Corso di Dottorato di Ricerca in Scienze della Vita e dell'Ambiente, Ciclo XXXVIII

Unveiling the Mystery of Endocannabinoid system: Its Role in Human Bone Homeostasis and Male Reproduction

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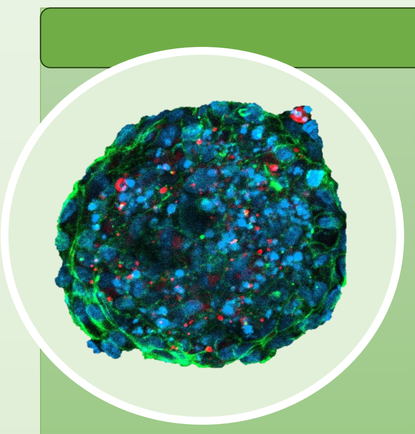
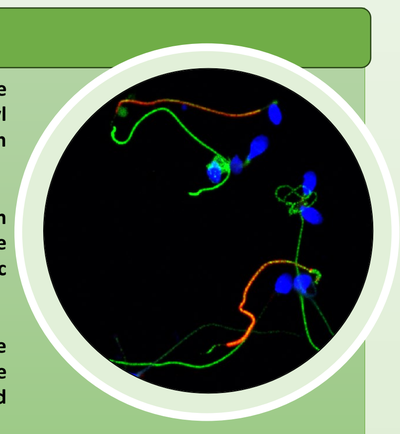
BACKGROUND



Endocannabinoids are lipid-signal molecules that are endogenous ligands for cannabinoid receptors, and together with enzymes responsible for their synthesis and degradation, they form the endocannabinoid system (ECS). ECS plays an important role in physiological and pathological processes in the human body. The two best characterized endocannabinoids are N-arachidonoyl ethanolamine (AEA, anandamide) and 2 arachidonoyl glycerol (2-AG). The endocannabinoids bind to and activate their target receptors, mainly CB1 and CB2, causing several biological effects on different target cells.

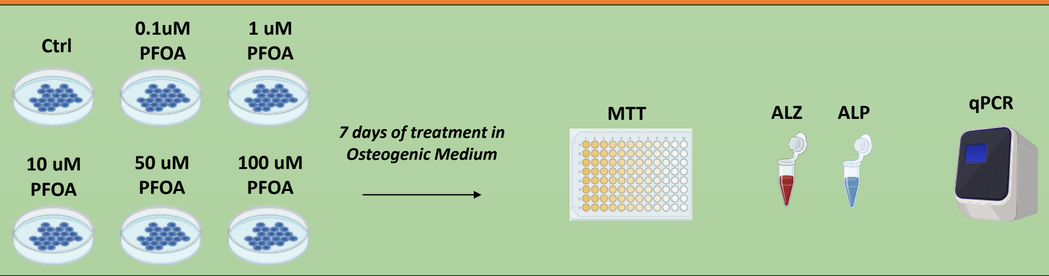
Following the discovery of ECS, many studies about its expression and function in male reproductive system have been carried out. All the components of the ECS have been identified in mammalian germ cells, from spermatogonia to spermatozoa, in the reproductive fluids and tracts. Evidence has been accumulated that endocannabinoid concentrations and alterations in their levels affect the functioning of spermatozoa. In this context, the effects of AEA and its agonist ACEA through CB1 on sperm capacitation, acrosome reaction, DNA damage and epigenetic changes in normospermic (NZS) and asthenozoospermic (ASZ) donors were investigated.

In addition, several evidences are available about the ECS presence in bone and synovial tissues and its important role in bone metabolism. Endocannabinoids have been shown to regulate bone formation, bone loss and bone turnover and they could alleviate the development of arthritis, prevent osteoporosis (OP), inhibit bone tumor cell proliferation, reduce bone cancer pain and improve fracture healing. Recently, it has been observed that the ECS could be a target of endocrine disruptor chemicals (EDCs). Our aim is to assess the effect of PFOA as EDC on bone proliferation and differentiation in 2D and 3D hFOB1.19 cell model to elucidate the involvement of ECS in bone homeostasis and its alteration triggered by EDC exposure.



