UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
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REGULACIÓN DE LAS ENZIMAS CYP11A1, CYP17A1, CYP19A1, 3β-HSDI Y 17β-HSD3 POR EL CALCITRIOL EN CULTIVOS DE TROFOBLASTO HUMANO

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PRESENTA:
NANCY NOYOLA MARTINEZ

DIRECTOR DE TESIS
DR. DAVID BARRERA HERNÁNDEZ
FACULTAD DE MEDICINA
COMITÉ TUTOR
DR. MARCO ANTONIO JUAREZ OROPEZA
FACULTAD DE MEDICINA
M. en C. CRISTINA LEMINI GUZMAN
FACULTAD DE MEDICINA

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Este trabajo está dedicado a las personas más importantes de mi vida, mi familia.

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ABREVIATURAS

°C	grados Celsius
17β-HSDI	17beta-hidroxiesteroide deshidrogenasa tipo 3
3β-HSDI	3beta-hidroxiesteroide deshidrogenasa I
AMPc	adenosin monofosfato cíclico
CYP	citocromo P450
CYP11A1	citocromo P450scc
CYP17A1	17β-hidroxilasa/17,20 liasa
CYP19A1	aromatasa
D.E.	desviación estándar
DHEA	deshidroepiandrosterona
DMEM-HG	medio Dulbecco alto en glucosa
cDNA	ácido desoxirribonucleico complementario
E_2	estradiol
g	gramos
h	hora
HBSS	solución salina balanceada de Hank
hCG	gonadotropina coriónica humana
HSD	hidroxiesteroide deshidrogenasa
kDa	kilodaltones
L	litro
LDL	lipoproteínas de baja densidad
mg	miligramo
min	minutos
mL	mililitro
p/v	relación peso/volumen
P_4	progesterona
PBS	solución amortiguadora de fosfatos
PREG	pregnenolona
qPCR	reacción en cadena de la polimerasa en tiempo real
RNA	ácido ribonucleico

RNAm	RNA mensajero
RPM	revoluciones por minuto
SDHEA	sulfato de deshidroepiandrosterona
SFT	suero de feto de ternera
SPREG	sulfato de pregnenolona
TBS-T	solución salina tamponada con tris y polisorbato 20
Vh	vehículo
μM	micromolar
μL	microlitro

RESUMEN

La placenta es un órgano temporal, con estructura discoidal-hemocorial formada principalmente por células deciduales de origen materno y por células de trofoblasto de origen fetal. Este órgano lleva a cabo una gran cantidad de funciones; entre ellas se encarga del metabolismo esteroidogénico. Dicha función cobra relevancia; ya que el desequilibrio en la producción de hormonas esteroides está asociado con complicaciones en el embarazo. Las hormonas esteroides son producidas por enzimas que se dividen en dos grandes grupos; las hidroxiesteroide deshidrogenasas (HSDs) y los citocromos P450 (CYPs). Considerando que la síntesis de esteroides depende del correcto funcionamiento de sus enzimas, la relevancia de estudiar los compuestos que las regulan ha sido de interés para nuestro grupo de trabajo. El calcitriol regula la producción de péptidos, citocinas y hormonas en los trofoblastos, además modifica la expresión y la actividad de algunas enzimas esteroidogénicas de manera tejido especifico. Sin embargo, los efectos del calcitriol sobre la expresión de las enzimas involucradas en la síntesis de esteroides sexuales en la placenta humana, aún no han sido totalmente estudiados. Por lo cual, el principal objetivo de este estudio fue evaluar el efecto del calcitriol sobre la expresión de 3 citocromos (CYP11A1, CYP17A1 y CYP19A1) y 2 deshidrogenasas (3β-HSDI y 17β-HSD3) en cultivos primarios de trofoblastos de placenta humana. Para cumplir este propósito, se obtuvieron células de placentas de mujeres gestantes clínicamente sanas, con un embarazo normoevolutivo a término y sometidas a cesárea. La expresión génica y proteica se evaluó por RT-qPCR y por “western blot”, respectivamente. Los resultados obtenidos mostraron que: 1) la expresión basal de todas la enzimas se incrementó significativamente durante la diferenciación de citotrofoblastos a sinciciotrofoblastos ($p<0.05$); 2) la expresión génica de la 3β-HSDI en sinciciotrofoblastos fue mayor en comparación con las demás enzimas ($p<0.05$); 3) el calcitriol estimuló la expresión génica del CYP11A1, del CYP19A1 y de la 17β-HSD3 a las 3 h del tratamiento, mientras que la 3β-HSDI se indujo a las 6 h ($p<0.05$). sin embargo, también se observó en los resultados una variable dependiente del tiempo; 4) la abundancia proteica del CYP11A1 y de la 3β-HSDI no se modificó significativamente por el calcitriol, no obstante, la expresión del CYP19A1 se inhibió 24 h después del tratamiento. En conclusión, el calcitriol afecta de manera dependiente del tiempo y del fenotipo celular la expresión de las enzimas que metabolizan esteroides en los trofoblastos de placenta humana.

ABSTRACT

Placenta is a temporary organ with hemochorial-discoidal structure; it is shaped by decidual cells from mother and trophoblast cells from fetal. Placental tissue is necessary for hormones steroids production, they are synthetized through two kind of enzymes; the hydroxysteroid dehydrogenase (HSD) and the P450 cytochromes (CYP). Keeping the steroid biosynthesis in adequate rate to guarantee the pregnancy. Several works shown that unbalanced sex steroids production is associated with pregnancy diseases; this information highlighted the importance to evaluate the factors that regulate the enzymes involved in hormonal product. The calcitriol modulate peptides, cytokines, and hormones production in trophoblast cells. In addition, calcitriol regulate the expression and activity of several steroidogenic enzymes, in a tissue specific manner. Nonetheless, the effects of calcitriol on the expression of enzymes involved in steroid biosynthesis in the human placenta has not been totally studied. So, the first goal of this work was investigated the effect of calcitriol on the gen and protein expression of steroidogenic enzymes; CYP11A1, CYP17A1, CYP19A1, 3β-hyidroxysteroid dehydrogenase type I (3β-HSDI) and 17β-HSD3 in trophoblast cells cultured from human placenta. Trophoblast cells were obtained from placentas collected after cesarean section of healthy pregnant women, who had normal term pregnancy. The effects of calcitriol at transcriptional and protein levels were evaluated by real time RT-qPCR, and Western blot, respectively. The results showed that: 1) basal expression of all enzymes was increased significantly when the cytotrophoblasts was differentiated to syncytiotrophoblast ($P < 0.05$); 2) from basal expression values of the five genes studied, 3β-HSDI had the higher expression ($P < 0.05$); 3) the presence of calcitriol in cultured trophoblast cells generally resulted in a stimulatory effect on CYP11A1, CYP19A1 and 17β-HSD3 gene expression at 3 h of treatment; whereas 3β-HSDI was induced at 6 h ($P < 0.05$). However, a time-dependent effects were also observed; 4) protein expression of CYP11A1 and 3β-HSDI were not modified significantly by calcitriol, however that of CYP19A1 was regulated in similar fashion as gene expression. In conclusion, calcitriol affected in a time dependent manner the expression of steroid metabolizing enzymes in cell cultures derived of human placental.

1 INTRODUCCIÓN

1.1 Generalidades de la placenta humana

La placenta es un órgano temporal, con estructura discoidal-hemocorial formada principalmente por células deciduales de origen materno y por células de trofoblasto de origen fetal [1]. Los trofoblastos son la unidad funcional de la placenta que forman estructuras conocidas como troncos vellosos o vellosidades coriónicas, cuya función es mantener activo el intercambio de nutrimentos y gases entre la madre y el feto, así como la depuración de los desechos fetales [2].

Los trofoblastos pueden ser vellosos o extravellosos. El trofoblasto extravelloso es el encargado de invadir el tejido endometrial y de remodelar las arterias espirales del útero materno [3, 4] mientras que el trofoblasto velloso sigue un proceso de diferenciación de células independientes llamadas citotrofoblastos que al fusionarse entre sí, forman estructuras multinucleadas denominadas sinciciotrofoblastos, los cuales proveen a la madre de las cantidades necesarias de hormonas esteroides para mantener el embarazo. Generalmente, las hormonas esteroides se catalogan en 5 grupos principales llamados; progestinas, andrógenos, estrógenos, glucocorticoides y mineralocorticoides, entre ellos las progestinas, los andrógenos y los estrógenos son considerados esteroides sexuales, mientras que los dos últimos como adrenales.

Cabe mencionar que entre la semana 8 y 10 de la gestación los trofoblastos placentarios aportan a la circulación materna la mayor cantidad de estrógenos así como de la progestina llamada progesterona (P_4). Además, estas células son capaces de producir más de 100 péptidos y hormonas peptídicas que tienen un papel central en la gestación [5, 6].

Aunque, los glucocorticoides y los mineralocorticoides tienen una participación fundamental en la maduración pulmonar fetal, la placenta no expresa a las enzimas requeridas para su síntesis [7]; por lo anterior, este trabajo se enfocó en

estudiar las principales enzimas involucradas en la biosíntesis de las progestinas, los andrógenos y los estrógenos.

1.2 Estructura de las hormonas esteroides

Las hormonas esteroides, son moléculas que comparten como característica estructural un núcleo tetracíclico denominado ciclopentanoperhidrofenantreno, formado por tres ciclohexanos y un ciclopentano. A partir de este núcleo las hormonas difieren entre sí, principalmente por el número de átomos de carbono unidos a su estructura de cuatro anillos, en sus grupos funcionales y en su estado de oxidación [8], lo cual les confiere diferente afinidad a sus receptores específicos para ejercer múltiples funciones biológicas.

La biosíntesis de las hormonas esteroides ocurre a partir del colesterol, una molécula de 27 átomos de carbono. A partir del colesterol, los esteroides son producidos por medio de enzimas que se dividen en dos grupos, los citocromos P450 (CYPs) y las hidroxiesteroide deshidrogenasas (HSDs), enzimas que se localizan en la membrana mitocondrial y/o en la membrana del retículo endoplásmico, sitios donde ocurren oxidaciones o reducciones específicas para la biosíntesis esteroidal [8].

1.3 Biosíntesis de las hormonas esteroides en la unidad feto-placenta-madre

En una mujer embarazada, la P_4 y los estrógenos son los principales esteroides producidos por los trofoblastos. Sin embargo, para su síntesis requieren del colesterol proveniente de la madre así como de la aportación de los andrógenos biosintetizados por el feto. El colesterol se obtiene de las lipoproteínas de baja densidad (LDL) maternas, las cuales reconocen a su receptor específico en la placenta, quien las internaliza, libera al colesterol y lo traslada hacia la mitocondria, lugar donde se encuentra el citocromo P450scc (CYP11A1). Este citocromo rompe la cadena lateral del colesterol para dar origen a una molécula de 21 átomos de carbono llamada pregnenolona (PREG). La PREG formada, sirve de sustrato para la enzima 3beta-hidroxiesteroide deshidrogenasa I (3β-HSDI), quien

isomeriza el doble enlace en posición Δ5 a Δ4 y ocurre la deshidrogenación del grupo hidroxilo del carbono 3 convirtiéndolo en grupo cetónico, dando lugar a la P_4 [9, 10].

Mediante una ruta alterna, parte de la PREG producida en los trofoblastos pasa a las adrenales del feto, lugar donde las sulfotransferasas sulfo-conjugan a la molécula esteroidal en el carbono 3, formando sulfato de pregnenolona (SPREG) [7]. Posteriormente, el SPREG es biotransformado en sulfato de dehidroepiandrosterona (SDHEA) por acción de la 17β-hidroxilasa/17,20 liasa (CYP17A1), que hidroxila al SPREG en el carbono 17 con la subsecuente pérdida de 2 átomos de carbono. A partir del SDHEA, por hidroxilación en el carbono 16, se forma al sulfato de 16α-SDHEA, que junto con el SDHEA restante se trasladan a la placenta para ser sustratos de la 3β-sulfatasa, de la 3β-HSDI y de la 17β-HSD, dando lugar a la biosíntesis de los andrógenos: androstendiona y testosterona que son sustrato inmediato de la aromatasa (CYP19A1), la cual elimina el grupo metilo unido al carbono 10 y aromatiza el anillo A de la molécula esteroidal para dar lugar a los correspondientes estrógenos; estrona, estradiol (E_2) y estriol [11] (ver figura 1).

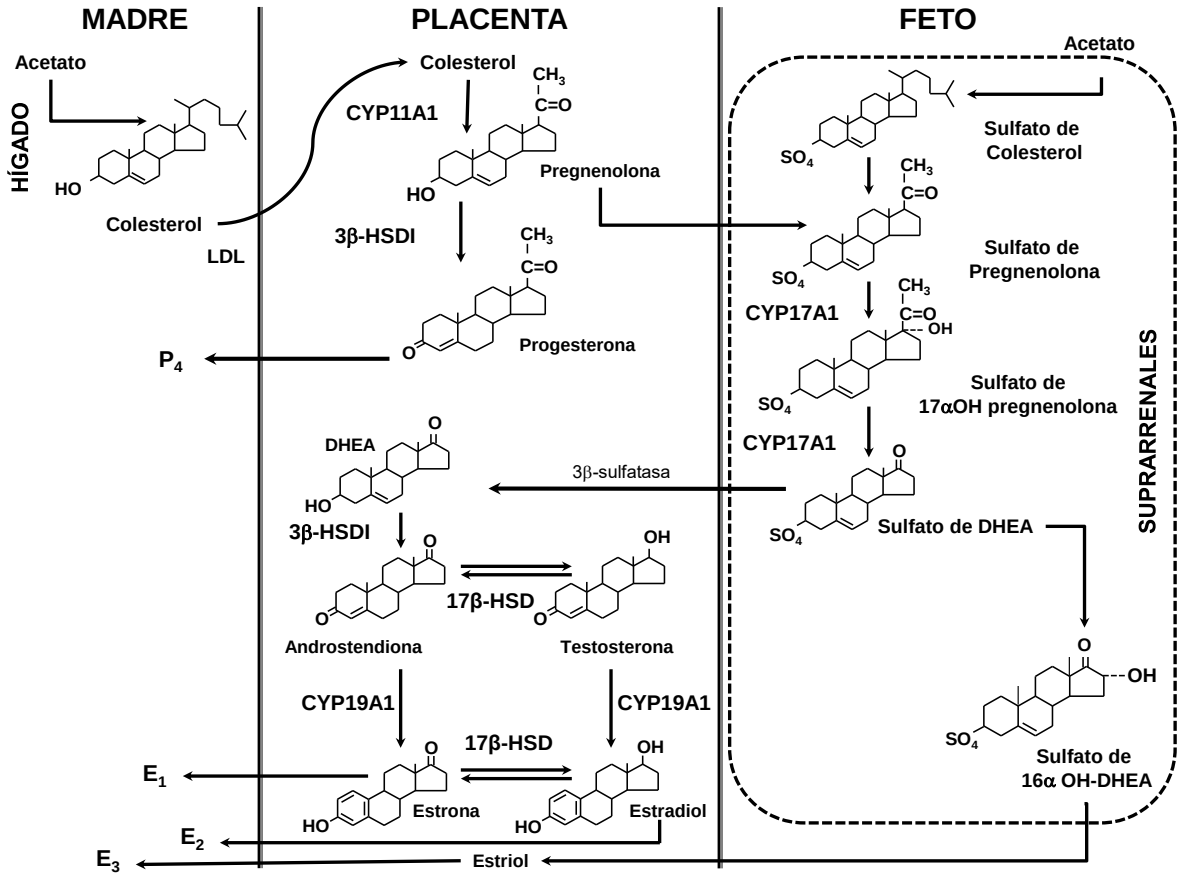

Figura 1. Biosíntesis clásica de las hormonas esteroides sexuales en la unidad madre-placenta-feto. Esquema modificado de Strauss, J. F. [12].

Desde la década de los años 60, se demostró que el tejido placentario expresa abundantemente al CYP11A1, a la 3β-HSDI y al CYP19A1; las enzimas clave en la biosíntesis de progestinas y estrógenos, mientras que la presencia del CYP17A1 encargado de la biosíntesis de los andrógenos, ha generado gran controversia. En efecto, reportes previos indicaban que la placenta era incapaz de biosintetizar andrógenos debido a la ausencia del CYP17A1 [13-15]. No obstante, Loganath y cols., en el año 2002, encontraron que explantes de placenta de 10 a 12 semanas de gestación pueden producir DHEA a partir de PREG [16]. Al siguiente año, Pezzi y cols., detectaron la expresión del gen del CYP17A1 en la placenta [17]. Asimismo, Escobar y cols., mostraron tanto el transcrito como la actividad del CYP17A1 en cultivos de trofoblastos y en la línea celular de coriocarcinoma JEG-3 [18] (ver figura 2).

