumed to be safe for use by pregnant women.

For our studies of Ibuprofen (Figure 1b), doses of 50.0 to 6.2 mg/ml had a negative effect on SM9.1 cell viability; while doses lower than 3.1 mg/ml seemed to have no effect on this trophoblast cell line (Figure 1b). Two previous studies have shown that Ibuprofen can also alter trophoblast physiology (DUTTAROY et al., 2002; EMOTO et al., 2002), but did not address how these alterations could affect pregnancy.

Finally, the results with Cephalexin show that for concentrations up to 12.5 mg/ml (Figure 1c) all doses were toxic to the SM9.1 cells and doses lower than 3.1 mg/ml had no cytotoxic effect.

For all the drugs tested, the concentration levels used were high (Figure 1); in all experiments, the doses were always in mg/ml levels, but the amount of effective medication available after intake is on the order of μg/ml, 1000x smaller than our doses. Therefore, it is hard to believe that any drug tested would affect trophoblasts *in vivo*. But cytotoxicity is only one of the many ways these drugs could interfere with pregnancy, and therefore further studies are needed to address their safe use.

2. Medication effects on Treg maintenance/conversion by SM9.1 cells

If all 3 tested drugs only had deleterious toxic effects on trophoblasts at very high doses, this would not imply that they couldn't also interfere with other important biological process during pregnancy. One well-known biological process that must occur in a successful pregnancy is the maintenance and/or conversion of Tregs. It has been established that trophoblast cell lines can induce/maintain Treg cells *in vitro* (FRACCAROLI et al., 2009; POLOSKI et al., 2016; RAMHORST et al., 2012) and, to the best of our knowledge, no data is available about medication interference in this process. Therefore, we studied whether commonly administered drugs (Paracetamol, Ibuprofen, and Cephalexin) could cause any disturbance in Treg maintenance/conversion in co-cultures between thymocytes/spleen cells and SM9.1 cells (mouse trophoblast cell line).

When we evaluated Paracetamol effect on thymocytes (Figure 2, a and c), we could observe that any tested dose has no effect on Treg maintenance/conversion by SM9.1 cells, in 24 or 48 h cell cultures. However, when we evaluated the effect of Paracetamol on spleen cells (Figure 2, b and d), we observed a reduction in Treg numbers, 48 h after incubation with this drug (Figure 2d). Taken together, these results indicate that Tregs from different organs can respond differentially to the presence of each drug. In fact, one study describes thymic naturally-occuring Tregs (nTregs) compared to peripherally-induced Tregs (iTregs), in the presence of anti-apoptotic mechanisms (SINGH et al., 2015). According to these authors, the nTregs expressing Helios had a higher expression of anti-apoptotic proteins than Neuropilin-1+ nTregs (SINGH et al., 2015). It is also interesting that independent of the Paracetamol dose used, the same pattern was observed (Figure 2).

This absence of a dose-response effect with Paracetamol was not observed in cell cultures stimulated with Ibuprofen (Figure 3). There, high Ibuprofen doses were always deleterious for Tregs as they were for SM9.1 cells (Figure 1), and low doses increased the amount of Tregs in cultures where thymocytes were seeded (Figure 3, a and c). Spleen cells under the same conditions did not alter their amount of Tregs (Figure 3, b and d). It is tempting to assume this low Ibuprofen dose has a small proliferative effect on SM9.1 cells (Figure 1b), which influences Treg maintenance/conversion, or this Ibuprofen dose has itself proliferative effects on nTregs. In either case, it is important to note that Ibuprofen it is not the first choice for treating women with problems that can be treated with Paracetamol. This raises a question: how is an increase in Treg numbers, induced by small Ibuprofen dose, deleterious to fetal implantation and maintenance?