BONE

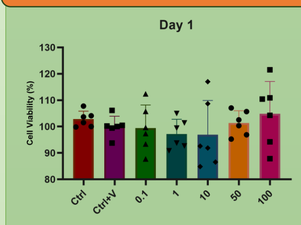
Experimental Design 2D



Aim

To assess the toxicity of PFOA on bone homeostasis and its ability to deregulate the ECS during the osteoblast hFOB1.19 differentiation phase

Results



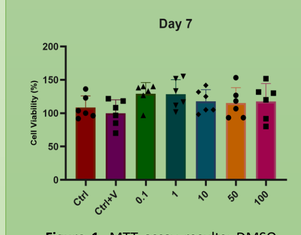


Figure 1. MTT assay results: DMSO treatment as positive control (Ctrl+V) at the concentrations of 0.02% in day 1 and in day 7. The human fetal osteoblast (hFOB1.19) were treated with concentration of 0.1, 1, 10, 50 and 100 uM for 7 days in ostegenic medium.

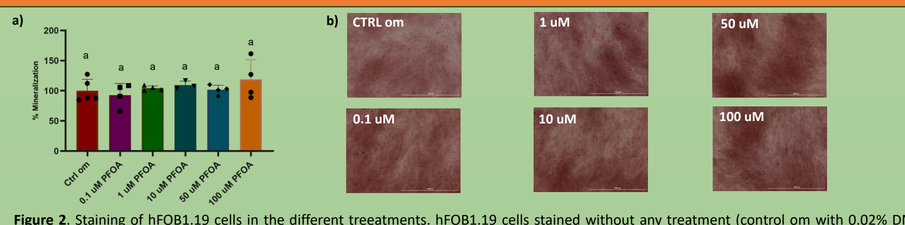


Figure 2. Staining of hFOB1.19 cells in the different treeatments. hFOB1.19 cells stained without any treatment (control om with 0.02% DMSO) and treated with 0.1, 1, 10, 50 and 100 uM PFOA. **a)** Preliminary quantitative analysis of the AR concentration indicative of the ECM mineralization following the different treatments by measuring the absorbance using spectroscopy. The graph shows the mean ± standard deviation. One-way ANOVA was used to analyzed the differences among the groups. **b)** Representative images of the different treatments applied to osteblastic cells for 7 days after AR staining.

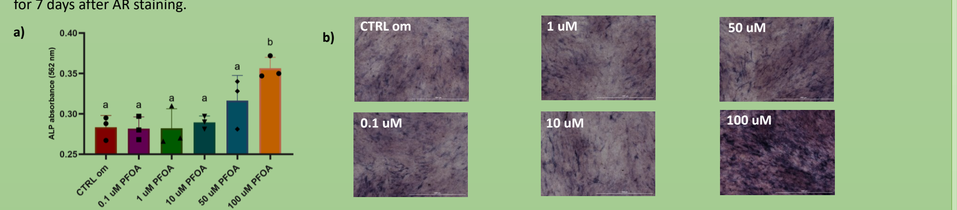


Figure 3. Staining of hFOB1.19 cells in the different treeatments. hFOB1.19 cells stained without any treatment (control om with 0.02% DMSO) and treated with 0.1, 1, 10, 50 and 100 uM PFOA. **a)** Preliminary quantitative analysis of the ALP concentration indicative of the ECM mineralization following the different treatments by measuring the absorbance using spectroscopy. The graph shows the mean ± standard deviation. One-way ANOVA was used to analyzed the differences among the groups.. **b)** Representative images of the different treatments applied to osteblastic cells for 7 days after ALP staining.

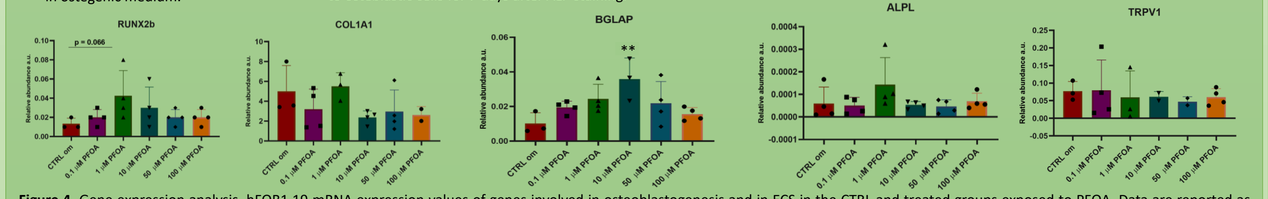
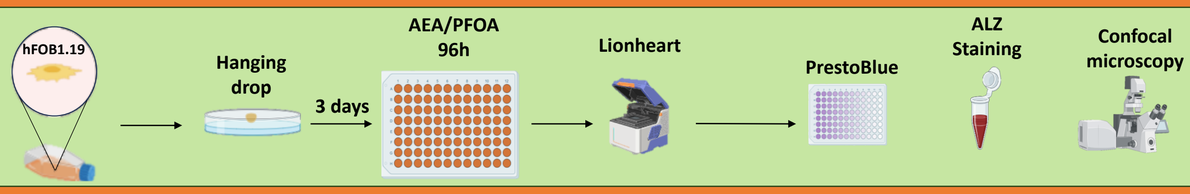