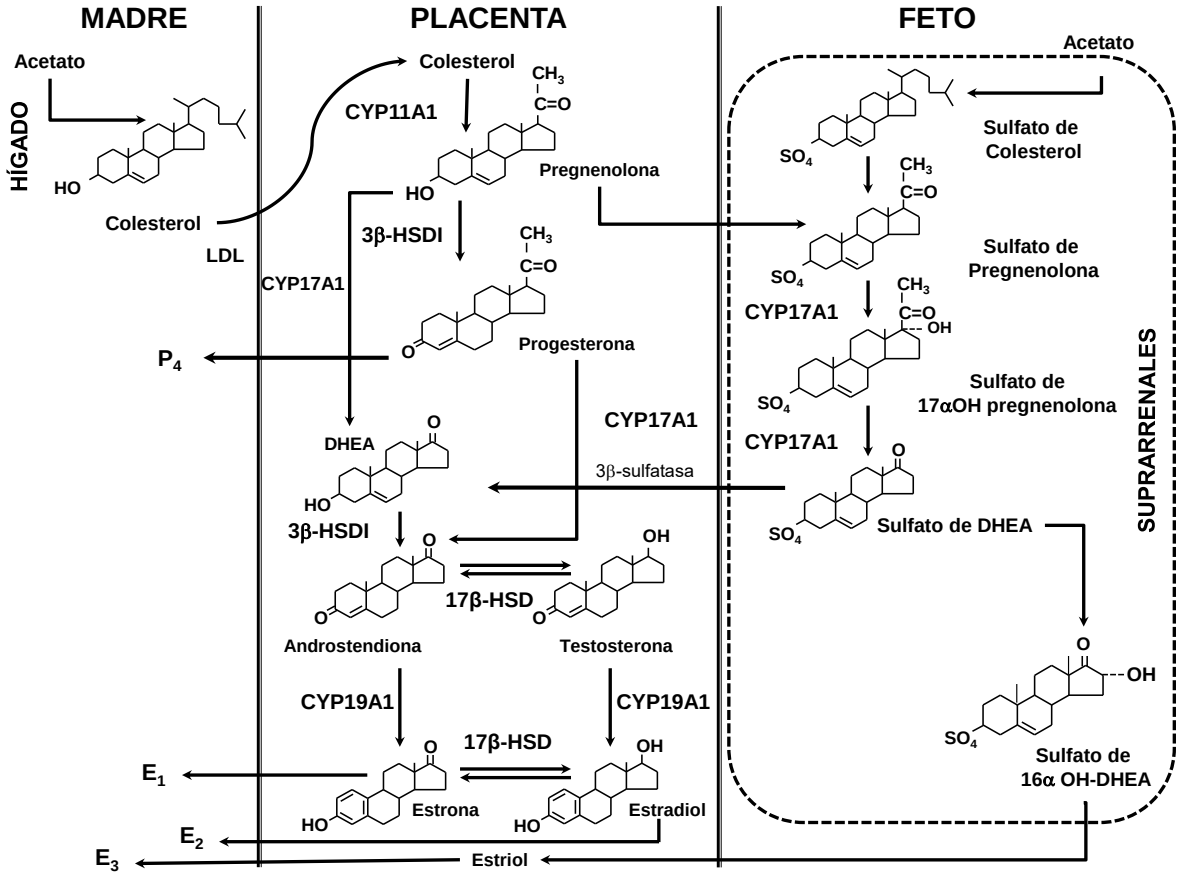

Figura 2. Propuesta más reciente de la biosíntesis de las hormonas esteroides sexuales en la unidad madre-placenta-feto. Esquema que incluye la presencia del CYP17A1 en el tejido placentario.

Lo anterior, modificó el concepto relacionado con la nula producción de andrógenos en la placenta. Sin embargo, la concentración generada aún es desconocida, por lo tanto, este es un tema que requiere más estudios para determinar la posibilidad de que parte de los andrógenos biosintetizados directamente en este tejido pudieran ejercer alguna función biológica localmente o en la interfase materno-fetal antes de ser biotransformados a los correspondientes estrógenos, aunque esta posibilidad existe, son necesarias más evidencias experimentales para corroborarlo.

1.4 Funciones de la progesterona, los andrógenos y el estradiol en el embarazo

En Trabajos pioneros realizados por Tulchinsky y cols., se demostró que las concentraciones de P_4 y E_2 en el suero materno se incrementan paulatinamente durante todo el embarazo, y que las concentraciones séricas óptimas de estas hormonas oscilan entre los 150-175 ng/mL para la P_4 y entre 15-17 ng/mL para el E_2 [19, 20]. En cuanto a los andrógenos, se sabe que su concentración fluctúa entre 1 y 5 ng/mL [21-24].

La P_4 es considerada como el esteroide preponderante para el mantenimiento de la gestación [19, 25]. Entre sus funciones esta modular el sistema inmunológico materno, favoreciendo la tolerancia hacia el alo-injerto fetal. A este respecto, la P_4 favorece la biosíntesis de citocinas anti-inflamatorias como la interleucina (IL)-3, la IL-4 y la IL-10 en los linfocitos T maternos [26]. Además, de que estimula la expresión génica de las moléculas HLA-G en la línea celular JEG-3, con lo que evita la activación de las células NK uterinas [9]. Por otra parte, esta hormona dilata las arterias del útero de la madre lo que favorece el flujo sanguíneo y disminuye la tensión arterial [10]. La P_4 también incrementa la síntesis del óxido nítrico en las células endoteliales de la vena umbilical [27] y media la invasión de los trofoblastos a las arterias espirales del útero materno inhibiendo la actividad de las metaloproteinasas de matriz [28]. Asimismo, favorece la decidualización del endometrio cuando incrementa la expresión de HOXA10, creando un ambiente propicio para la implantación del embrión [29]. Adicionalmente, la P_4 disminuye la producción de las prostaglandinas E2 y F2α, así como la formación de uniones comunicantes o “gap” en el miometrio materno mientras que incrementa la actividad de la sintetasa de óxido nítrico en el útero [26, 30]. Al mismo tiempo, la P_4 junto con otras progestinas disminuye la actividad contráctil del miometrio humano [31], manteniendo así la quiescencia del útero para favorecer la implantación y el desarrollo del feto.

Aunque durante el embarazo los andrógenos son considerados únicamente como precursores de los estrógenos, estudios realizados en modelos animales y humanos mostraron que la DHEA, la testosterona y algunos metabolitos 5α- y 5β-reducidos, inhiben la contractilidad uterina [21, 32], participan en la maduración del cérvix al término de la gestación y modulan la vasculatura materna a través de la inducción de óxido nítrico [15, 16]. También, la testosterona favorece el tono vascular promoviendo la proliferación de las células endoteliales [17]. Por otra parte, en células de placenta la testosterona inhibe la síntesis de estradiol, la expresión de la aromatasa y la del receptor de estrógenos [33]. De manera interesante, este andrógeno inhibe e incrementa al CYP27B1 y al CYP24A1 respectivamente, citocromos implicados en el metabolismo del calcitriol [34].

Por su parte los estrógenos tienen efectos sobre los vasos maternos y fetales a través de la inducción de óxido nítrico, favorecen la angiogénesis en la placenta al incrementar la expresión del factor de crecimiento del endotelio vascular, la síntesis de conexina 43 y la formación de uniones comunicantes en el miometrio [28, 30, 35]. Por otra parte, los estrógenos estimulan la expresión del CYP11A1 en los trofoblastos humanos, la expresión del receptor de lipoproteínas de baja densidad (LDL) en la placenta de primates no humanos [36-38], así como la secreción baso lateral y apical de la apoB y el LRP2 (miembro de la familia proteica relacionada al receptor LDL) en células BeWo [39]. Por su parte, Lou y cols., sugirieron que el E_2 al activar a la quinasa regulada por glucocorticoides y suero (SGK1) suprime la apoptosis mediada por lipopolisacáridos y promueve la respuesta anti-inflamatoria TH2 en células estromales de la decidua contribuyendo al éxito del embarazo [40]. Previo al trabajo de parto el E_2 incrementa la concentración del receptor de oxitocina y de agentes α-adrenérgicos, modula la apertura de canales iónicos dependientes de calcio en la membrana celular y además estimula la expresión de los receptores de las prostaglandinas E2 y F2α en el útero para inducir el trabajo de parto [30, 41].

Los antecedentes nos muestran la gran importancia de los esteroides desde el comienzo, durante y al término de la gestación, en la cual se requieren cantidades adecuadas de esteroides para que se lleven a cabo todas las funciones descritas para garantizar el éxito del embarazo.

Lo anterior toma relevancia debido a que diversos estudios muestran que el desequilibrio en la producción de esteroides, antes de la concepción y/o durante el embarazo, está asociado con patologías como los abortos, la preeclampsia (PE), el síndrome de HELLP y los partos prematuros [42], con lo cual se destaca la importancia de investigar con mayor profundidad los factores que regulan la esteroidogénesis en la placenta humana.

1.5 Regulación de las enzimas encargadas de la biosíntesis de esteroides sexuales en la placenta humana

La producción de los esteroides depende de la biodisponibilidad de sus precursores, así como de la expresión, regulación y funcionalidad de las enzimas esteroidogénicas. En particular, en los trofoblasto, la expresión y la actividad de las enzimas son modificadas por factores presentes dentro del microambiente placentario, así como por variaciones espacio-temporales. Por lo anterior, la regulación de la expresión del gen, de la proteína o de la actividad de las enzimas involucradas es muy compleja y merece ser estudiada con mayor atención.

Los factores que modifican la síntesis hormonal han sido motivo de estudio por varios grupos de investigación, los cuales han demostrado que diversos compuestos naturales o sintéticos exógenos como pesticidas, entre otros, afectan tanto la expresión como la actividad de las enzimas esteroidogénicas en la placenta humana [33, 43-55]. En particular, nuestro grupo de trabajo se ha enfocado en el estudio de los efectos del calcitriol en la regulación de diferentes moléculas producidas por los trofoblastos, incluyendo su impacto sobre la síntesis de hormonas tanto glicoproteicas como esteroides (Tabla 1).

Tabla 1. Factores que regulan la expresión y/o actividad de las enzimas esteroidogénicas en la placenta humana

Factor	Blanco	Efectos	Células	Ref.
P_4 y E_2	3β-HSDI CYP11A1	Incrementan mRNA sin cambiar la abundancia proteica	Citotrofoblastos	[43]
E_2	CYP19A1	Incrementa mRNA	Sinciciotrofoblastos	[55, 56]
T	CYP19A1	Disminuye mRNA y producción de E_2	JEG-3	[33]
Calcitriol	CYP19A1	Incrementa mRNA y actividad enzimática	JEG-3	[57]
		Incrementa la producción de P_4 y E_2	Sinciciotrofoblastos	[46]
hCG y LH	CYP11A1	Incrementa mRNA	JEG-3	[58]
Prolactina		Incrementa y disminuye la secreción de P_4 y E_2, respectivamente	Explantes placentarios	[59]
IL-1α e IL-1β	CYP19A1	Incrementa actividad enzimática y producción de estrógeno	Citotrofoblastos	[60]
IL-1β		Incrementa producción de P_4	TCL-1	[49]
TNF-α		Disminuye producción de P_4 e incrementa la de E_2	JEG-3	[50]
		Incrementa producción de P_4		[61]
	3β-HSDI CYP19A1	Disminuye mRNA	Citotrofoblastos	[47]
TGF-β1		Disminuye producción de P_4 y E_2	JEG-3 y NPC	[45]
	CYP19A1	Disminuye mRNA y actividad enzimática		
		Disminuye actividad enzimática	Sinciciotrofoblastos	[62]
Activina	CYP19A1	Incrementa actividad enzimática	Sinciciotrofoblastos	[62]
Insulina e Inositolglicano mediatores de insulina	3β-HSDI	Incrementa actividad enzimática	Citotrofoblastos	[63, 64]
	CYP19A1	Disminuye actividad enzimática		
IGF-I	CYP11A1	Incrementa actividad enzimática	Citotrofoblastos	[63]
	CYP19A1	Disminuye actividad enzimática		
IGF-II	CYP11A1 3β-HSDI	Incrementa actividad enzimática	Citotrofoblastos	[65]
	CYP19A1	Disminuye actividad enzimática		

Factor	Blanco	Efectos	Células	Ref.
VEGF y Dexametasona	CYP19A1	Disminuye mRNA, sin cambios en actividad enzimática	Citotrofoblastos	[66]
Forskolina y PMA		Incrementa mRNA		
ATP/Mg^{2+}/Ca^{2+}	CYP19A1	Incrementan mRNA y actividad enzimática	JEG-3	[67]
Ca^{2+}	3β-HSDI CYP11A1 17β-HSD1	Incrementa mRNA	JEG-3	[44]
Hipoxia	CYP19A1	2 % O_2: disminuye actividad enzimática y previene inducción de mRNA	Transición de citotrofoblastos hacia sinciciotrofoblastos	[68]
		1% O_2: disminuye mRNA y proteína	JEG-3	[69]
c-Myc miR-17 miR-19b miR-106a	CYP19A1	Disminuye mRNA	Citotrofoblastos	[48]
Bisfenol A	CYP19A1	Disminuye mRNA, proteína y actividad enzimática	JEG-3	[70]
		Disminuye producción de P_4 y E_2	JEG-3 y Sinciciotrofoblastos	[71]
	CYP11A1 CYP19A1	Disminuye mRNA		
Alquilfenoles	CYP19A1	Inhibe actividad enzimática	JEG-3	[72]
Sulfonato de perfluorooctano	CYP19A1	Disminuye mRNA y actividad enzimática	Sinciciotrofoblastos	[54]
Fungicidas: azoles y organitinas	3β-HSDI y/o CYP19A1	Inhibe actividad enzimática y producción de P_4/E_2	JEG-3	[55]

hCG: gonadotropina coriónica humana, LH: hormona luteinizante, miR: microRNA, ATP: trifosfato de adenosina, Mg^{2+}: magnesio, Ca^{2+}: calcio, VEGF: factor de crecimiento del endotelio vascular, IGFs: factor de crecimiento similar a la insulina, PMA: forbol miristrato acetato.

1.6 Calcitriol

La 1α,25-dihidroxivitamina D (calcitriol), es el metabolito activo de la vitamina D (VD). El calcitriol se diferencia estructuralmente de los esteroides por conservar la cadena lateral del colesterol a partir del carbono 17 y por el rompimiento del

enlace que está entre el carbono 9 y 10 del anillo B del núcleo esteroidal, por lo cual también se clasifica en un subgrupo llamado secoesteroides. La ruta de la síntesis y degradación del calcitriol ha sido extensivamente reportada [73].

El calcitriol lleva a cabo sus efectos biológicos a través de dos vías de acción bien descritas, brevemente:
La primera es una vía denominada genómica mediada por el receptor especifico de la vitamina D (VDR), el cual es un factor de transcripción que se encuentra en el núcleo celular en espera del ligando. Una vez que el calcitriol se une al VDR, se recluta al receptor X de retinoides (RXR) y forma un heterodímero con él. Así, el complejo VDR-RXR se empalma, junto con proteínas co-activadoras o co-represoras de la transcripción, a los elementos de respuesta específicos de la vitamina D presentes en genes sensibles a la regulación de su expresión por el calcitriol [73] .

En el segundo mecanismo, el calcitriol regula la expresión de distintas moléculas a través de una vía de señalización denominada no genómica. A este respecto, el calcitriol puede unirse a un VDR de membrana o a las proteínas de respuesta rápida a esteroides asociadas a la membrana (MARRS, por sus siglas en ingles) de diferentes tipos celulares. En general, mediante este mecanismo se generan segundos mensajeros como $Ca^{2+,}$ AMP cíclico, ácidos grasos y 3-fosfoinositidos como el fosfatidilinositol 3,4,5 trisfosfato, entre otros. Tras el estímulo del calcitriol, los mensajeros activan proteínas quinasas como la proteína quinasa A (PKA), la proteína quinasa activada por mitógeno (MAPK), la proteína quinasa C (PKC) y la Ca^{2+}-calmodulina quinasa. Asimismo, las acciones no genómicas incluyen también la apertura o cierre de canales iónicos [74].

1.7 Funciones del calcitriol en el embarazo

El calcitriol es una hormona versátil con múltiples funciones biológicas en el organismo, sus efectos incluyen no solo la regulación del metabolismo mineral

sino también la de eventos de proliferación, diferenciación y apoptosis en múltiples tipos celulares [75].

En el embarazo, la cantidad del calcitriol aumenta conforme avanza la gestación [76] y aunque dicho incremento es atribuido principalmente al calcitriol producido por los riñones de la madre, tanto la decidua como los trofoblastos también aportan este secoesteroide que se suma al aumento observado. De manera interesante, la deficiencia de calcitriol se asocia con eventos reproductivos adversos como la infertilidad, las infecciones urogenitales bacterianas, los abortos recurrentes, la restricción del crecimiento intrauterino del feto, el parto pre-término, así como con un alto riesgo de desarrollar PE [73], patologías que también se han asociado a un desequilibrio hormonal.

En particular, usando como modelo experimental el cultivo primario de trofoblastos humanos nuestro grupo de trabajo mostró que el calcitriol regula tanto la expresión como la secreción de la hCG de una manera dependiente del tiempo [77], y que induce la secreción de P_4 y E_2 en los trofoblastos en cultivo [46]. Asimismo, media la síntesis de las proteínas transportadoras de calcio CDK9 y CDK28, de los péptidos anti-microbianos catelicidina y defensina 1 y 2, así como de las citocinas tanto anti- como pro-inflamatorias (IL-1β, IL-10, IL-6, TNF-α y el IFN-γ) [47, 78-81], regulando así a diferentes componentes tanto del sistema endocrinológico como inmunológico en la placenta humana. Lo anterior, resalta la importancia que tiene esta hormona para modular moléculas clave producidas en los trofoblastos.

