EFFECT OF ESTRADIOL AND PROGESTERONE ON OVINE AMNIOTIC EPITHELIAL CELLS.

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Introduction

Amniotic-derived epithelial cells (AECs), an emerging source of fetal stem cells, has recently attracted the attention of researchers for their great regenerative potential.[1,2] Because of their fetal origin ,these cells exhibit elevated proliferation rates and plasticity, as well as, immune tolerance and anti-inflammatory properties, making AECs suitable for both allogenic and xenogenic transplantation.[3,4]

Methods

Treatments and differentiation : oAECs were cultured as previously reported[5] and treated with 12.5µM and 25µM of E2 or P4, alone or in both combination, for three passages. Untreated cells were marked as control (CTR). At 70% confluency, cells were detached for doubling time (DT) evaluation. Cells at fourth passage were differentiated for 21 days in osteogenic media (DM)[6] without steroid treatment. Alizarin Red and Alcian-Blue staining's were performed.

Real Time PCR : RNA and cDNA was obtained as previously reported.[3] Real Time for *NANOG*, *SOX2* ,*OCT4* stemness genes, ACAN, COL2A1 and SOX9 chondrogenic and OCN osteogenic genes expression was performed by SensiFast SYBR using specific primers.[6] Protocol was: 5 min at 95°C, 30 cycles at 95°C for 15 sec, 60°C for 30 sec, 72°C for 15 sec. Comparative Ct $2^{-\Delta\Delta C(t)}$ normalized to *GAPDH* was applied.

Immunohistochemical (IHC) Analysis. IHC analyses were carried out for Cytokeratin 8 and αSMA expression as previous report.[5]

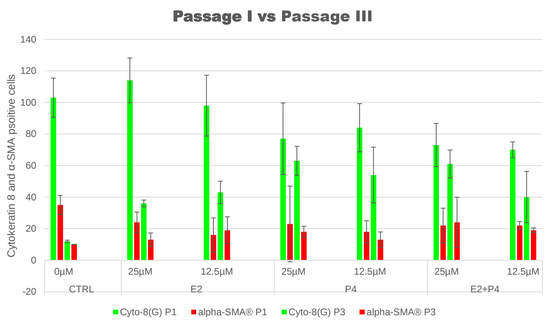
Statistical analysis. Data expressed as mean (±SD), compared by one-way ANOVA followed by Tukey's test (GraphPad Prism 5). Significant values for $p < 0.05$.

Results

A **Doubling Time**

STEROIDS	DOSAGES	PASSAGE I	PASSAGE II	PASSAGE III
CTRL	0µM	36.06	20.13	14.67
ESTRADIOL(E2)	25µM	31.93	19.98	13.48
	12.5µM	29.28	19.08	16.11
PROGESTERONE(P4)	25µM	35.90	20.29	15.38
	12.5µM	36.64	21.55	16.27
E2+P4	25µM	35.64	19.33	14.44
	12.5µM	32.37	20.49	15.26