Figure 4. Gene expression analysis. hFOB1.19 mRNA expression values of genes involved in osteoblastogenesis and in ECS in the CTRL and treated groups exposed to PFOA. Data are reported as mean ± SD. One way ANOVA was used to analyzed the difference between the groups. ** P<0.002

Conclusions

- **The PFOA delays the differention phase acting on ALP production and *RUNX2b* gene expression**
- **The PFOA alters the mineralization process through the upregulation of *BGLAP* gene expression**

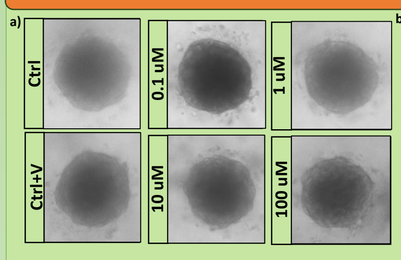
Experimental Design 3D



Aim

To assess: a) toxicity of PFOA in bone homeostasis and in the ECM deposition and its involvement in ECS deregulation in the human fetal osteoblast spheroids (3D); b) the effects of endogenous cannabinoid Anandamide (AEA) on bone homeostasis and ECM deposition during differentiation phase in 3D cells

Results



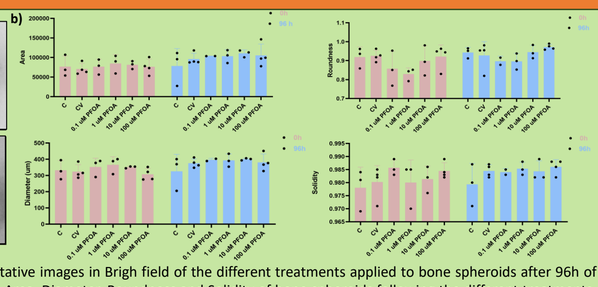


Figure 1. Bone spheroids treated with PFOA. **a)** Representative images in Brigh field of the different treatments applied to bone spheroids after 96h of PFOA exposure **b)** Preliminary quantitative analysis of the Area, Diameter, Roundness and Solidity of bone spheroids following the different treatments. The graph show the mean and standard deviation as error bars. One-way ANOVA was used to analyzed the difference between the groups..

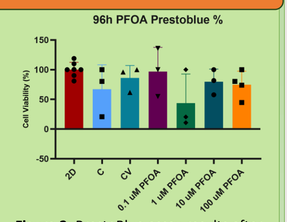
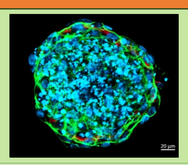


Figure 2. PrestoBlue assay results after 96h: DMSO treatment as positive control (CV) at the concentrations of 0.02%. Spheroids were treated with concentration of 0.1, 1, 10 and 100 uM PFOA for 96h

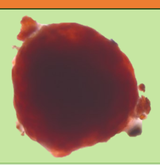
Conclusions

- **Preliminary results evidence that PFOA exposure does not alter the viability and morphology of bone spheroids**