1.8 Efectos del calcitriol sobre la expresión y la actividad del CYP11A1, CYP17A1, CYP19A1, 3β-HSD y 17β-HSD

El calcitriol regula a las enzimas implicadas en la biosíntesis de hormonas esteroides en una amplia variedad de tejidos. Por ejemplo, favorece la bioconversión de E_2 a estrona en los queratinocitos [82], regula su propio metabolismo al modular a las enzimas que están involucradas en su síntesis e

inactivación manteniendo así la biodisponibilidad del calcitriol en cantidades apropiadas [29].

Por otra parte, el calcitriol modifica la expresión y la actividad de otros citocromos, así como de HSDs de manera diferencial y dependiente del tejido. A este respecto, Merhi y cols., mostraron que el calcitriol incrementa la actividad de la enzima 3β-HSDI en células de la granulosa [83], mientras que Yagishita y cols., mostraron que induce la expresión génica de la 3β-HSDI así como la del CYP11A1 en las células Gi-1 de glioma humano [84]. De manera similar, diferentes isoformas de la 17β-HSD también son blanco del calcitriol ya que la expresión génica de la 17β-HSD tipo 2, 4 y 5 es estimulada de manera dependiente de la concentración y del tiempo, en líneas celulares de cáncer de próstata [85].

El calcitriol también incrementa la expresión del gen del CYP11A1 y del CYP17A1 en las células adrenocorticales humanas NCI-H295R [86], y la expresión génica y/o la actividad del CYP19A1 en osteoblastos, próstata y coriocarcinoma humano, así como en células de Sertoli y de la de granulosa de rata [57, 87-89]. Interesantemente, Kinuta y cols., demostraron que la expresión del gen del Cyp19a1 y su actividad está disminuida en los ovarios, los testículos y el epidídimo de ratones mutantes nulos para el VDR, lo que indica que el calcitriol es un factor estimulador de esta enzima [90]. Contrario a lo reportado por Kinuta en los ratones, se encontró que el calcitriol disminuye la expresión génica y la actividad del CYP19A1 en macrófagos humanos y en la línea celular MCF-7 de cáncer de mama [91, 92].

Con los antecedentes descritos en este estudio nos resultó interesante indagar a fondo si el calcitriol es capaz de regular de manera puntual la expresión génica y la cantidad de proteína de las principales enzimas involucradas en la biosíntesis de esteroides sexuales en la placenta humana a término.

2 JUSTIFICACIÓN

Considerando la relevancia de las hormonas esteroides para el éxito de la gestación y que el calcitriol regula de manera diferencial la expresión y la actividad de las enzimas esteroidogénicas en múltiples tipos celulares, nos resultó interesante determinar el efecto del calcitriol sobre la expresión de las enzimas CYP11A1, CYP17A1, CYP19A1, 3β-HSDI y 17β-HSD3, en los trofoblastos placentarios humanos en cultivo.

Lo anterior cobra relevancia ya que la deficiencia del calcitriol se ha asociado con complicaciones del embarazo como la preeclampsia, un síndrome de etiología desconocida, en la cual existe desequilibrio hormonal. Por lo tanto, los resultados mostrarán la participación de este secoesteroide como un mediador de la expresión enzimática necesaria para la síntesis de hormonas esteroides en la placenta.

3 HIPÓTESIS

El calcitriol modula la expresión de las enzimas que participan en la biosíntesis de las hormonas esteroides sexuales en la placenta humana de una manera dependiente del tiempo.

4 OBJETIVOS

4.1 Objetivo General

Conocer el efecto del calcitriol sobre la expresión de las enzimas involucradas en la biosíntesis de las hormonas esteroides en trofoblastos placentarios en cultivo.

4.2 Objetivos Específicos

1) Determinar el patrón de expresión génica basal de las enzimas *CYP11A1, CYP17A1, CYP19A1, 3β-HSDI* y *17β-HSD3* durante la diferenciación de los trofoblastos en cultivo.

2) Comparar la expresión génica basal entre las diferentes enzimas estudiadas.

3) Investigar el efecto del calcitriol en la expresión del gen y de la proteína de CYP11A1, CYP17A1, CYP19A1, 3β-HSDI y 17β-HSD3 en los sinciciotrofoblastos durante una cinética de tiempo.

4) Establecer el efecto del calcitriol sobre la expresión génica del *CYP11A1, CYP17A1, CYP19A1, 3β-HSDI* y *17β-HSD3* durante la diferenciación de los trofoblastos.

5 MATERIALES Y MÉTODOS

Para realizar este proyecto se contó con la aprobación del Comité de Ética del Instituto Nacional de Perinatología “Isidro Espinosa de los Reyes” (No. de registro 212250-21131), del Hospital General Dr. Manuel Gea González (No. de registro 11-57-2016) y del Instituto Nacional de Ciencias Médicas y Nutrición “Salvador Zubirán” (No. de registro BRE-535-12/15-1).

En este estudio se procesaron placentas humanas que se obtuvieron previo consentimiento informado de mujeres gestantes sometidas a cesárea por elección, por desproporción céfalo-pélvica o por circular del cordón umbilical al cuello del feto. El estudio incluyó la placenta de donadoras sanas con edad entre los 18 y los 40 años quienes cursaron un embarazo normoevolutivo con un solo producto. Las muestras fueron colectadas al término de la gestación (37 a 41 semanas de embarazo) registrando en bitácoras las características clínicas de la madre y de su recién nacido. Se excluyeron del estudio las placentas de mujeres con antecedentes de tratamiento hormonal para lograr la concepción y de mujeres que presentaron complicaciones durante la gestación.

5.1 Cultivo primario de trofoblastos placentarios

El cultivo primario de los trofoblastos se hizo con base en el método establecido por Kliman y colaboradores en 1986 [93]; con algunas modificaciones implementadas en nuestro laboratorio, según se describe brevemente a continuación.

Las muestras se obtuvieron inmediatamente después del alumbramiento y se transportaron del hospital al laboratorio dentro de un contenedor metálico hermético y estéril. Una vez en el laboratorio, la placenta se colocó en un contenedor estéril para retirar la decidua, los restos de cordón umbilical y las membranas corioamnióticas con material quirúrgico. Posteriormente, el exceso de sangre de los cotiledones se retiró mediante lavados con abundante solución fisiológica estéril (cloruro de sodio al 0.9% (p/v)). Después, los cotiledones se

seccionaron y pesaron en tres porciones de 40 g. Cada porción, se colocó por separado en un matraz Erlenmeyer de 1 L y las muestras se procesaron mediante tres digestiones enzimáticas en baño María usando solución salina balanceada de Hank (HBSS) libre de calcio y de magnesio. Para disgregar el tejido se usó tripsina de páncreas de bovino (Sigma, T9201) y para fragmentar el DNA genómico liberado por la ruptura celular se utilizó DNasa de páncreas de bovino (Sigma, DN25), las cantidades empleadas se muestran en la Tabla 2.

Tabla 2. Condiciones de las digestiones enzimáticas para la obtención de los trofoblastos

Digestión / Tiempo/ temperatura	Solución Salina 1X (mL)	Tripsina (153.8 mg/mL)	DNAsa I (100 mg/mL)
1ª / 20 min / 37°C	150	780	100
2ª / 20 min / 37°C	100	480	50
3ª / 20 min / 37°C	75	250	50

Una vez transcurrido el tiempo de cada digestión, las células disgregadas en la solución salina se obtuvieron depositando 45 mL de muestra en tubos cónicos (2 tubos por cada matraz). La actividad enzimática de la tripsina y la DNasa se detuvo adicionando a cada tubo 5 mL de suero fetal de ternera (SFT) inactivado. La muestra colectada se centrifugó a 3000 rpm por 10 min, y el botón celular resultante en cada tubo se resuspendió en 600 μL de medio DMEM-HG (medio mínimo esencial de Dulbecco, alto en glucosa). El medio con las células de cada digestión se juntó en un solo tubo y se mantuvieron a 37°C en baño María. La suspensión celular total recolectada se mezcló y se colocó sobre un gradiente de

percoll del 5 al 70 % de densidad previamente elaborado (ver tabla 3). El gradiente se centrifugó a 3000 rpm durante 20 min y así se obtuvieron las células de interés separadas por densidad.

Tabla 3. Gradiente de percoll. Volumen necesario de percoll y de HBSS 1X para la preparación del gradiente de densidad 5-70 %

No. De tubo	% de Percoll	mL de Percoll 90 %	HBSS 1x
1	70	2.33	0.67
2	65	2.17	0.83
3	60	2.00	1.00
4	55	1.83	1.17
5	50	1.67	1.33
6	45	1.50	1.50
7	40	1.33	1.67
8	35	1.17	1.83
9	30	1.00	2.00
10	25	0.83	2.17
11	20	0.67	2.33
12	15	0.50	2.50
13	10	0.33	2.67
14	5	0.17	2.83

Solución 90 % Percoll: 18 mL de Percoll al 100 % + 2 mL de HBSS 10X con rojo fenol.

Después de la centrifugación, se aspiró y se desechó la parte superior del gradiente mientras que la fase ubicada entre 1.048 y 1.062 g/mL que contenía a los citotrofoblastos se recuperó en tubos nuevos (figura 4). Finalmente, los citotrofoblastos recuperados se lavaron y se centrifugaron obteniendo un nuevo botón celular. En condiciones de esterilidad el medio de lavado se decantó y las células se resuspendieron en medio DMEM-HG suplementado con 10 % SFT y 1

% de antibióticos, mezcla de penicilina y estreptomicina 10 000 U/mL (Gibco 15140-122). Las células totales se cuantificaron en cámara Neubauer usando la tinción con azul tripán.

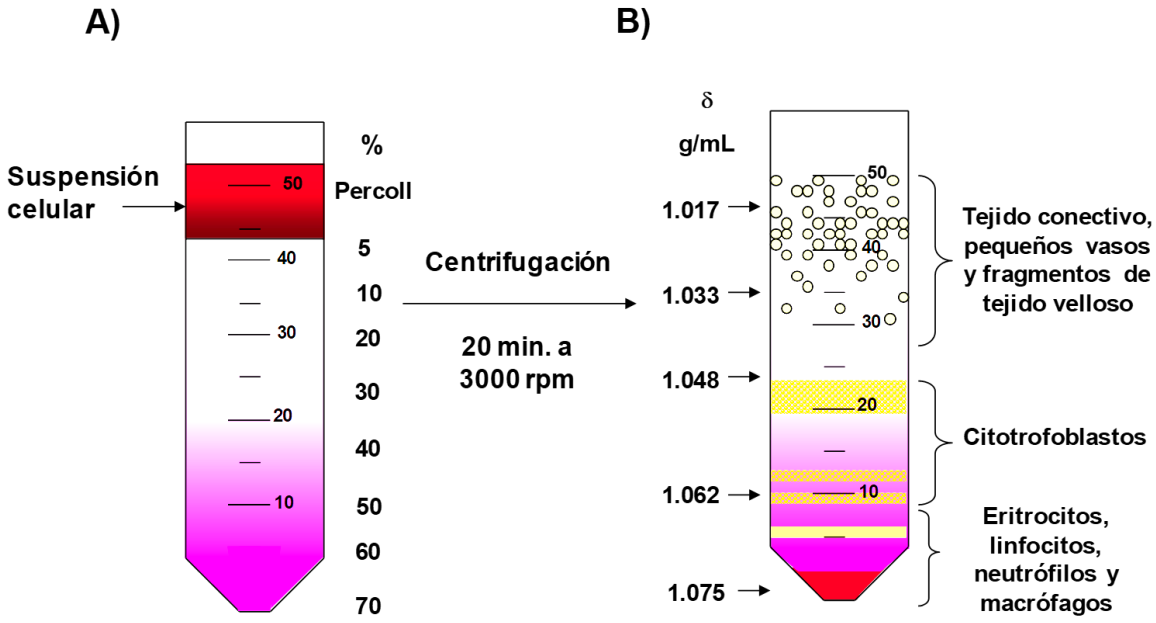

Figura 3. Separación de los citotrofoblastos. A) Suspensión celular total (rojo) colocada sobre el gradiente de Percoll B) Separación por densidad: la banda superior 5-29 % se desechó. Los citotrofoblastos ubicados del 30-50 % de densidad se colectaron y procesaron. La banda inferior 51-70 % también fue desechada. δ: densidad

Para la evaluación de los genes se sembraron 3 x 10^6 células por botella de 25 cm^2 y para la cuantificación de la proteína se sembraron 9 X 10^6 por caja Petri. Los trofoblastos se mantuvieron por 3 h a 37°C, 95 % de humedad y 5 % de CO_2 en medio DMEM suplementado para permitir que las células se adhirieran a la superficie de cultivo. Transcurrido ese tiempo, para retirar las células muertas y las no adherentes, las cajas se lavaron con HSSB, se colocó medio de cultivo nuevo y se dejó incubando a los trofoblastos hasta el momento de llevar a cabo los tratamientos.

5.2 Tratamientos experimentales

Aunque los trofoblastos en cultivo proliferan escasamente, estos se diferencian paulatinamente de un fenotipo mononuclear (citotrofoblastos) hacia estructuras de agregados celulares llamados sinciciotrofoblastos [93]. En el cultivo primario de trofoblastos vellosos se puede encontrar la presencia de ambos fenotipos en diferentes momentos.

Con base únicamente en las observaciones hechas en cada cultivo se estima que a las 3 h de siembra hay un 100 % de citotrofoblastos, a las 24 h 80 % citotrofoblastos y 20 % sinciciotrofoblastos, a las 48 h el 30% de las células son citotrofoblastos y el 70 % sinciciostrofoblastos. Finalmente, a las 72 h de siembra el 95-100 % de los trofoblastos presentes son sincicios. Considerando lo anterior, se determinó el patrón de la expresión génica basal del *CYP11A1, CYP17A1, CYP19A1, 3β-HSDI* y *17β-HSD3* durante la diferenciación de los trofoblastos, evaluando así los genes de interés a las 24, 48 y 72 h de siembra. Por otra parte, sabiendo que los sinciciotrofoblastos son considerados como las estructuras placentarias con mayor capacidad para producir esteroides [94] adicionalmente se evaluó y comparó la expresión basal del *CYP11A1, CYP17A1, CYP19A1, 3β-HSDI* y *17β-HSD3* en los sincicios.

Para cumplir uno de los principales objetivos de este trabajo se observó el efecto del calcitriol sobre la expresión de las enzimas esteroidogénicas de interés mediante una cinética de tiempo. Una vez que se sembraron los trofoblastos se mantuvieron en incubación por 48 h y a ese tiempo se aplicó el tratamiento con calcitriol. Las células permanecieron por 3 h, 6 h, 12 h o 24 h con el respectivo tratamiento. Transcurrido el tiempo establecido los trofoblastos se cosecharon para evaluar cada gen.

Por último, para indagar si el efecto del calcitriol dependía del fenotipo celular se determinó su efecto durante la diferenciación de los trofoblastos. Para ello, a un conjunto de células se les adicionó el tratamiento a las 3 h de siembra. Otro grupo

se mantuvo en incubación y a las 24 h de siembra se les puso el calcitriol. Finalmente, 48 h después de la siembra se trató a un conjunto distinto de células. En todos los casos, los trofoblastos permanecieron 24 h en presencia de calcitriol. Cabe señalar, que los tratamientos con calcitriol 1 nM y 100 nM o con su vehículo (Vh, etanol 0.1%) se hicieron por triplicado en medio F-12 libre de SFT suplementado con antibióticos. Los métodos utilizados para valorar la expresión de los genes, se describen brevemente en el siguiente apartado.

5.3 Estudios de expresión génica

5.3.1 Extracción de RNA

Una vez que las muestras estaban listas se les aspiró el medio de cultivo y se procesaron para aislar el RNA total de los trofoblastos utilizando la técnica descrita por Chomczynski y Sacchi [95], bajo algunas modificaciones realizadas por nuestro grupo de trabajo (tabla 4).

Tabla 4. Procedimiento para la extracción de RNA

Número de células por muestra $3x10^6$ **Reactivos**	**Volumen (μL)**	**Centrifugación rpm/min a 4ºC**
Trizol	900	-
Cloroformo	200	13500 / 20
Recuperar fase superior		
Isopropanol	500	13500 / 20
Incubar toda la noche		
Etanol	900	13500 / 20
Agua tratada con dietilpirocarbonato	22	-

Para determinar la cantidad y calidad del RNA total, se tomaron 2 µL de la muestra y se leyó su absorbancia a la longitud de onda de 260 y 280 nm en un espectrofotómetro. Una relación 260/280 igual a 2.0 implica que se tiene 100 % de RNA y 0 % de proteínas, por lo tanto en nuestros ensayos se descartaron aquellas muestras con pureza menor a 1.7.

5.3.2 Retrotranscripción

El RNA total se utilizó para sintetizar DNA complementario (cDNA) por retro-transcripción, para lo cual se usó el estuche comercial *Transcriptor First Strand cDNA Synthesis* de Roche, siguiendo las instrucciones del fabricante.

Para esta técnica se tomó una concentración constante de 2 µg de RNA total por cada muestra. Así, en tubos de Eppendorf se mezcló el RNA, 1µL de Oligo dT 50 pmol/µL y se ajustó a un volumen final de 15 µL con agua grado PCR. Los tubos se colocaron en un termociclador (Veriti, Applied Biosystems) a 65°C por 10 min, posteriormente, los tubos se retiraron del equipo y se mantuvieron a 4°C. A cada muestra se le agregaron 7µL de la mezcla de reacción previamente preparada con: 4 µL de solución amortiguadora 5X, 0.5 µL de inhibidor de RNasas, 2 µL de mezcla de desoxinucleótidos y 0.5 µL de transcriptasa reversa. Una vez agregada la mezcla a cada tubo, las muestras se regresaron al termociclador para terminar la reacción a 55°C durante 30 min y 85°C por 5 min. Finalmente, el DNAc sintetizado se mantuvo a 4°C hasta su uso.