Finally, we also tested Cephalexin effect on Treg cells (Figure 4), revealing interesting results. At 24 h, high Cephalexin doses induced an increase in Treg numbers, in cultures

with SM9.1 cells and thymocytes (Figure 4a) or spleen cells (Figure 4b). Moreover, in 24 h co-cultures with thymocytes (Figure 4a), low Cephalexin doses were also capable to induce and increase in Treg numbers. In 48 h cell cultures (Figure 4, c and d), thymocyte cell co-cultures only were able to increase their Treg numbers with low Cephalexin doses (Figure 4c); high Cephalexin doses in these same conditions were deleterious for Tregs (Figure 4c). Increase in Treg numbers were not seen in co-cultures with spleen cells and Cephalexin (Figure 4d). Considering the relatively safe use of Cephalexin, it is interesting to observe this medication has a positive effect on Tregs.

Conclusions

In conclusion, we have shown that different doses of 3 types of drugs can affect the cell viability of a trophoblast cell line (SM9.1 cells) and the amount of Tregs these cells can maintain/induce. If we consider the safety of each medication together with our results, it becomes clear that a better understanding of what parameters these drugs alter in different cells types during pregnancy are needed. Also, studies with very low medication doses, but during longer times of cultures, are needed to understand chronic effects of these drugs.

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Figures

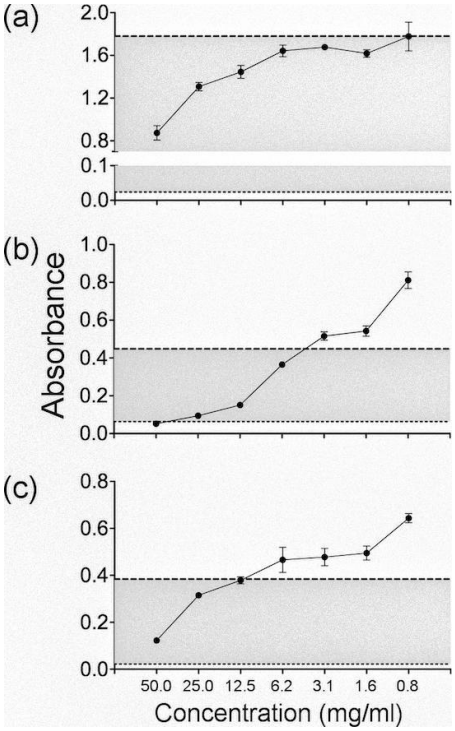

Figure 1 – Cytotoxic effect of medications on trophoblast cell cultures. Cell cultures of trophoblast cell line SM9.1 were grown in the presence of medications for 24 h and subjected to a MTT viability test. (a) SM9.1 cells cultured with Paracetamol. (b) SM9.1 cells cultured with Ibuprofen. (c) SM9.1 cells cultured with Cephalexin. The traced and dotted lines represent the average absorbance of unstimulated live cells (viability positive control) and cells killed by DMSO (viability negative control). Each point represents the average and standard deviation of quadruplicates of culture. These results are representative of 2 – 3 independent experiments.