Future prospectives

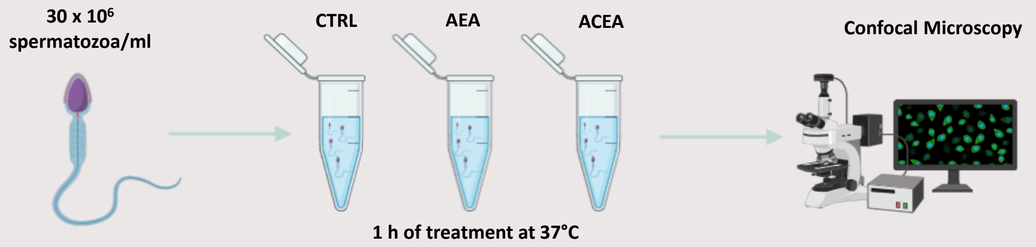


SCAN ME



REPRODUCTION

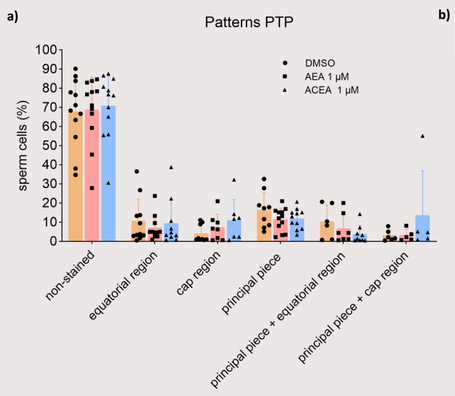
Experimental Design



Aim

To assess whether CB1 is involved in human sperm capacitation, acrosome reaction, apoptosis and H4 acetylation in both normospermic (NZS) and astenozoospermic (ASZ).

Results



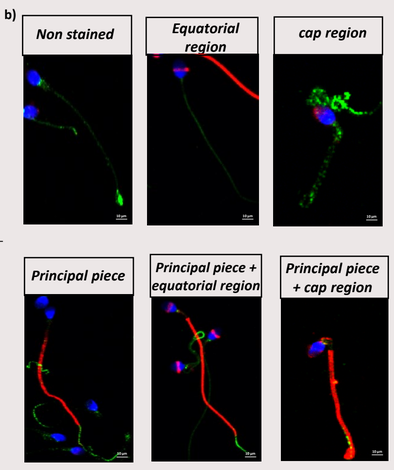


Figure 1. **a)** Representative graph of six PTP patterns in human spermatozoa. **b)** Representative immages of eight PTP patterns in human spermatozoa that were detected employing PyT antibody (red). Nuclei were counterstained with DAPI (blue)

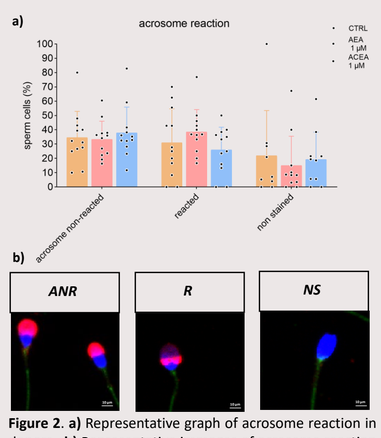


Figure 2. **a)** Representative graph of acrosome reaction in donors. **b)** Representative immages of acrosome reaction in human spermatozoa that were detected employing PNA staining (red). Nuclei were counterstained with DAPI (blue)