5.3.3 Reacción en cadena de la polimerasa en tiempo real

Para amplificar la expresión de los genes en estudio se empleó PCR en tiempo real (RT-qPCR) usando el estuche *LightCycler 480 Probe Master* que utiliza el sistema de sondas de hidrólisis Taqman. La sonda específica para cada gen se adicionó a una mezcla de reacción que contenía: 4.7 µL de agua grado PCR, 0.1 µL del iniciador [20 µM] correspondiente al gen en estudio y 3.5 µL de máster mix, obteniendo 9 µL de mezcla. El sistema utiliza placas de polietileno de 96 pozos y en cada uno de ellos se introdujo la mezcla de reacción descrita más 1 µL de

DNAc obteniendo un volumen final de 10 μL en cada pozo. Posteriormente, la placa se agitó cuidadosamente, se centrifugó a 1000 rpm durante 30 segundos y se colocó en un termociclador 480 de Roche previamente programado bajo las condiciones que se muestran en la tabla 5.

Tabla 5. Condiciones para la PCR en tiempo real

	Número de ciclos	**Temperatura (°C)**	**Tiempo (segundos)**
Activación de la enzima *Taqman*	1	95	600
PCR **Desnaturalización** **Alineación** **Elongación**	45	 95 60 72	 10 30 1
Enfriamiento	1	40	10

Los resultados obtenidos para cada muestra en estudio, se compararon con la expresión del gliceraldehído 3-fosfato deshidrogenasa (GAPDH) usado como gen constitutivo. Los iniciadores y las sondas específicas se diseñaron usando el *Universal Probe Library Assay Design Center* de Roche obteniendo las secuencias que se muestran en la tabla 6.

Tabla 6. Secuencias de los iniciadores y sondas utilizadas en la RT-qPCR

Gen blanco	Orientación de los oligonucleótidos (secuencia 5´------3´)		# Sonda
	Sentido	Anti-sentido	
CYP11A1	AGG AGG GGT GGA CAC GAC	TTG CGT GCC ATC TCA TAC A	59
CYP17A1	GCA TCA TAG ACA ACC TGA GCA A	GGG TTT TGT TGG GGA AAA TC	64
CYP19A1	GAA TTC ATG CGA GTC TGG ATC T	TCA TTA TGT GGA ACA TAC TTG AGG A	55
3β-HSDI	CGG ACC AGA ATT GAG AGA GG	GAA TGG CTC ATC CAG AAT GTC	11
17β-HSD3	AAC TTG CAG GCT TAG AAA TTG G	GGT GCG TTC AGG AAA TGG	7
hCGβ5	GCT CAC CCC AGC ATC CTA T	CAG CAG CAA CAG CAG CAG	79
GAPDH	AGC CAC ATC GCT GAG ACA C	GCC CAA TAC GAC CAA ATC C	60

5.4 Cuantificación de las proteínas de interés por "Western Blot"

Para evaluar el efecto del calcitriol sobre la abundancia protéica del CYP11A1, CYP17A1, CYP19A1 y 3β-HSD en sinciciotrofoblastos. Las células se incubaron 48 h para luego ser tratadas con calcitriol 100 nM o su Vh en medio F-12 libre de SFT suplementado con antibióticos. Después de permanecer 6 o 24 h con el tratamiento, los trofoblastos se lavaron con HBSS y la proteína se liberó con solución de lisis (9.1 mM fosfato dibásico de sodio, 1.7 mM fosfato monobásico de sodio, 150 mM cloruro de sodio, 1 % Nonidet P40, 0.1 % SDS, pH 7.4). La proteína total se cuantificó por el método de Bradford [96], la cual se utilizó para evaluar la presencia de los citocromos y la deshidrogenasa como se describe a continuación.

La proteína de interés se separó por electroforesis vertical en geles con 10 % de poliacrilamida usando 30 µg de proteína total. Después de la electroforesis, la

proteína se transfirió a una membrana de nitrocelulosa de 0.45 μM, la cual se bloqueó durante 1 h con solución amortiguadora a base de Tris más un detergente (TBS-T) más 5 % de leche en polvo libre de grasa. Posteriormente, las membranas se lavaron 3 veces con TBS-T y se dejaron incubar a 4°C toda la noche en presencia del anticuerpo primario; anti-CYP11A1 (50 kDa, Cell Signaling) o anti-CYP19A1 (51 kDa, Cell Signaling) diluidos 1:1000, o del anti-CYP17A1 (55 kDa, Santa Cruz) o anti-3β-HSD (42 kDa, Santa Cruz) diluidos 1:200. Tras la primera incubación, las membranas se lavaron en 3 ocasiones con TBS-T y se incubaron a temperatura ambiente con el anticuerpo secundario (acoplado a HPR, diluido 1:8000) durante 1 h. Después de lavar nuevamente las membranas la proteína específica se visualizó usando el reactivo luminiscente Clarity Western ECL Substrate (Bio-Rad), en el fotodocumentador de imágenes ChemiDoc XRS+ (Bio-Rad) y el programa Image Lab (Bio-Rad). La cantidad de proteína detectada en cada caso, fue normalizada con el GAPDH (32 kDa, Millipore, 1:8000) usado como control de carga.

6 ANÁLISIS DE LOS DATOS

Los tratamientos para evaluar la expresión de las enzimas en presencia de calcitriol o su Vh se hicieron por triplicado en cada cultivo. Cada gráfica es el resultado de por lo menos tres cultivos diferentes y los datos se expresaron como el promedio $\pm$ la desviación estándar (DE). Las diferencias entre cada grupo fueron significativas con una $P \leq 0.05$ usando la prueba estadística de ANOVA de una vía. La comparación por pares se realizó por el análisis post hoc Holm-Sidak; con el programa estadístico Sigma Plot 11.0.

7 RESULTADOS

7.1 Diferenciación y funcionalidad del cultivo primario de trofoblastos

Los trofoblastos placentarios en cultivo forman sincicios de manera dependiente del tiempo. Este proceso es el resultado de la fusión entre los citotrofoblastos mononucleares adyacentes para dar origen a sinciciotrofoblastos multinucleados, es por eso que la diferenciación de los trofoblastos y su viabilidad se determinaron mediante microscopia óptica siguiendo el cambio morfológico de las células durante 72 horas. Como se aprecia en las imágenes, las células mononucleares se fusionaban entre sí para formar sincicios (Figura 4A-D).

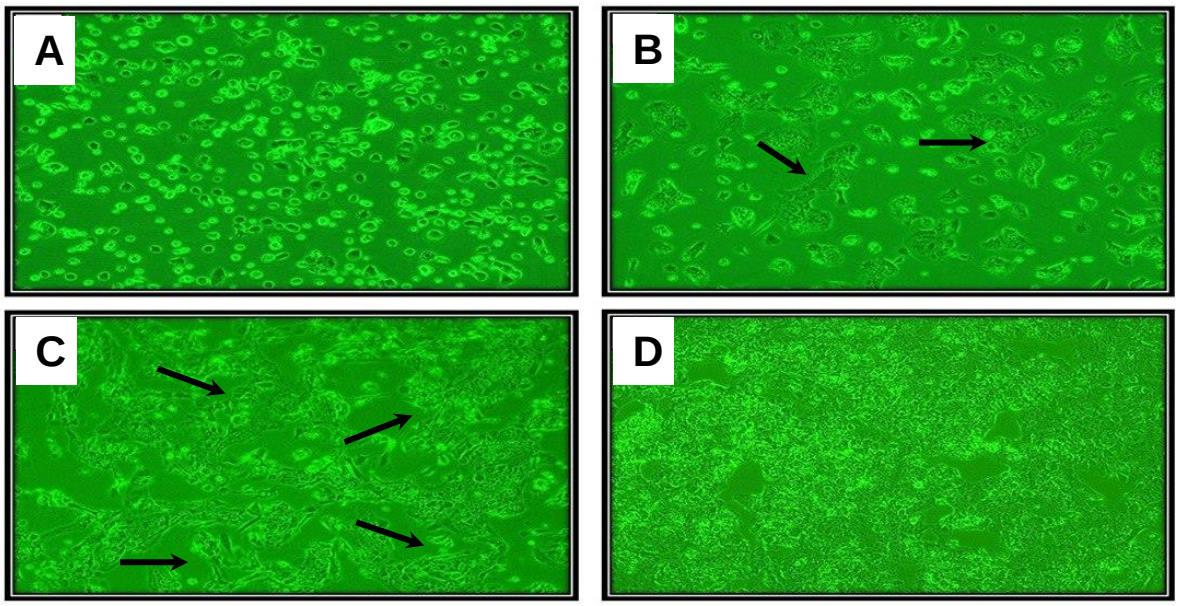

Figura 4. Cambio morfológico de los trofoblastos en cultivo.
A) Citotrofoblastos recién sembrados (3 h), B) 24 h de cultivo, los citotrofoblastos empiezan a tener proyecciones citoplasmáticas, C) 48 h de cultivo, transición avanzada de cito- a sinciciotrofoblastos, D) 72 h de cultivo exclusivamente sinciotrofoblastos. Imágenes tomadas con un objetivo 10X. Las flechas señalan los sinciciotrofoblastos.

Adicionalmente, el fenotipo endocrino de los trofoblastos se corroboró evaluando la expresión de la hCG. Como se aprecia en la figura 5, a lo largo de la diferenciación se observó un incremento en la expresión génica (figura 5A) y en la secreción de la subunidad beta de la hCG (figura 5B). Estos resultados respaldan nuestro modelo experimental garantizando las mismas condiciones del cultivo al realizar las evaluaciones correspondientes.

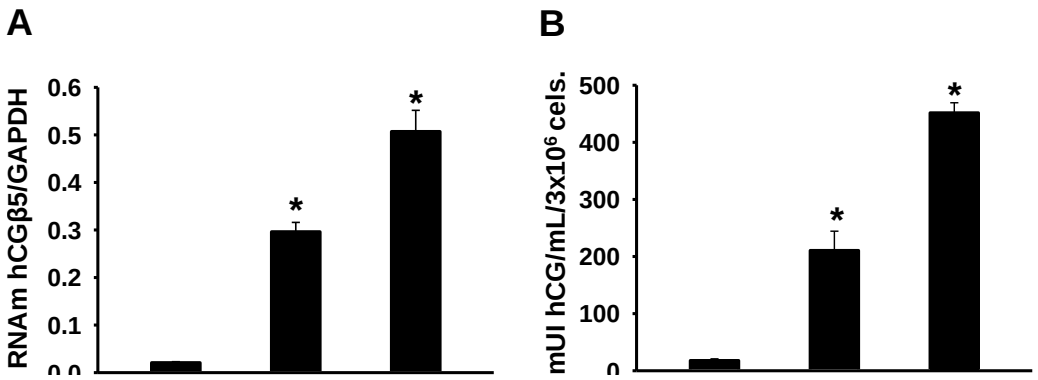

Figura 5. Expresión basal de la hCG durante 3 días de cultivo.
En la figura se muestra el incremento paulatino tanto del A) gen como de la B) proteína de la hCG evaluada a las 24, 48 y 72 h de cultivo. La expresión génica se obtuvo por RT-qPCR y la secreción de la subunidad β5 de la hCG por ELISA. *P < 0.05 *vs* 24 h.

Para comprobar que las células responden a la presencia del calcitriol se evaluó un gen altamente regulado por este secoesteroide: el CYP24A1. Como se esperaba, el calcitriol estimuló la expresión génica de dicho citocromo (Anexo 1).

7.2 Expresión génica basal de *CYP11A1, CYP17A1, CYP19A1, 3β-HSDI y 17β-HSD3* durante la diferenciación de los trofoblastos

Se ha descrito que los citotrofoblatos carecen de las enzimas necesarias para producir hormonas esteroides atribuyendo esta importante función únicamente a los sincicios [97]. Por ese motivo, en este estudio se muestra el patrón de expresión de *CYP11A1, CYP17A1, CYP19A1, 3β-HSDI y 17β-HSD3* a lo largo de la diferenciación de los trofoblatos (figura 6).

Como era de esperarse, y similar a lo que sucede con la hCG, la mayor expresión de las enzimas ocurrió en los siciciotrofoblatos. Sin embargo, desde las 24 h de siembra los trofoblastos expresan a las 5 enzimas esteroidogénicas. No obstante, como se aprecia en la figura 6, todas las enzimas alcanzan su máxima expresión a las 72 h de cultivo ($P < 0.05$ *vs* 24 h) y en particular, se observó que la expresión de las HSDs y del CYP19A1 fue significativamente mayor desde las 48 h con respecto a las 24 h de cultivo, lo que sugiere que ambos tipos celulares podrían contribuir con los requerimientos de P_4 y estrógenos en el embarazo.

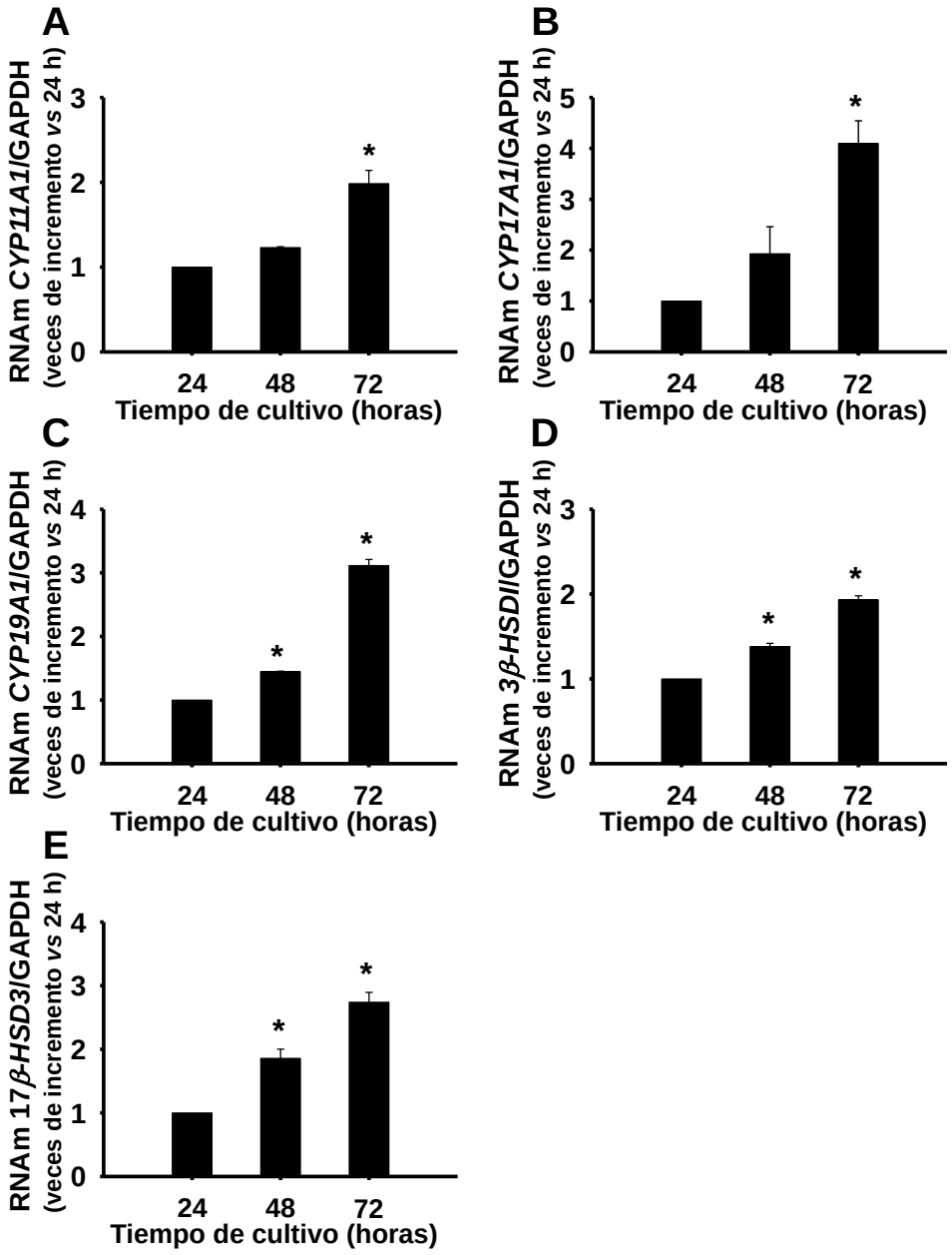

Figura 6. Expresión basal de los CYPs y HSDs en 3 días de cultivo.
Patrón de expresión génica de A) *CYP11A1*, B) *CYP17A1*, C) *CYP19A1*, D) *3β-HSDI* y E) *17β-HSD3* durante la diferenciación de los trofoblastos. n = 3. A los datos obtenidos a las 24 h de cultivo se les asignó el valor de 1. *P < 0.05 *vs* 24 h.