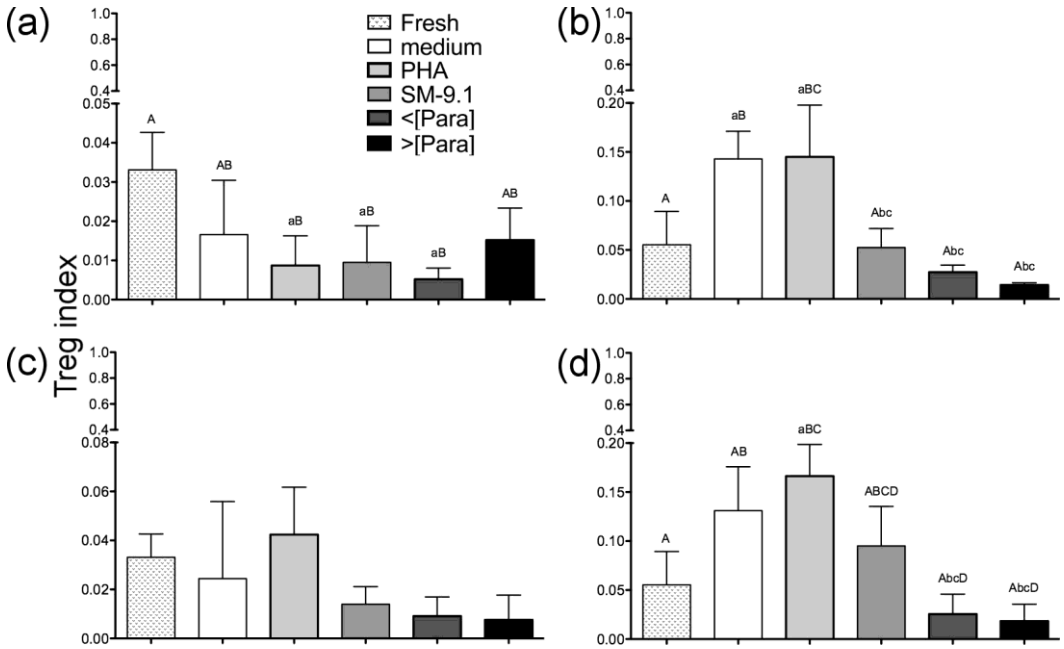

Figure 2 – Paracetamol effect on Treg maintenance/conversion by SM9.1 cells. Thymocytes os spleen cells from Swiss female mice were cultured for a total of 48 h, with or without SM9.1 cells, and with or without 2 different doses of Paracetamol (>[Para] = 50.0 mg/ml; <[Para] = 6.2 mg/ml). (a) Thymocytes with Paracetamol for 24 h. (b) Spleen cells with Paracetamol for 24 h. (c) Thymocytes with Paracetamol for 48 h. (d) Spleen cells with Paracetamol for 48 h. Each column represents the average and standard deviation of triplicates of culture. Equal letters represent no difference while different letters represent difference among groups. These results are representative of 2 independent experiments.