B **Cytokeratin and α-SMA expression**



C **Stemness Gene Profile**

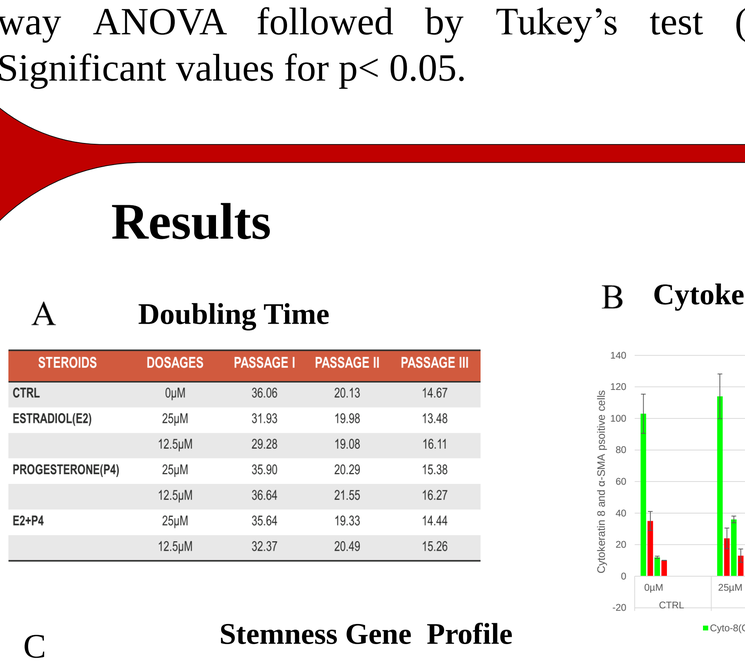
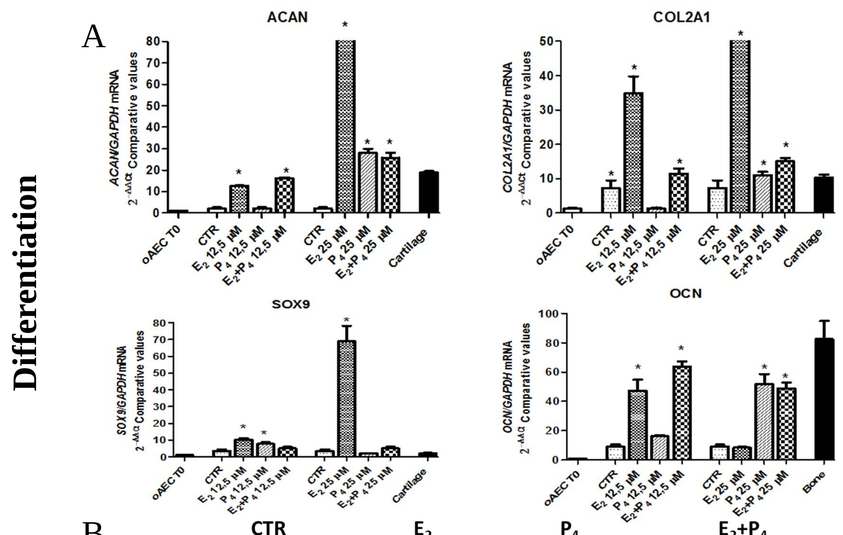


Fig.1. A, Evaluation of proliferation (doubling time) of cells treated with 12,5µM and 25µM Steroids concentration until Passage 3 of culture in growth medium.
*B, Fluorescence Quantification of Cytokeratin 8 and αSMA positive cells after Steroid treatments at Passage 1 and Passage 3. * P<0.05 vs CTRL.*
C, Real Time PCR for OCT4 ,SOX2 and NANOG stemness gene expressions for 12.5µM and 25µM steroid concentration until Passage 3 of culture.
** P<0.05 vs CTR*

A



B

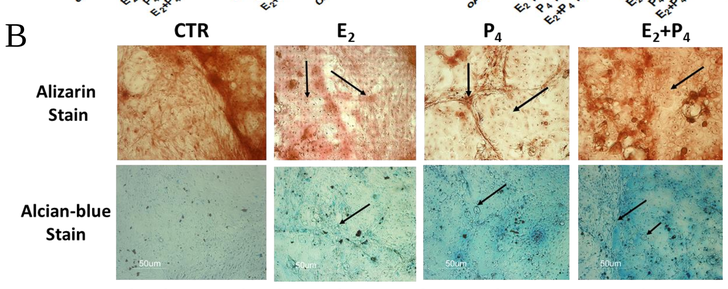


*Fig. 2. A, Real Time of Chondrogenic and Osteogenic gene expression in CTR and steroids pre-treated oAECs at 4th Passage of cell culture and in osteogenic media. * P<0.05 vs AEC T0. 2.B, Alizarin Red and Alcian-Blue positive osteogenic and chondrogenic cells for 25µM steroid pre-treated cells. Scale bar 50µm.*

Results

Steroids treated ovine AECs proliferate with a significant differences between concentrations (Fig.1A).

While P4 treated cells showed cuboidal shape and Cytokeratin expression until third passage, CTR shows a rapid downregulation of the proteins. E2 treated cells also show a rapid downregulation of Cytokeratin along with an unchanging αSMA expression. oAECs with E2+P4 showed both cell type morphology (Fig.1B).

Steroids modified stemness genes depending on the concentration. 12.5 µM E2, 25µM P4 and 25µM of both E2+P4 treatments maintained higher *OCT4*, *NANOG* and *SOX2* expressions in treated cells despite their progressive downregulation in the CTR (Fig1C).

Moreover, differently to CTR mineralization observed after Alizarin staining, steroids pretreated cells suffer morphological change under osteogenic media acquiring Alcian-Blue positive chondrogenic-like morphology as confirming by the induction of specific *ACAN*, *COL2A1* and *SOX9* chondrogenic genes expression (Fig.2**)**

Conclusions

AECs stemness properties and plasticity can be modified by prolonged high dosage of steroids treatment. These data improves our knowledge thus opening new prospective on AEC use in stem cell-based therapy.

Future Work

- Confirming presence of chondrogenic genes like
- 3D cell culture

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Acknowledgments

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