7.3 Comparación de la expresión basal de los genes del *CYP11A1, CYP17A1, CYP19A1, 3β-HSDI* y *17β-HSD3* en sinciciotrofoblastos

Tomando en cuenta que en los sinciciotrofoblastos se detectó la mayor expresión de las enzimas en estudio, la expresión basal de cada una de ellas se evaluó y comparó a las 72 h de la siembra.

Como se aprecia en la Figura 7, el análisis reveló que la *3β-HSDI* es la enzima que más se expresa en nuestros cultivos. En contraste, la enzima de menor expresión fue el CYP17A1 (*P < 0.05). En conjunto, la comparación mostró el siguiente patrón de expresión: *3β-HSDI* > *CYP19A1* = *CYP11A1* > *17β-HSD3* ≥ *CYP17A1*.

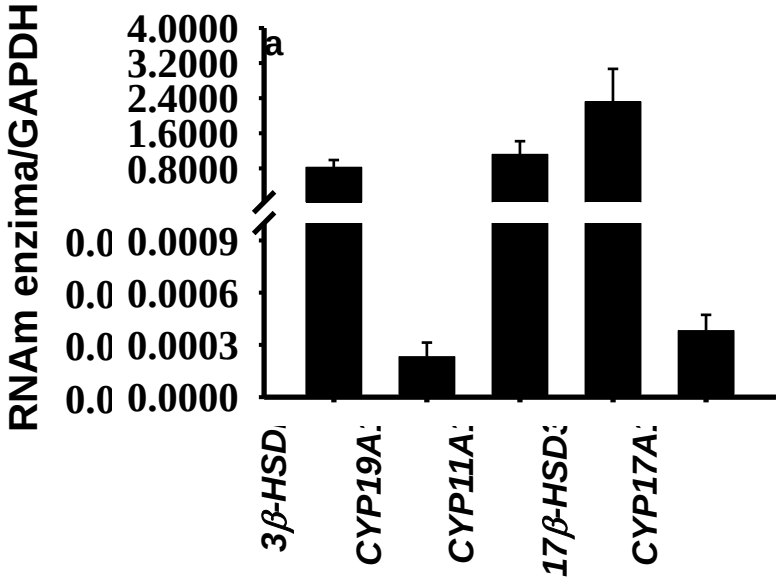

Figura 7. Expresión basal de las enzimas esteroidogénicas en sinciciotrofoblastos. Las barras representan el promedio ± D. E. de n = 4. Las letras iguales señalan que no hay diferencias significativas entre la expresión de las enzimas.

7.4 Cinética de tiempo de los efectos del calcitriol sobre la expresión génica de las enzimas esteroidogénicas en sinciciotrofoblastos

Cabe mencionar, que tras realizar curvas con diferentes concentraciones de calcitriol (Anexo 1) se decidió utilizar 1 nM y 100 nM para evaluar sus efectos. Estas concentraciones se eligieron considerando que 1 nM fue la menor concentración a la que la hormona tuvo efectos y a que es la más cercana a las concentraciones fisiológicas reportadas, mientras que 100 nM se eligió por ser una concentración farmacológica que se usa comúnmente y no es tóxica para los trofoblastos.

Previamente, nuestro grupo de trabajo demostró que el calcitriol induce la secreción de P_4 y E_2 a tiempos cortos, pero se desconoce si este efecto involucra la expresión de las enzimas encargadas de su síntesis. Por tal motivo, se realizó una cinética de tiempo (3, 6, 12 y 24 h).

De manera interesante, como se muestra en las gráficas 8A y 8C, el calcitriol (100 nM), incrementó la expresión de *CYP11A1* y *CYP19A1* después de 3 h de tratamiento. Sin embargo, este estímulo fue descendiendo conforme pasaron las horas, disminuyendo significativamente su expresión entre las 12 y las 24 h post-tratamiento. En contraste, la expresión del *CYP17A1* (Figura 8B) disminuyó a las 6 h pero fue incrementándose conforme pasaban las horas, alcanzando un pico máximo de estímulo a las 24 h. En cuanto a las deshidrogenasas, la expresión de 17β-HSD3 y *3β-HSDI* se estimuló a las 3 y 6 h, respectivamente. No obstante, este efecto se perdió paulatinamente estabilizándose sin cambios significativos 24 h después.

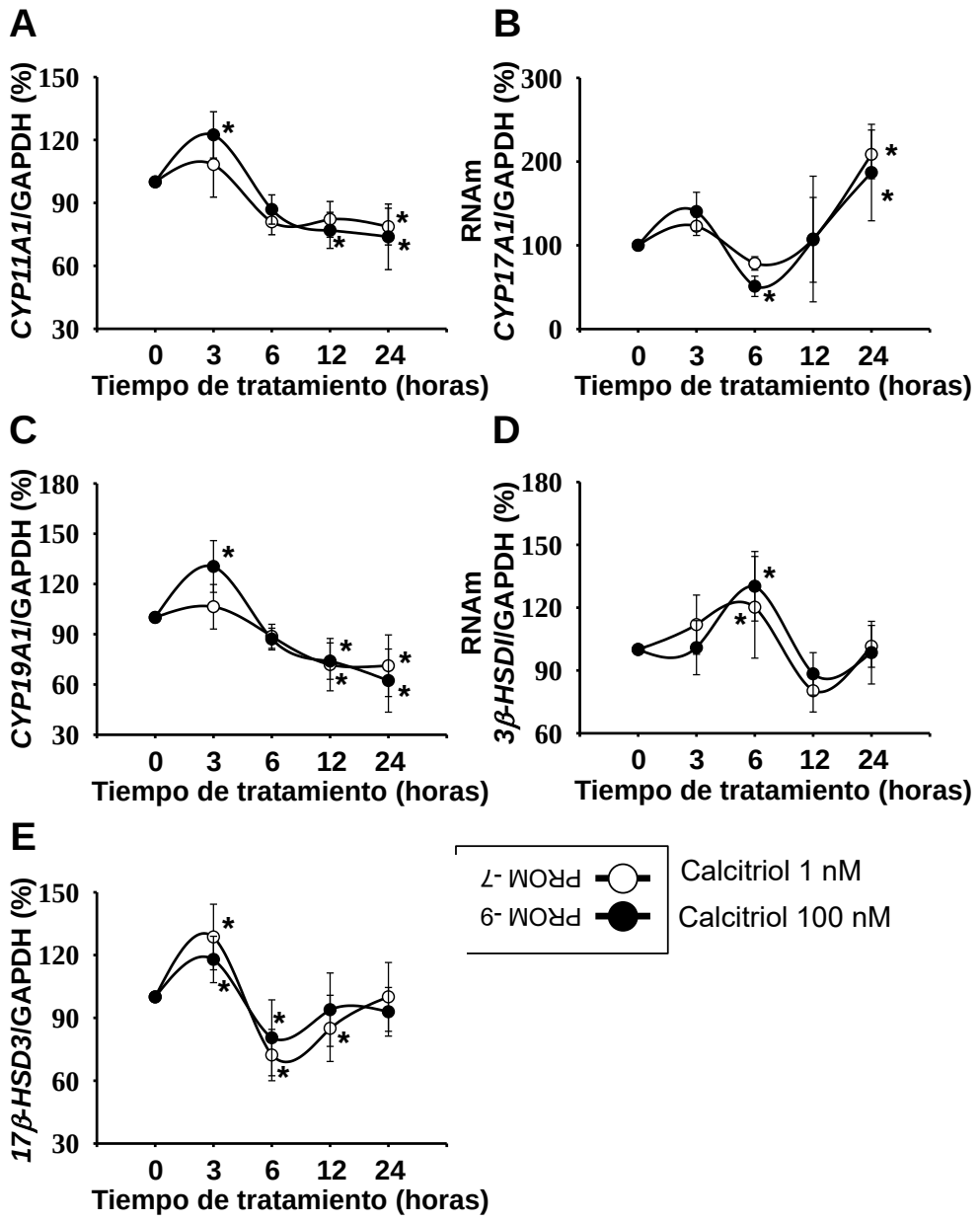

Figura 8. Regulación del calcitriol sobre los CYPs y HSDs en sinciciotrofoblastos. La expresión génica de A) *CYP11A1*, B) *CYP17A1*, C) *CYP19A1*, D) *3β-HSDI* y E) *17β-HSD3* se evaluó por RT-qPCR; 3, 6, 12 y 24 h después de los tratamientos con calcitriol 1 nM (círculos blancos) y 100 nM (círculos negros). Los puntos representan el promedio ± D. E. de n ≥ 3. Los resultados son expresados como el porcentaje de cambio de las células tratadas con calcitriol vs 0 h, tiempo al que se le asignó el valor del 100 %. *P < 0.05 *vs* 0 h.

7.5 Efectos del calcitriol sobre la abundancia protéica del CYP11A1, CYP19A1 y 3β-HSD en sinciciotrofoblastos

El efecto del calcitriol sobre la abundancia de la proteína del CYP11A1, CYP19A1 y 3β-HSDI, se muestra en la Figura 9. Como se aprecia la densidad relativa de la proteína del CYP11A1 y de la 3β-HSDI no fue significativamente diferente ni a las 6 h ni a las 24 h de tratamiento con calcitriol (Figura 9A y 9C). Sin embargo, la abundancia de CYP19A1 se incrementó a las 6 h mientras que disminuyó a las 24 h de incubación (Figura 9B), ambas observaciones coinciden con la expresión génica de esta enzima. Cabe mencionar que aunque la proteína del CYP17A1 se evaluó no se pudo detectar usando el método y las condiciones descritas en este trabajo.

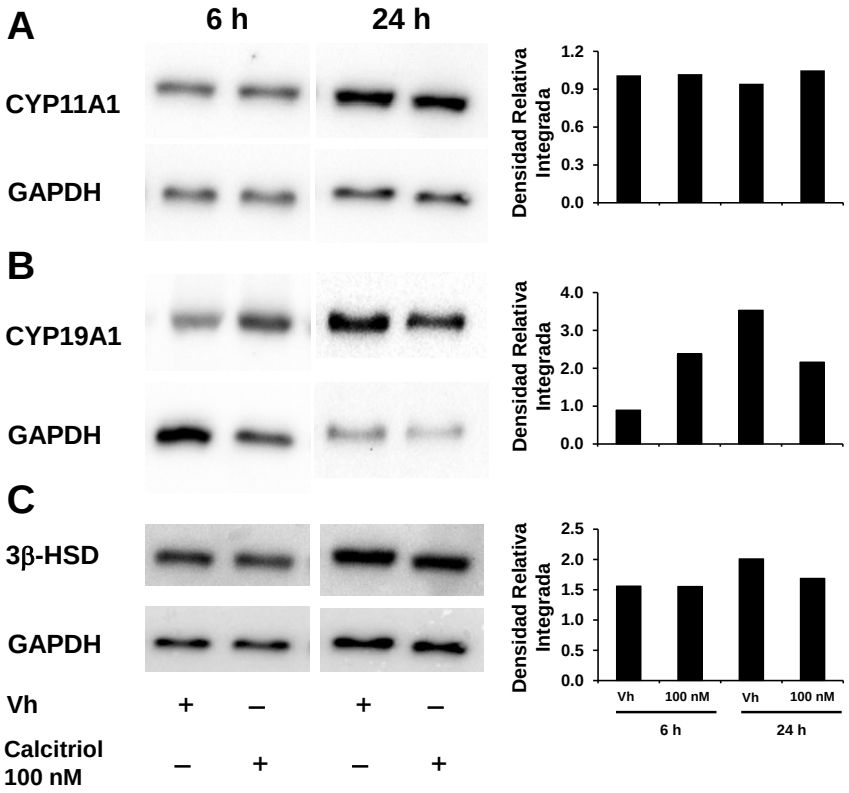

Figura 9. Efecto del calcitriol sobre la proteína de los CYPs y HSDs en sinciciotrofoblastos.
La proteína de A) CYP11A1, B) CYP19A1 y C) 3β-HSD se cuantificó por WB. Los histogramas son la representación cuantitativa de la densidad relativa integrada de las enzimas evaluadas a las 6 y 24 h en ausencia o presencia de calcitriol. La imagen es representativa de 3 experimentos independientes.

7.6 Efectos del calcitriol en la expresión génica de las enzimas esteroidogénicas durante la diferenciación de cito- a sinciciotrofoblastos

Como se muestra en la figura 10, el calcitriol inhibió la expresión génica del *CYP11A1* (Figura 10A) en cada día de la diferenciación celular y al *CYP19A1* (Figura 10C) solo ocurrió en una etapa tardía que corresponde a los sinciciotrofoblastos. En contraste con los otros citocromos el *CYP17A1* se incrementó significativamente desde las 48 h de cultivo en presencia de calcitriol (Figura 10B). Por su parte, la expresión de ambas deshidrogenasas en presencia del calcitriol no fue diferente en los tiempos evaluados.

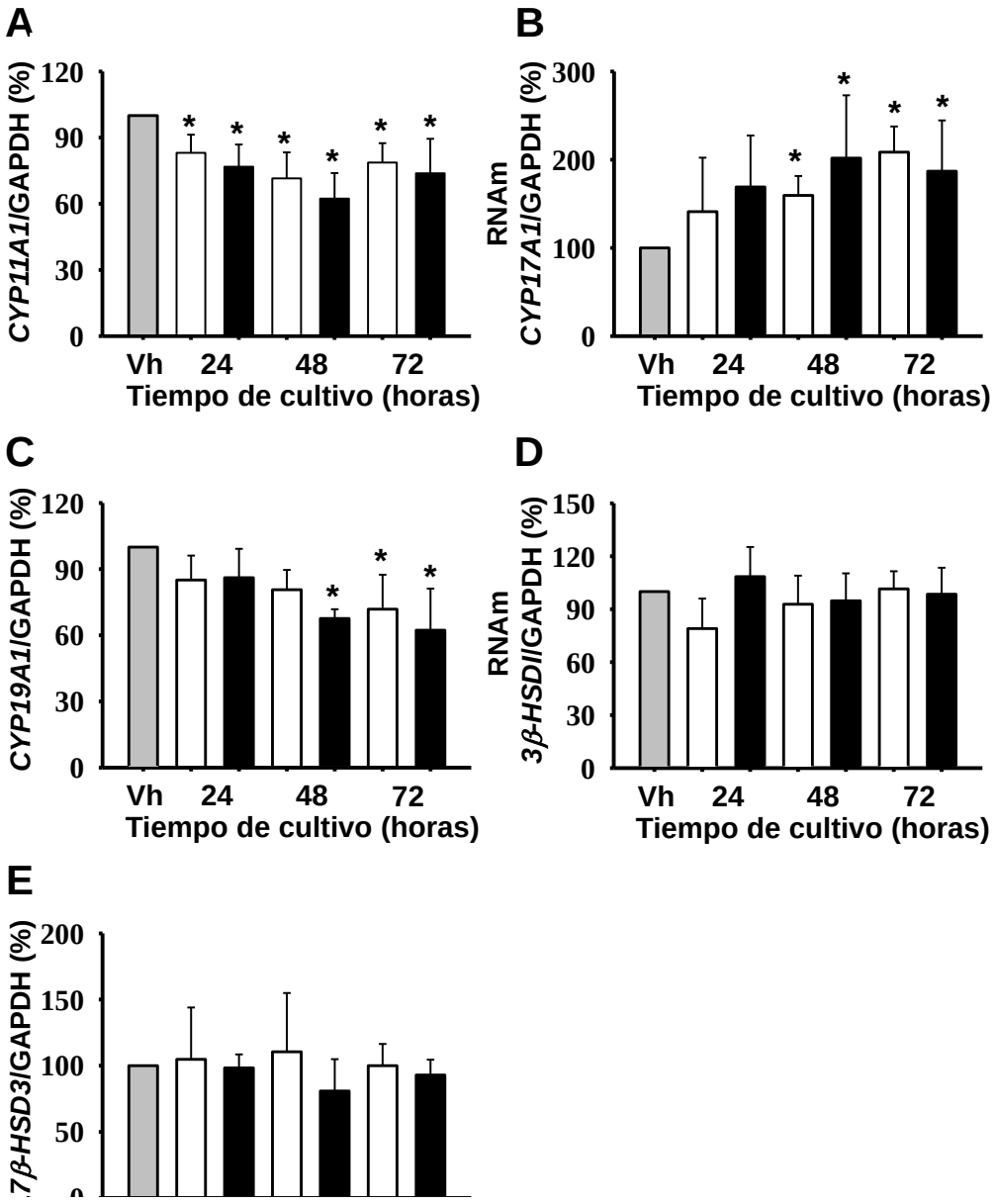

Figura 10. Efecto del calcitriol sobre la expresión de las enzimas esteroidogénicas durante la diferenciación de los trofoblastos.

La expresión génica se evaluó por RT-qPCR a las 24, 48 y 72 h de cultivo. Cada barra representa el promedio ± la D. E. de n $\geq$ 3. Los resultados son expresados como el porcentaje de cambio de las células tratadas con calcitriol 1 nM (barras blancas) y 100 nM (barras negras) *vs* vehículo (Vh) (barras grises), al que se le asignó el valor del 100 %. *P < 0.05 vs Vh.

8 DISCUSIÓN

El embarazo es una condición fisiológica asociada con cambios en las concentraciones de una amplia variedad de compuestos como hormonas esteroides, glicoproteicas, factores de crecimiento, citocinas y péptidos que garantizan el éxito de la gestación. En particular, las enzimas placentarias que median las fluctuaciones de los niveles hormonales durante este periodo juegan un papel central en la producción de cada esteroide para así asegurar la quiescencia uterina, la tolerancia inmunológica y la adaptación tanto de la madre como del feto durante el embarazo. Es por ello que nos resulta de gran relevancia evaluar el patrón de expresión basal de cada una de ellas e identificar y estudiar con más detalle a las moléculas implicadas en la regulación de estas enzimas en los trofoblastos.