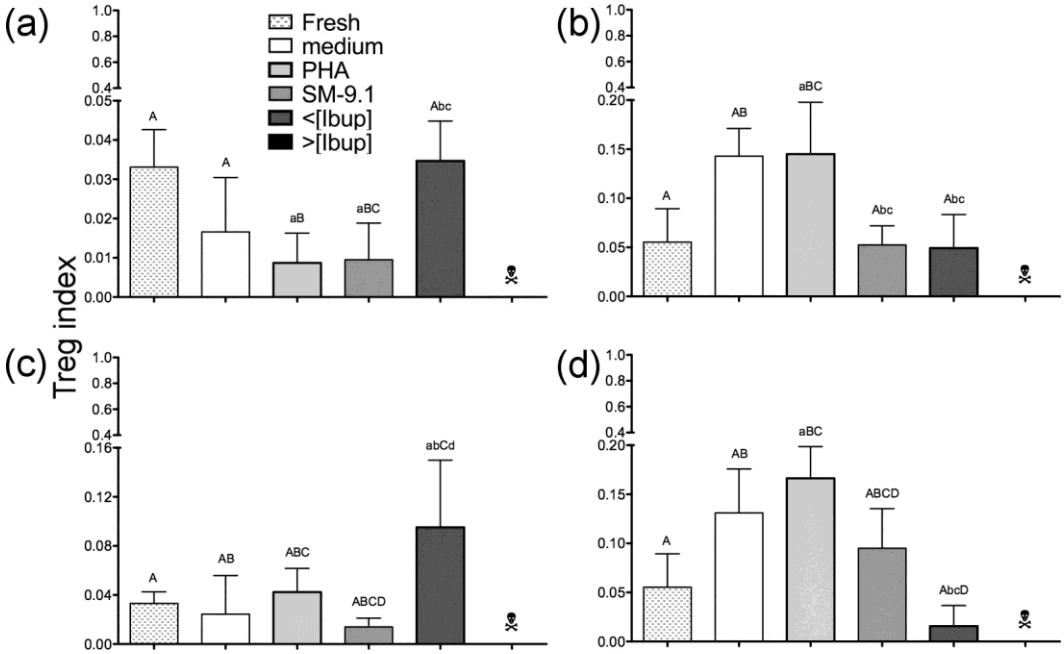

Figure 3 – Ibuprofen effect on Treg maintenance/conversion by SM9.1 cells. Thymocytes os spleen cells from Swiss female mice were cultured for a total of 48 h, with or without SM9.1 cells, and with or without 2 different doses of Ibuprofen (>[Ibup] = 6.2 mg/ml; <[Ibup] = 3.1 mg/ml). (a) Thymocytes with Ibuprofen for 24 h. (b) Spleen cells with Ibuporfen for 24 h. (c) Thymocytes with Ibuprofen for 48 h. (d) Spleen cells with Ibuprofen for 48 h. Each column represents the average and standard deviation of triplicates of culture. Equal letters represent no difference while different letters represent difference among groups. These results are representative of 2 independent experiments.

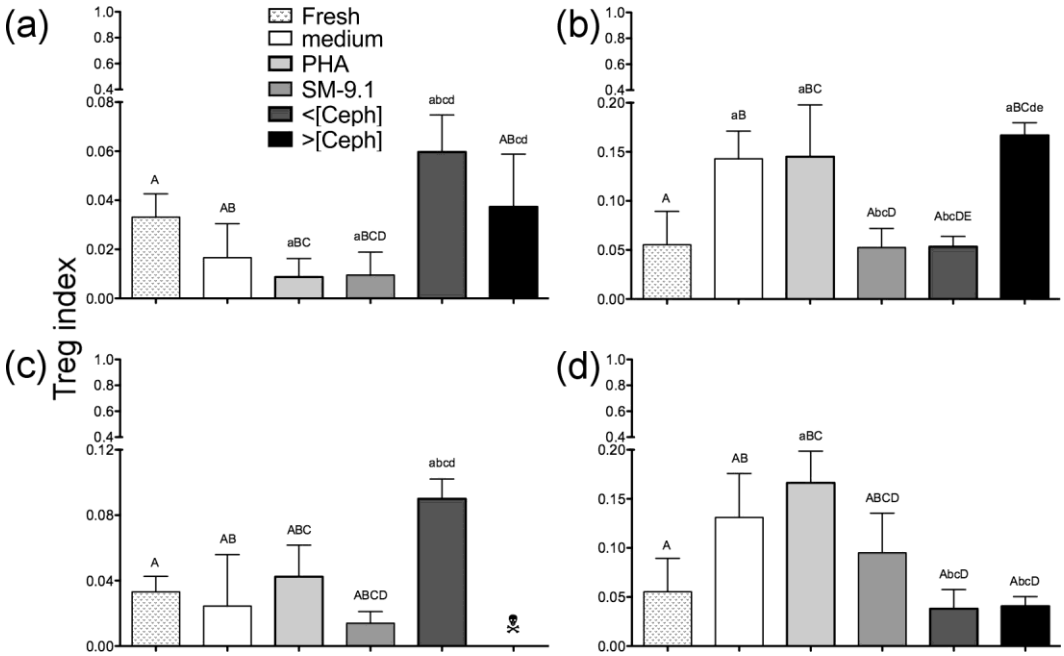

Figure 4 – Cephalexin effect on Treg maintenance/conversion by SM9.1 cells. Thymocytes os spleen cells from Swiss female mice were cultured for a total of 48 h, with or without SM9.1 cells, and with or without 2 different doses of Cephalexin (>[Ceph] = 12,5 mg/ml; <[Ceph] = 6,2 mg/ml). (a) Thymocytes with Cephalexin for 24 h. (b) Spleen cells with Cephalex for 24 h. (c) Thymocytes with Cephalexin for 48 h. (d) Spleen cells with Cephalexin for 48 h. Each column represents the average and standard deviation of triplicates of culture. Equal letters represent no difference while different letters represent difference among groups. These results are representative of 2 independent experiments.