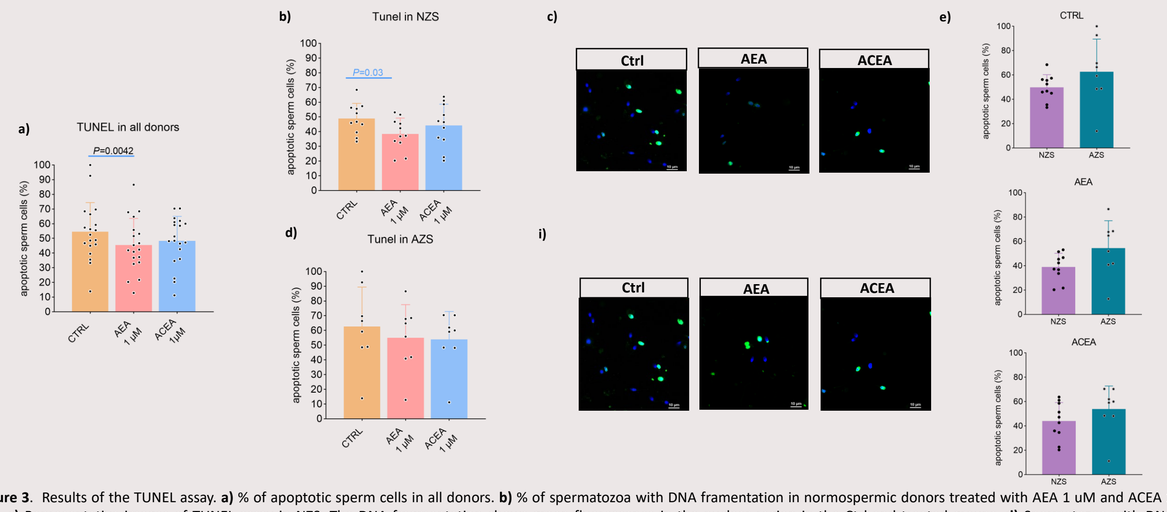


Figure 3. Results of the TUNEL assay. **a)** % of apoptotic sperm cells in all donors. **b)** % of spermatozoa with DNA framentation in normospermic donors treated with AEA 1 uM and ACEA 1 uM. **c)** Representative images of TUNEL assay in NZS. The DNA fragmentation shows green fluorescence in the nuclear region in the Ctrl and treated groups. **d)** Spermatozoa with DNA fragmentation in asthenozoospermic donors treated with AEA 1uM and ACEA 1 uM. **i)** Representative images of TUNEL assay in AZS. The DNA fragmentation shows green fluorescence in the nuclear region in the Ctrl and treated groups. e) % of apoptotic sperm cells between NZS and AZS donors in the CTRL, AEA and ACEA groups. Positive TUNEL: green, DAPI:blue. Scale bar, 10 µm. Nested t test. $p < 0.05$, dots representing the number of sample (N) per group of donors and per treatment.

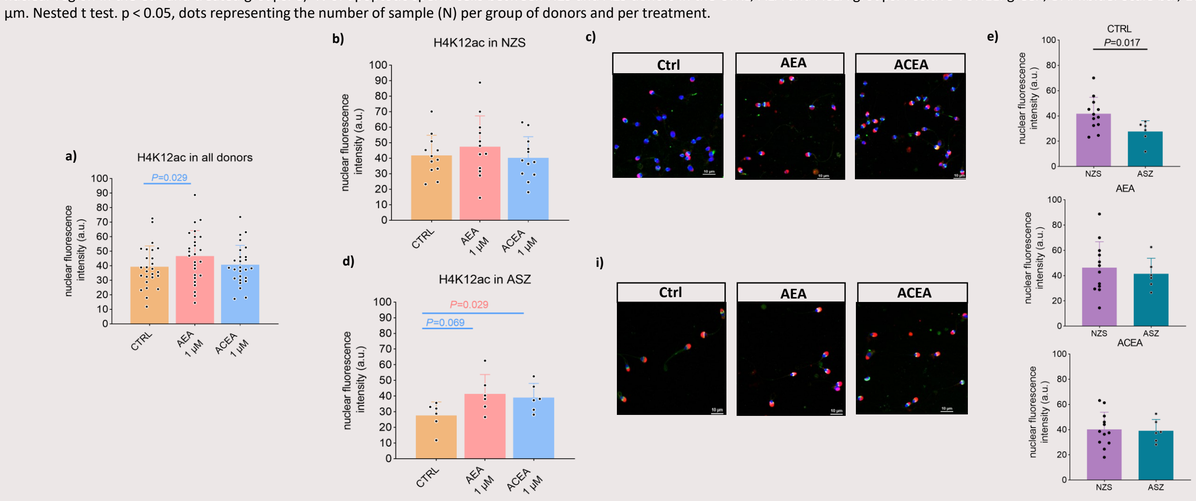


Figure 4. Histone modifications of the H4K12ac in human spermatozoa. **a)** nuclear fluorecence intensity (a.u.) of H4K12ac signal in spermatozoa of all donors. **b)** H4K12ac signal intensity of normospermic donors treated with AEA 1 uM and ACEA 1 uM. **c)** Representative images of H4K12ac immunostaining in the Ctrl and treated groups of NZS donors. **d)** nuclear fluorescence intensity (a.u.) of asthenozoospermic donors treated with AEA 1uM and ACEA 1 uM. **i)** Representative images of H4K12ac immunostaining in the Ctrl and treated groups of NZS donors. e **)** nuclear fluorecence intensity (a.u.) of H4K12ac signal in spermatozoa NZS and AZS donors in the CTRL, AEA and ACEA groups. H4K12ac: green, sperm nucleus_ DAPI, blue, Acrosome: PNA,red. Scale bar, 10 µm. Data were analyzed with RM one-way ANOVA, and the Welch's test. $p < 0.05$.

Conclusions

- **AEA and ACEA do not influence human spermatozoa capacitation**
- **In NZS spermatozoa the treatment with AEA reduces the apoptotic sperm cells suggesting that the regulation of AEA signalling may be associated with apoptosis pathway in human spermatozoa.**
- **In ASZ spermatozoa the epigenetic modification of H4K12ac is significantly increased when treating the human spermatozoa with AEA and ACEA 1 uM. Interestingly, H4K12ac level is lower in AZS than in NZS, the treatment with AEA and ACEA increases the H4K12ac level to that of NZS suggesting the involvement of CB1 in human sperm histone 4 acetylation.**