Considerando lo anterior, en este trabajo se comparó la expresión génica basal de los CYPs (CYP11A1, CYP17A1 y CYP19A1) así como de las HSDs (3β-HSDI y 17β-HSD3) en sinciciotrofoblastos. En particular, el análisis de la expresión basal de los citocromos evaluados en nuestro modelo mostró que el mensajero del CYP19A1 y del CYP11A1 son más expresados comparados con el CYP17A1. En cuanto a las deshidrogenasas, la expresión de la 3β-HSDI fue mayor que la 17β-HSD3 y también significativamente más expresada que los demás citocromos. Estos resultados coinciden parcialmente con lo reportado por Pezzi y cols., [17] quienes encontraron una mayor expresión del CYP19A1 seguida por la 3β-HSDI, mientras que nosotros observamos lo contrario para estos dos genes. La discrepancia en la mayor presencia de una u otra enzima podría deberse a que las muestras procesadas fueron obtenidas con una metodología y en una edad gestacional distintas a las empleadas en el presente estudio. No obstante, se conserva el predominio de la expresión de las enzimas encargadas de biotransformar a la PREG hacia P_4 y del citocromo que biotransforma a los andrógenos hacia los correspondientes estrógenos siguiendo el patrón que se muestra a continuación:

3β-HSDI > CYP19A1 = CYP11A1> 17β-HSD ≥ CYP17A1

Adicionalmente, los resultados del patrón de expresión génica basal de los CYPs y de las HSDs durante el proceso de diferenciación de los trofoblastos sugieren que las cinco enzimas se expresan en los trofoblastos de placentas humanas a término tanto en los citotrofoblastos como en los sincicios. Además como se esperaba, la expresión génica de cada enzima se incrementó paulatinamente conforme fueron adquiriendo el fenotipo de agregados celulares multinucleados, lo cual coincide con estudios previos donde se ha reportado que los sinciciotrofoblastos son los principales productores de esteroides en la placenta [43, 94, 98]. Estos resultados junto a la producción de hCG muestran que los trofoblastos en cultivo conservan su fenotipo hormonal validando nuestro modelo para el propósito principal del estudio.

Por lo anterior, nos enfocamos a investigar el efecto del calcitriol sobre la expresión del gen y la abundancia de la proteína de los CYPs y las HSDs a diferentes tiempos de incubación. Los resultados mostraron que el calcitriol estimula la expresión génica del *CYP11A1*, *CYP19A1* y de la *3β-HSDI*. Estos hallazgos concuerdan con lo reportado previamente por nuestro grupo, quien demostró que el calcitriol favorece de manera dependiente de la concentración la bioconversión de PREG a P_4 y de androstendiona a E_2 [46]. Sin embargo, en ese trabajo se detectó un efecto bifásico dependiente del tiempo en la expresión del CYP11A1 y CYP19A1, encontrando que su expresión se inhibió a las 24 h de tratamiento mientras que el estímulo sobre la 3β-HSDI se perdió. Esta regulación diferencial se ha reportado también en otros modelos biológicos incluyendo líneas celulares de cerebro, de próstata y en células adrenocorticales [84, 86].

El efecto inhibitorio del calcitriol sobre sobre la expresión del CYP11A1 se ha descrito previamente en células T $CD8^+$ activadas con IL-4 [99]. En ese trabajo, los autores reportaron siete sitios de unión al receptor de la vitamina D con actividad transcripcional represora, lo cual justifica el efecto observado. Sin embargo, se

desconoce si un promotor similar pudiera ser el responsable del efecto observado en nuestro modelo experimental, lo que abre una alternativa de estudio para un futuro proyecto.

Curiosamente, aunque la expresión génica del CYP11A1 fue inhibida, la abundancia proteica no fue modificada. Estos resultados sugieren que alguna modificación post- transcripcional o traduccional podría estar regulando diferencialmente la expresión protéica. Resultados similares han sido reportados por Beaudoin y cols., quienes mostraron que aunque la actividad transcripciónal de la 3β-HSDI es inducida por P_4 y E_2 ninguna de las dos hormonas modificó la abundancia proteica de esta enzima [43]. Por lo tanto, nosotros sugerimos que después de la inducción de estos esteroides por el calcitriol [46], la expresión proteica podría ser preservada y la actividad enzimática afectada positivamente por mecanismos directos del calcitriol sobre la enzima. Lo anterior puede ser fortalecido considerando que el efecto observado está relacionado con la generación de segundos mensajeros y/o la síntesis de otras hormonas, como la síntesis de la P_4 inducida por el calcitriol, la cual se ha reportado que también incrementa tanto la expresión de la 3β-HSDI como la del CYP11A1 [43].

Con respecto a CYP19A1, el calcitriol inhibió su expresión después de 24 h, lo cual concuerda con algunos estudios en otros tejidos [56, 87, 91, 92]. Asimismo, la abundancia proteica de esta enzima se modificó de manera similar a su expresión génica. Nos resulta difícil explicar este efecto, sin embargo, se sabe que las enzimas; CYP11A1, 3β-HSDI y CYP19A1, son estimuladas por respuestas rápidas mediadas por segundos mensajeros como el AMPc y se ha reportado que el calcitriol estimula la producción del AMPc en tiempos cortos en los trofoblastos en cultivo [46]. Por lo tanto, al transcurrir el tiempo es probable que el efecto del calcitriol a través del AMPc disminuya y por lo tanto el estímulo a nivel transcripcional podría estarse perdiendo en tiempos mayores. Sin embargo, en un escenario *in vivo*, cabe la posibilidad de que el efecto inductor del calcitriol sobre esta enzima pueda mantenerse por la aportación constante de calcitriol a lo largo

de la gestación o ser mantenido por otras hormonas o factores del entorno local, así como, *in vivo,* por otros tipos celulares encontrados en el microambiente placentario lo cual, no descarta una modificación por el entrecruzamiento con otras vías de señalización.

Interesantemente, se ha demostrado que tanto el factor de crecimiento del endotelio vascular como la desamexatosona disminuyen la expresión génica del CYP19A1 sin afectar su actividad [66]. Por lo tanto, los cambios sobre la expresión génica no siempre son consistentes con aquellos observados en la actividad catalítica de la enzima, un fenómeno que al parecer también ocurre con el calcitriol y el CYP11A1.

Por otra parte, en este estudio también se evaluó la expresión génica del CYP17A1 y de la 17β-HSD3 dos enzimas que están estrechamente relacionadas en la biosíntesis de los andrógenos, mostrando que su transcrito se encuentran tanto en cito- como en sinciciotrofoblastos de placentas a término, aunque en menor proporción comparado con las otras enzimas evaluadas. En cuanto al efecto del calcitriol sobre la expresión génica del CYP17A1 y la 17β-HSD3, nuestros resultados demostraron que en tiempos cortos la expresión del CYP17A1 es inhibida mientras que en un lapso de 24 h es estimulada por el calcitriol.

El efecto inductor del calcitriol sobre este citocromo había sido previamente reportado en células adrenales por Lundqvist y cols., [86]. Sin embargo, los autores encontraron que a pesar de observar un aumento en la expresión génica de la enzima, la producción de andrógenos estaba disminuida. Los autores señalan que lo observado se debía a que el CYP17A1 media dos actividades diferentes; la actividad de 17α-hidroxilasa que es regulada a nivel de la expresión génica, y la actividad de la 17,20-liasa que es regulada por mecanismos post-transcripcionales [100]. Por lo tanto, la expresión incrementada del CYP17A1 por el calcitriol podría no resultar en más biosíntesis de andrógenos, sino estar relacionada con la producción de intermediarios como la 17α-hidroxipregnenolona

y/o la 17α-hidroxiprogesterona, metabolitos que podrían permanecer latentes y/o sulfoconjugados listos para cubrir los requerimientos de la madre, o bien para ser trasladados al compartimento materno o fetal para ser biotransformados por otra vía de síntesis hacia glucocorticoides [101-103]. En cuanto a la proteína del CYP17A1 esta no se pudo detectar en nuestro modelo, lo cual sugiere muy poca abundancia en las placentas a término.

Por otro lado, es conocido que los trofoblastos expresan varias isoenzimas de la 17β-HSD incluidas la 1, 2, 4, y 5 [98, 104, 105]. Sin embargo, a la fecha del estudio no se había reportado la presencia de la 17β-HSD3 en este tejido. Estos resultados son importantes debido a que reportes en la literatura indican que la 17β-HSD3 es quien cataliza el último paso en la biosíntesis de la testosterona, reduciendo el grupo ceto de la 4-androstene-3,17-diona a grupo hidroxilo [8]. De hecho, se ha reportado que deficiencias en su expresión durante la gestación impactan en la formación de la testosterona en los testículos fetales y en el proceso de la diferenciación sexual del feto [106]. Cabe destacar que en nuestro cultivo primario de trofoblastos mostramos que la 17β-HSD3 no solo se expresa sino que su expresión aumenta junto con el proceso de diferenciación.

De los resultados anteriores no se descarta totalmente que la producción de andrógenos sea posible en la placenta, los cuales aunque en limitadas cantidades pudieran tener alguna posible acción biológica como ha sido sugerido por trabajos previos [102, 107]. Además, los resultados de que la expresión génica de estas enzimas es baja apoyan la hipótesis de que su presencia es insuficiente para asegurar que se produzcan andrógenos en cantidades significativas que pudieran afectar la diferenciación del feto y más bien lo que se llegara a producir transitoriamente serían sustrato inmediato de la enzima aromatasa, quien se encuentra abundantemente en la placenta, para convertir los compuestos androgénicos en los correspondientes estrógenos.

9 CONCLUSIONES

1. La diferenciación celular hacia sinciciotrofoblastos favorece la expresión basal de todas las enzimas.

2. Las enzimas *CYP11A1, CYP19A1* y *3β-HSDI* fueron las de mayor expresión génica basal en los trofoblastos.

3. El *CYP11A1, CYP19A1* y *3β-HSDI* son inducidas por el calcitriol a tiempos cortos.

4. La regulación de la expresión de los genes no corresponde, forzosamente, a lo observado en la proteína.

5. El efecto del calcitriol sobre las enzimas evaluadas es diferencial, dependiente del tiempo y del fenotipo celular.

En conclusión, se mostró que el calcitriol es un regulador *in vitro*, de la expresión de las enzimas involucradas en la producción de hormonas esteroides en los trofoblastos, por lo que sugerimos que la continua e incrementada producción de calcitriol durante el embarazo, podría preservar la regulación inicial de la expresión de las enzimas en estudio. Por lo anterior, la suplementación con vitamina D, en aquellas pacientes deficientes de esta hormona, podría ofrecer una alternativa con acción terapéutica natural para pacientes con desequilibrio en su biosíntesis hormonal para restablecer el balance endócrino y así prevenir o aminorar complicaciones en el embarazo como los abortos recurrentes, los partos pre-término y la preeclampsia, que son enfermedades asociadas con el desbalance hormonal.

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ANEXOS

Anexo 1. Controles Experimentales

Curva de concentraciones de calcitriol.

El calcitriol disminuye de manera dependiente de la concentración la expresión de CYP19A1 en el cultivo primario de trofoblastos. Con base en estos resultados se eligieron las concentraciones de trabajo. La expresión se evaluó por qPCR a las 24 h de cultivo.

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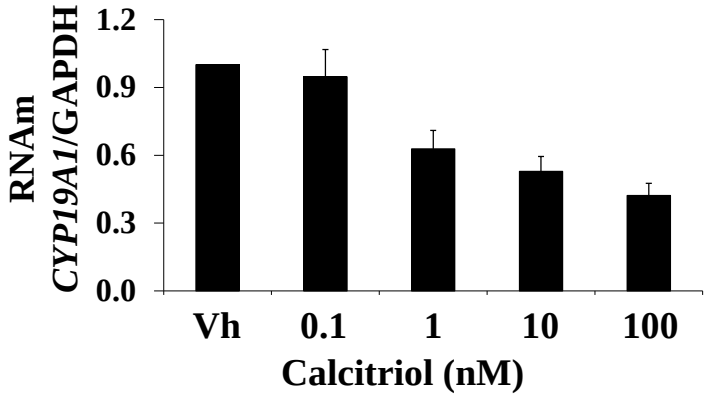
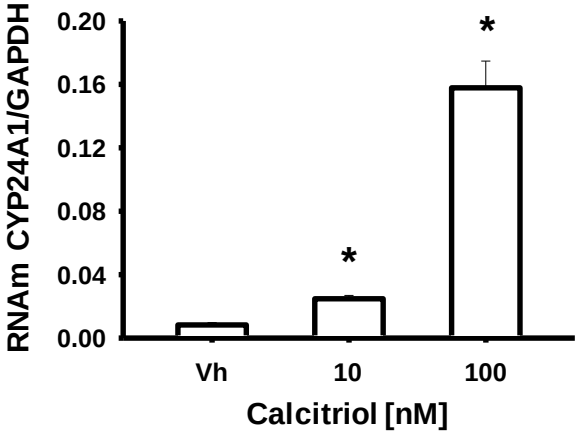


Control de funcionalidad del calcitriol en los cultivos de trofoblastos

El calcitriol estimula la expresión de CYP24A1 en los trofoblastos. La expresión se evaluó por qPCR a las 24 h de cultivo.



Anexo 2. Articulo requerido para la obtención del grado de Doctora en Ciencias

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A time-course regulatory and kinetic expression study of steroid metabolizing enzymes by calcitriol in primary cultured human placental cells

Nancy Noyola-Martínez[a], Ali Halhali[a], Verónica Zaga-Clavellina[b], Andrea Olmos-Ortiz[a], Fernando Larrea[a], David Barrera[a,*]

[a] Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Av. Vasco de Quiroga No. 15, Col. Belisario Domínguez, Sección XVI, México, D.F. 14080, Mexico
[b] Departamento de Inmunobioquímica, Instituto Nacional de Perinatología Isidro Espinosa de los Reyes, Montes Urales No. 800, Miguel Hidalgo 11000, México, D.F., Mexico

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ABSTRACT

1,25-dihydroxivitamin D_3 (calcitriol), is a secoesteroid involved in several placental functions. In particular, we and others showed that calcitriol regulates peptides, proteins, cytokines and hormones production in human trophoblastic cells. On the other hand, calcitriol modifies the activity and expression of some steroidogenic enzymes, a process that is considered tissue-specific. However, the effects of calcitriol on the expression of enzymes involved in the synthesis of sex steroids in placental tissue have not yet been entirely studied. The aim of the present study was to investigate the effects of calcitriol upon gene expression of several steroid enzymes such as cytochrome P450scc (*CYP11A1*), type 1 3β-hydroxysteroid dehydrogenase(*3β-HSDI*), *17β-HSD3*, 17α-hydroxylase/17,20 lyase (*CYP17A1*) and aromatase (*CYP19A1*) in primary cultures of human placental cells. Cell cultures were performed using placentas obtained immediately after delivery by caesarean section from normotensive healthy women and calcitriol effects were evaluated, at level of transcription, by qPCR. The results showed that: 1) from basal expression values of the five genes studied, 3β-HSDI was the most expressed gene ($P < 0.05$); 2) basal expression of all enzymes was significantly higher in cultured syncytiotrophoblast than in cytotrophoblasts ($P < 0.05$); 3) the presence of calcitriol in cultured trophoblast cells generally resulted in a stimulatory effect of *CYP11A1*, *CYP19A1* and *17β-HSD3* gene expression at 3 h of treatment whereas *3β-HSDI* was induced at 6 h ($P < 0.05$). However, a time-dependent variable was also observed; 4) protein expression of CYP11A1 and 3β-HSDI were not modified significantly by calcitriol, however that of CYP19A1 was regulated in similar fashion as gene expression. In conclusion, calcitriol affected in a time-dependent manner the expression of steroids metabolizing enzymes in human placental cell cultures.

1. Introduction

Calcitriol, is a secosteroid with both calcemic and non-calcemic biological activities [1]. Similar to steroids hormones, calcitriol is metabolized by human placenta, a tissue that is also considered a target for its hormonal actions. Indeed, this hormone regulates the expression or production of calbindins, cytokines, antimicrobial peptides as well as human chorionic gonadotropin (hCG), progesterone (P_4) and estradiol (E_2) in enriched trophoblastic cell preparations (ETC) [2–5]. In addition, it is well known that calcitriol differentially regulates CYP27B1 and CYP24A1, the two cytochromes involved in calcitriol synthesis and metabolism [6]. Likewise, its role in the regulation of other steroidogenic enzymes in a tissue-specific manner has been also reported [7].

Steroid hormones are synthetized from a common precursor called cholesterol and the steroidogenic enzymes involved are members of both the cytochrome (CYP) P450 superfamily and hydroxysteroid dehydrogenases (HSD) [8,9]. During pregnancy, the fluctuations of maternal steroids depend mainly of the placenta, and the major pathways involved in their synthesis have been well established [8]. In human placenta, after the conversion of

* Corresponding author at: Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, Tlalpan 14000, México, D.F, Mexico.
E-mail addresses: barrera1912@gmail.com, vidadav@hotmail.com (D. Barrera).

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cholesterol to pregnenolone by cytochrome P450 cholesterol side chain cleavage (P450scc; CYP11A1), pregnenolone is bioconverted mainly to P_4 by means of the type 1 3β-hydroxysteroid dehydrogenase (3β-HSDI). On the other hand, it well known that androgens from fetal tissues are necessary to produce the corresponding estrogens in the placenta means aromatase enzyme (CYP19A1). However, it has been showed *in vitro* the placental capability to express and synthetized androgens *de novo* by 17α-hydroxylase/17,20 lyase (CYP17A1) presence, a topic that is still discussed controversially [10,11].

Steroidogenic enzymes regulation is very complex [8,12], and the identification of factors regulating their expression has long been sought. At this respect, it has been demonstrated that some enzymes are target of the biological effects of calcitriol. In fact, it has been showed that calcitriol increased the 3β-HSDI activity in granulosa cells [13] and induces *3β-HSDI* and *CYP11A1* transcription in human glioma GI-1 cells [14]. In addition, it has been demonstrated that calcitriol up regulates *17β-HSD* type 2, type 4 and type 5 gene transcription in human prostate cancer lines cells, and both *CYP11A1* and *CYP17A1* in human adrenocortical NCI-H295R cells [15–17]. On the other hand, it has been shown that calcitriol stimulates aromatase activity in diverse cell types such as prostate, osteoblasts, human choriocarcinoma, purified immature rat Sertoli and rat granulosa cells [18–21]. Interestingly, Kinuta et al., [22] have showed that the activity and gene expression of *Cyp19A1* are decreased in the ovary, testis and epididymis of VDR null mutant mice, which indicates that calcitriol is an additional stimulator of aromatase. However, the effects of calcitriol on aromatase gene expression and enzyme activity are controversial, since is mainly inhibitory in breast cancer cells and human macrophages [7,23]. These findings indicate the differential tissue-specific effects of calcitriol.

We have previously shown that calcitriol induce P_4 and E_2 secretion in ETC [2]; however, its effects on the enzymes involved in this process have not been studied. Therefore, the aim of this study was to investigate the effects of calcitriol on expression of steroidogenic enzymes in primary cultures of human placental cells.

2. Materials and methods

2.1. Reagents

Culture media, fetal bovine serum (FBS), Trizol and all oligonucleotides for real time polymerase chain reaction (PCR) were by Invitrogen (CA, USA). Light Cycler® 480 probes master kit, TaqMan Master reaction, TaqMan probes, 96 well PCR microplates, the reverse transcription (RT) system and Proteases inhibitor cocktail were from Roche (Roche Applied Science and Roche Diagnostics, IN, USA). Calcitriol (1α,25-dihydroxycholecalciferol) was kindly donated from Hoffmann-La Roche Ltd (Basel, Switzerland). Deoxyribonuclease I and trypsin enzymes from bovine pancreas used for cell cultures were from Sigma-Aldrich (MO, USA). Percoll was from GE Healthcare (Uppsala, Sweden). CYP11A1 and CYP19A1 antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) whereas CYP17A1 and 3β-HSDI antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and GAPDH antibody was from Millipore (Temecula, CA, USA).

2.2. Trophoblast cell culture

This protocol was approved by the Human Research Ethics Committee from the Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán (No. BRE-535-12/15-1) and the Internal Review Board of the Instituto Nacional de Perinatología "Isidro Espinosa de los Reyes" in Mexico City (No. 212250-21131). Written informed consent was obtained from all participants. All pregnant women were from an urban area of Mexico City, 18–39 years old, previously normotensive, with no history of diabetes mellitus or thyroid, liver, renal disease. Term placentae (39–41 weeks of gestation) were acquired following caesarean section. All women had uncomplicated pregnancies, without evidence of active labor, cervical dilation or loss of the mucus plug. In addition, none had any clinical or microbiological signs of chorioamnionitis or lower genital tract infection; twin pregnancy was excluded from this study.

Placental cotyledons were dissected free of decidua and fetal membranes. The ETC were cultured as previously described [6,19]. Briefly: Villous tissue was enzymatically dispersed and cells were separated on density percoll gradients. Before plating, the viability of the percoll-enriched cells was estimated by dye exclusion (0.4% trypan-blue). Cells were plated at a density of 3×10^6 cells in flasks of 25 cm^2 with 4 mL each of supplemented medium [(DMEM HG) 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 mg/ml Fungizone], containing 10% heat-inactivated-FBS. Incubations were performed in humidified 5% CO_2-95% air at 37 °C. In order to remove all non-adherent cells, after 3 h to incubation, culture medium was aspirated and trophoblasts cells were washing with Hank's Balanced Salt Solution (HBSS) 1X. Treatments with calcitriol (1 or 100 nM) or its vehicle (ethanol 0.1%) were added in supplemented medium F-12 with penicillin and streptomycin. Afterward, RNA was extracted from cells for gene expression studies. Expression of human chorionic gonadotropin (hCGβ5) was used as control of the cell culture system and CYP24A1 gene as control of calcitriol function.

2.3. Calcitriol effects on CYP11A1, CYP17A1, CYP19A1, 3β-HSDI and 17β-HSD3 gene expression

Calcitriol effects upon gene expression were studied by extracting total RNA from treated cells using Trizol reagent [24]. In all cases, the amount and quality of RNA were estimated spectrophotometrically at 260/280 nm and a constant amount of RNA (2 μg) was reverse transcribed using a RT assay. Primers and probes for PCR amplifications are shown in Table 1. Identical PCR conditions were performed for all genes and in all cases normalized against glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) used as housekeeping gene.

Real time PCR amplifications were carried on a LightCycler® 480 II (Roche), as described below: The specific probe for each gene was added to a reaction mixture with 5.2 μL of PCR grade water, 0.1 μL of primer corresponding to the gene of interest [20 μM] and 3.5 μL of 2X enzyme. The mixture was homogenized and placed in plates of 96 wells to PCR. In each well 9 μL of the mixture and 1 μL cDNA are placed. The plate was then sealed and centrifuged at 1000 rpm for 30 s. Finally, the plate was placed in the thermocycler LightCycler480 II, where occurred activation of Taq DNA polymerase and DNA denaturation at 95 °C for 10 min, proceeded by 45 amplification cycles of 10 s at 95 °C, 30 s at 60 °C, and 1 s at 72 °C.

2.4. Calcitriol effects upon CYP11A1, CYP17A1, CYP19A1 and 3β-HSDI protein expression

In order to evaluate the calcitriol effects upon CYP11A1, CYP17A1, CYP19A1 *and* 3β-HSDI protein expression these were studied by Western blot. Briefly: Syncytiotrophoblast were incubated in the absence or presence of calcitriol 100 nM during 6 and 24 h. Afterwards cells were pelleted and lysed with RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mMNaCl, 1% Nonidet P-40, 0.1% SDS, pH 7.4) in presence of a proteases inhibitor cocktail. Protein content

Table 1
Oligonucleotides and probes used for real time PCR analysis.

Gen	Upper primer	Lower primer	Amplicon (nt)	[a]Probe	Accession Number
CYP11A1	aggaggggtggacacgac	ttgcgtgccatctcataca	60	59	M14565.1
CYP17A1	gcatcatagacaacctgagcaa	gggttttgttggggaaaatc	75	64	NM_000102.3
CYP19A1	gaattcatgcgagtctggatct	tcattatgtggaacatacttgagga	76	55	NM_000103.2
3β-HSDI	cggaccagaattgagagagg	gaatggctcatccagaattc	88	11	M35493.1
17β-HSD3	aacttgcaggcttagaaattgg	ggtgcgttcaggaaatgg	85	7	NM_000197.1
CYP24A1	catcatggccatcaaaacaa	gcagctcgactggagtgac	65	88	NM_000782.3
hCGβ5	gctcaccccagcatcctat	cagcagcaacagcagcag	131	70	NM_000737.2
GAPDH	agccacatcgctcagacac	gcccaatacgaccaaatcc	66	60	NM_002046.3

[a] From the universal probe library (Roche).

was determined in the cell lysates and equal amounts (30 μg protein) were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and blocked overnight in TBST (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 5% skim milk. Then membranes were washed and incubated overnight at room temperature in the presence of anti-CYP11A1 (50 kDa) or anti-CYP19A1 (51 kDa) diluted 1:1000, or anti-CYP17A1 (55 kDa) or anti-3β-HSD (42 kDa) diluted 1:100 and 1:200 respectively, in TBST. After washing membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:8000), followed by chemiluminescent detection with Clarity Western ECL substrate (Bio-Rad laboratories, USA) according to the manufacturer's instructions. Visualization was achieved by Molecular Imager ChemiDoc XRS System and the densitometry was performed by Imagen Lab Software (Bio-Rad, Hercules, California, USA). The membranes was washed and incubated with Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific Inc. Rockford, IL, USA) to follow use with further antibody. For normalization, blots were incubated with anti-GAPDH (32 kDa, 1:8000) and processed as described above.

2.5. Statistical analysis

The results were described as the mean ± standard deviation (S. D.). Statistical differences for dose-response assays were determined by one-way ANOVA followed by appropriate post hoc tests (Holm-Sidak method for pair-wise comparisons), using a specialized software package (SigmaPlot 11.0, Jandel Scientific). For each experiment the final result was calculated considering the mean of triplicate from at least three separated cell cultures. Differences were considered statistically significant at $P < 0.05$.

3. Results

Trophoblast cells were seeded (3×10^6 cells per flask) and during three days of culture a progressive increase of *hCGβ5* gene expression was observed and used as a biological marker of placental differentiation from cytotrophoblasts to syncytiotrophoblast. Likewise, gene expression level of *CYP24A1* in ETC was used as a control of calcitriol bioactivity, as previously described [25].

3.1. Comparison of basal gene expression profiles of CYP11A1, CYP17A1, CYP19A1, 3β-HSDI and 17β-HSD3 enzymes in ETC

Fig. 1, shows the basal gene expression of each one of the steroidogenic enzymes studied. As depicted,the *3β-HSDI* transcript was the one with the highest ($P < 0.05$) expression level when compared with the other transcripts. In general, the expression profile level of these genes was as follows: *3β-HSDI* > *CYP19A1* ≥ *CYP11A1* > *17β-HSD3* ≥ *CYP17A1*.

3.2. Basal gene expression profiles during cell differentiation

Gene expression of steroidogenic enzymes was also studied during the differentiation process of trophoblast cells in culture. As shown in Fig. 2, 3 of the 5 enzymes showed a significantly higher expression on days 2 and 3 of cultures ($P < 0.05$ *vs* day 1). Gene expression of *CYP11A1* and *CYP17A1* reached a maximum and significant higher value at 72 h of plating ($P < 0.05$; Fig. 2).

3.3. Calcitriol effects upon gene expression of steroidogenic enzymes during 3, 6, 12 and 24 h of treatment in syncytiotrophoblast

Considering previous results from our laboratory, we decided to study the effects of calcitriol on gene expression of all enzymes at two different concentrations (1 and 100 nM) and for various times of incubation (3, 6, 12 and 24 h) as shown in Fig. 3. As depicted, after 3 h of treatment, 100 nM of calcitriol upregulated *CYP11A1* and *CYP19A1* gene expression whereas *CYP17A1* was down regulated after 6 h (Fig. 3A–C). Meanwhile,the relative gene expression of *3β-HSDI* was significantly increased around 6 h and *17β-HSD3* mRNA shown a biphasic effect between 3 and 6 h. On contrary, between 12 and 24 h of calcitriol treatment, *CYP11A1* and *CYP19A1* transcripts were decreased and *CYP17A1* gene transcription was increased whereas that the expression both *3β-* and *17β-HSDs* was stabilized since no further effects of calcitriol were observed after 12 h (Fig. 3D, E).

3.4. Calcitriol effects upon CYP11A1, CYP17A1, CYP19A1 and 3β-HSDI protein expression after 6 and 24 h of treatment in syncytiotrophoblasts

Calcitriol effects upon protein abundance of CYP11A1, CYP19A1 *and* 3β-HSDI are shown in Fig. 4. Protein expression of CYP11A1 and 3β-HSDI was not affected by treatment at 6 and 24 h (Fig. 4A

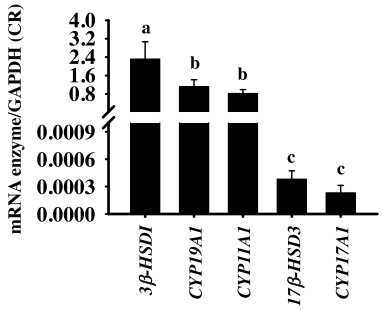

Fig. 1. Placental basal gene expression of steroidogenic enzymes. The data are expressed as concentration ratio (CR). Bars represent the data of at least 4 separated cell cultures. Different letters indicate significant differences at the $P < 0.05$.

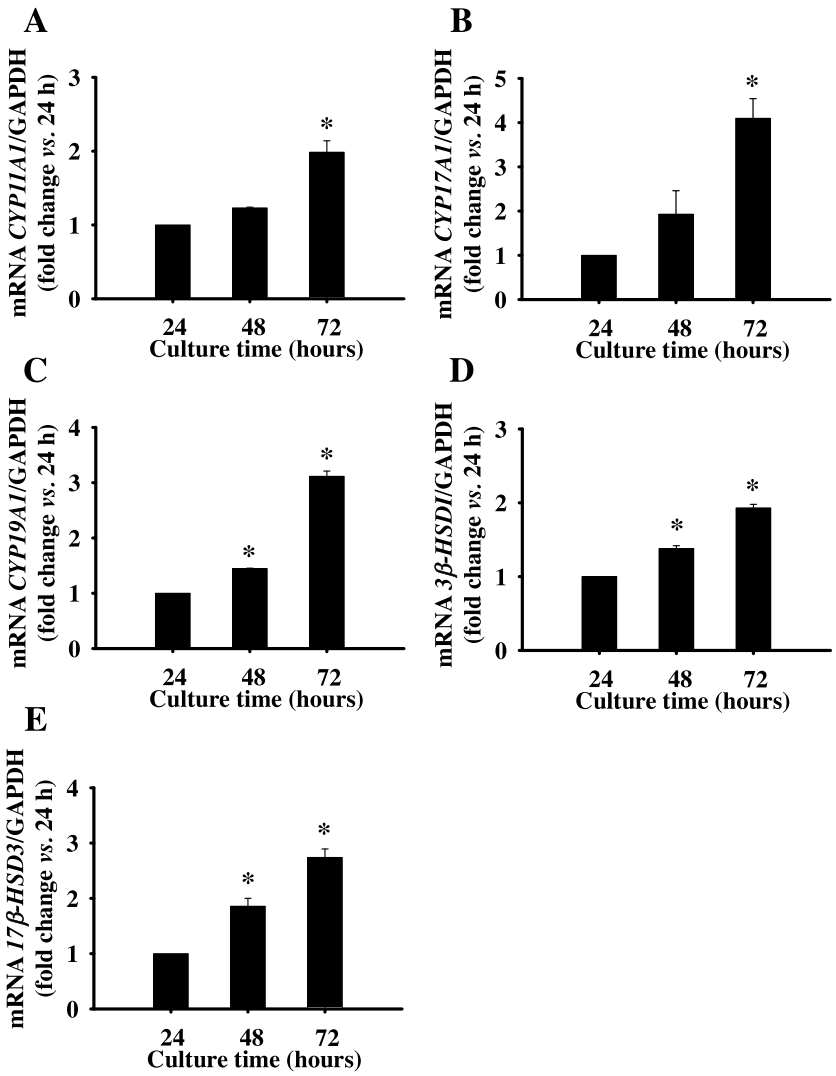

Fig. 2. Placental gene expression profiles of the indicated steroidogenic enzymes during differentiation process of trophoblast cell cultures. The data are expressed as fold changes as compared to 24 h of culture which value was arbitrarily set to 1. Bars represent the data of at least 3 separated cell cultures. $^{*}P < 0.05$.

and C). However, the abundance of CYP19A1 was increased at 6 h and decreased at 24 h of incubation which was in accordance with gene expression of this enzyme at the same times of treatment (Fig. 4B). Regarding CYP17A1, its protein expression was not detected using the methodology used in this study (data no shown).

3.5. Calcitriol effect upon gene expression of enzymes during each day of differentiation

Fig. 5 shows the role of the cell differentiation process on the calcitriol effects on gene expression of the enzymes studied. As depicted, calcitriol at two doses (1 nM and 100 nM) was added at 3, 24 and 48 h of culture times that corresponded to cytotrophoblasts transition into syncytiotrophoblast. At each time, calcitriol treatment was incubated for 24 h and gene expression of each enzyme was analyzed. As shown in Fig. 5, calcitriol at 1 nM and 100 nM concentrations decreased *CYP11A1* in each stage of cell differentiation. Similarly, *CYP19A1* was inhibited but only in the syncytiotrophoblast while *CYP17A1* transcription was upregulated also in this last phenotype cell. Regarding the dehydrogenases, their expression was not significantly affected at any dose of calcitriol and the day of cell culture differentiation studied.

4. Discussion

Human pregnancy is a physiological condition associated with several changes in the concentrations of maternal and fetal-placental hormones, cytokines and peptides, among others, that ensure and guarantee the maintenance of gestation. Specifically, steroidogenic enzymes in placental tissue plays a pivotal role to produce and increase in steroid hormones levels, which assure uterine quiescence, immunological tolerance and adaptation during this period [26–29]. Therefore, the regulation of these factors, particularly in placenta deserves to be further studied.

It has been shown that syncytiotrophoblast cells are the main steroidogenic cell type in placenta [30–32]. This is in line with our results, where this structure had significantly higher enzymatic expression than that observed in cytotrophoblasts. In this study, the basal level of gene expression of the cytochromes being investigated was as follows: *CYP19A1* ≥ *CYP11A1* > *CYP17A1*, these results are in accordance with those reported by Pezzi et al. [11]. Regarding the dehydrogenases, the expression of *3β-HSDI* was significantly higher than that of *17β-HSD3* but in contrast to Pezzi et al.*3β-HSDI* was also more expressed than the three other cytochromes evaluated. The discrepancy may be due to differences in gestational age and preparation of the biological samples.

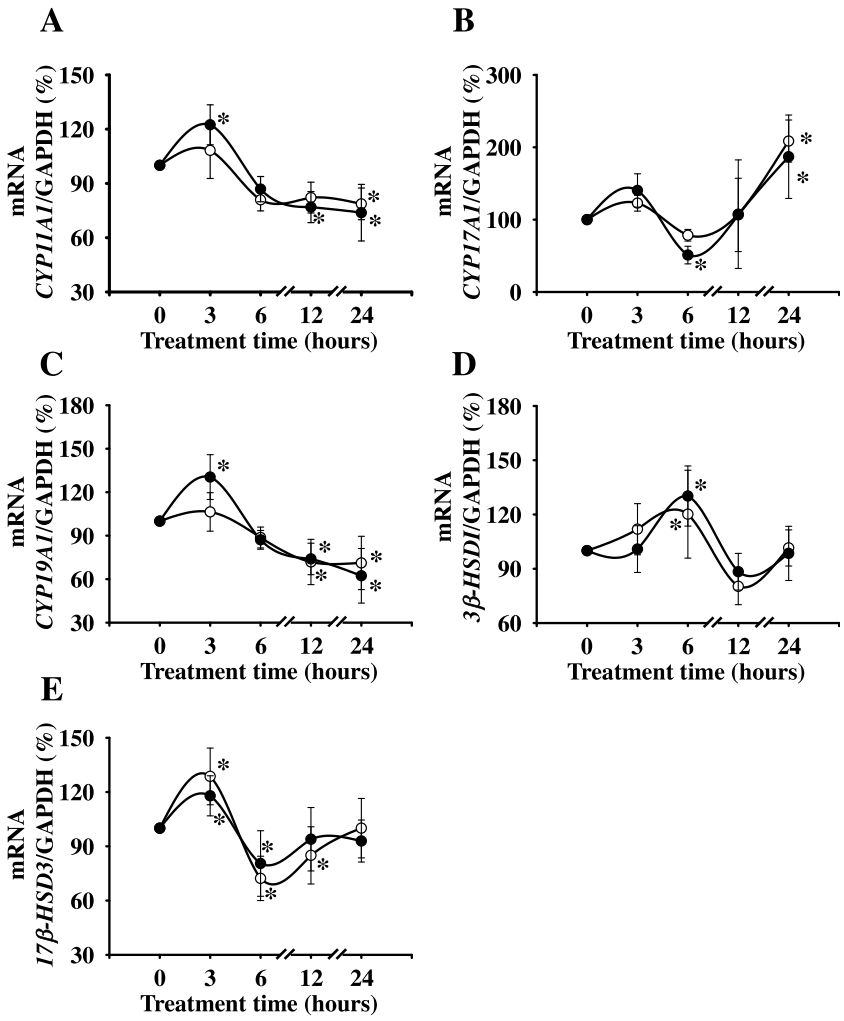

Fig. 3. Time-dependent effect of calcitriol treatment upon gene expression of the indicated steroidogenic enzymes in syncytiotrophoblast cells. Calcitriol 1 nM (open circles) and 100 nM (closed circles). The data are expressed as percent changes as compared to 0 h of treatment which value was arbitrarily set to 100. Bars represent the data of at least 3 separated cell cultures. *$P < 0.05$.

Indeed, we used cultured ETC obtained from term placentas while Pezzi et al. performed their study in placental homogenates obtained at 15–19 weeks of gestation after abortion induction. On the other hand, it has been demonstrated that placenta expresses several *17β-HSD* isozymes [32–34], but not the *17β-HSD3*. Interestingly, in our ETC culture model, we demonstrated that *17β-HSD3* is not only expressed but its expression increased along with the differentiation process. Overall, the results in the present study demonstrated that *CYP11A1*, *CYP19A1* and *3β-HSDI* are highly expressed in human placenta to provide steroids hormones such as P_4 and E_2 which are essential for the maintenance and adequate outcome of pregnancy [27,28].

These results demonstrated the hormonal phenotype of our culture and validated its use for the purposes of this study. The aim of this study was to investigate the role of calcitriol upon gene expression regulation of key steroidogenic enzymes involved in the synthesis of some steroids produced during normal pregnancy. It has previously shown the role of calcitriol in steroidogenesis; therefore, we thought of importance to evaluate if this hormone might be also involved in gene expression regulation of steroidogenic enzymes throughout different stages of placenta cell differentiation in culture and times of incubation.

The results in this study demonstrated that calcitriol upregulates *CYP11A1*, *CYP19A1* and *3β-HSDI* gene expression. In this regard, these results agreed with those previously reported by our laboratory showing the ability of calcitriol to stimulate in a dose-dependent manner both P_4 and E_2 secretion in ETC [2]. However, this effect was time-dependent according to the enzyme been studied. Similar results have been observed in other cell types from different sources, including established cell lines from brain and adrenocortical cells [14,17]. Interestingly enough was the observation of the down regulatory effect of calcitriol on *CYP11A1* and *CYP19A1* gene expression at 24 h of treatment. This inhibitory effect of calcitriol upon *CYP11A1* expression has been previously observed in mice IL-4-activated $CD8^+$ T cells by Schedel et al. [35]. These authors reported seven potential VDR-binding sites with repressor transcriptional VDR-dependent activity. Whether a similar promoter behavior is responsible of the inhibitory effects of calcitriol on *CYP11A1* and *CYP19A1* in this study deserves to be further investigated. Although the *CYP11A1* gene expression was altered, the protein abundance of this enzyme was not modified. This result may indicate that some translational mechanisms could be differentially regulated. Similarly, it has been reported by Beaudoin et al. [31] that P_4 and E_2 induce both *3β-HSDI* and

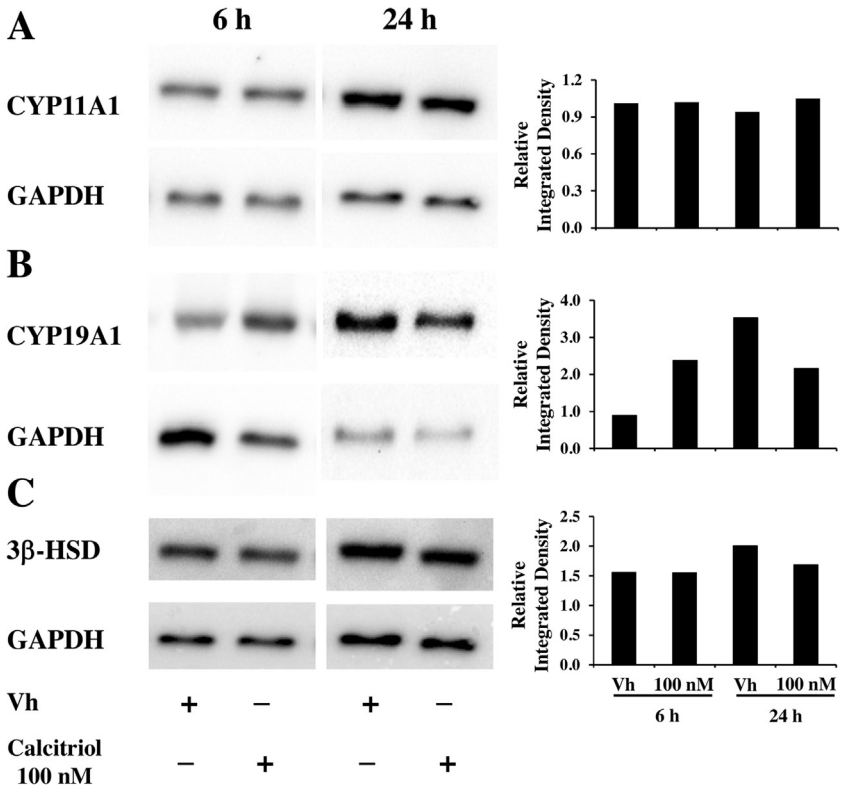

Fig. 4. Calcitriol effects upon syncytiotrophoblasts protein expression of the indicated steroidogenic enzymes at 6 and 24 h of treatment. Figures are representative of three independent experiments. Figures showed the abundance of proteins (left panel) and their respective relative integrated density (right panel).

CYP11A1 gene transcription but not the protein abundance of 3β-HSDI. Therefore, we suggest that after the induction of these steroid hormones by calcitriol [2], protein expression of these key enzymes could be preserved and the enzymatic activity affected positively.

In addition to its importance in steroid hormones biosynthesis, Slominski et al. [36] have demonstrated the participation of CYP11A1 in novels activities since this enzyme also metabolizes 7-dehydrocholesterol (7DHC), ergosterol, lumisterol 3 and hydroxylates the side chain of vitamin D_3 and D_2 [37–40]. In fact, it has been shown that this enzyme is able to convert vitamin D_3 to 20-hydroxyvitamin D_3 and 20,22-dihydroxyvitamin D_3 in female rat adrenal glands and human term placenta as well as vitamin D_2 form into 20-hydroxyvitamin D_2, 20,22-dihydroxyvitamin D_2 and 1,20-dihydroxyvitamin D_2 in several cells types such as epidermal keratinocytes, Caco-2 colon cells, adrenal glands and also in human placenta [36,37,41,42].

Regarding CYP19A1, its gene expression was inhibited after 24 h of treatment with calcitriol which is in accordance with others studies [7,18,23,43]. Interestingly, in the present study protein abundance of this enzyme was modified by calcitriol in similar fashion as its gene expression. In *in vivo* conditions, we cannot discard a modification of this effect of calcitriol upon this enzyme by the presence of others hormones, local environment factors, as well as others cells types found in human placenta. This possibility is not unlikely since both calcitriol and estrogen circulating levels increase during healthy pregnancy [44,45].

Additionally, we identified *CYP17A1* gene transcripts in both cyto- and syncytio-trophoblasts as previously reported [10,46,47]. However, the placental *CYP17A1* and *17β-HSD3* gene expression are very low in comparison with the others enzymes, which suggest minor production of androgens that could be biotransformed immediately to estrogens by CYP19A1 [10]. Indeed, calcitriol did induce *CYP17A1* mRNA after 24 h of treatment. This result is similar to the stimulatory effect previously described of calcitriol upon *CYP17A1* expression in adrenal cells; however those authors found that androgen production was decreased [17]. It is well known that *CYP17A1* mediates two different activities, 17α-hydroxylase activity that is regulated by *CYP17A1* gene expression and the 17,20-lyase activity that is regulated by post-transcriptional mechanisms [48]. Therefore, increased gene expression of *CYP17A1* observed in the present study by calcitriol could not result in more androgens synthesis but to be related also to production of 17α-hydroxypregnenolone and/or 17α-hydroxyprogesterone that are formed as intermediates to glucocorticoids [8,49–52], which merits further investigation.

Identifying the factors and molecules that regulate the steroideogenesis in the placental tissue is of importance, since pregnancy is dependent of steroids produced by this organ. In fact, several adverse pregnancy outcomes are associated with hormonal unbalance such as miscarried, preeclampsia and preterm delivery. In particular, it has been demonstrated in preeclamptic women that the androgens and estrogens production is abnormal as compared with normotensive pregnancy [53,54]. In addition, in this pathology the vitamin D status has been found deficient [55] and it has been demonstrated that the supplementation with vitamin D diminishes the risk of preeclampsia development.

In summary, we demonstrated that calcitriol is a placental regulator of expression of the enzymes involved in the sex steroids production in ETC and we suggest that continuous and increased production of calcitriol during pregnancy could preserve the initial regulation of the expression of these enzymes and that vitamin D supplementation could offer an alternative therapeutic action for patients with hormonal imbalance.

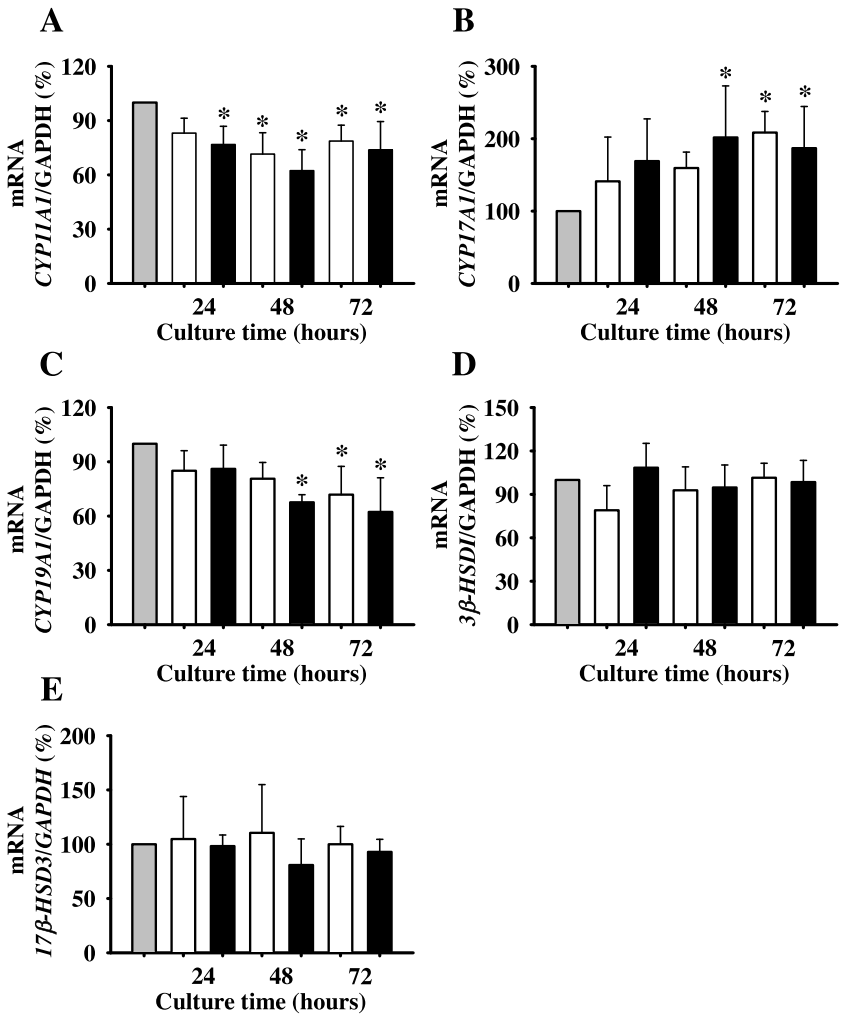

Fig. 5. Time-dependent effect of calcitriol treatment upon gene expression of the indicated steroidogenic enzymes during trophoblast cells differentiation. Calcitriol 1 nM (white bars) and 100 nM (black bars). Results are expressed as percent change of cells treated *vs* Vh (grey bars), which was assigned a value of 100%. $^{*}P < 0.05$ *vs* Vh. Bars represent the data of at least 3 separated cell cultures.

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Anexo 3. Artículos publicados durante los estudios de doctorado

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STEROID HORMONES AND PREGNANCY

Steroid hormones and pregnancy

Nancy Noyola-Martínez, Ali Halhali and David Barrera

Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, D.F. México, México

ABSTRACT

Pregnancy is associated with physiological adjustments in order to allow adequate growth and fetal development. In particular, steroids are necessary to maintain in balance numerous functions during gestation. Steroidogenesis in the maternal, placental and fetal compartments and the biological effects of progestins and estrogens that play a pivotal role before and during pregnancy are described. Although it is well-known that androgens are considered as substrate for estrogens biosynthesis, their biosynthesis and functionality in placental and other tissues have been questioned. As compared with healthy pregnancy, steroid hormones levels have been found altered in complicated pregnancies and hormonal treatments have been used is some pathologies. Therefore, the aim of this work was to review the biosynthesis, function and regulation of progestins, androgens and estrogens during gestation. Furthermore, steroid hormones concentrations during healthy and complicated pregnancy as well hormonal therapies for the prevention of miscarriages and preterm deliveries are discussed in the present review.

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Pregnancy; steroidogenic enzymes; endocrine disorder; hormonal therapies

Generalities of steroid hormones

Steroids are oxidized derivatives of cholesterol. These molecules share as a structural characteristic a tetracyclic nucleus, formed by three cyclohexanes and one cyclopentane. These hormones differ among them mainly in their number of carbon atoms attached to the four-ring structure, their functional groups and oxidation states that give them different biochemical functions and biological specificity and the steroidogenic enzymes involved in their synthesis are well-established [1].

In human male and female, pregnenolone (PREG), synthetized from cholesterol, is the substrate for gonadal production of progestins, androgens and estrogens or adrenal glucocorticoids and mineralocorticoids. During pregnancy, the adrenal steroids are important for fetal maturation and growth. Interestingly, placenta expresses 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) that inactivate maternal cortisol and corticosterone, avoiding fetal and placental disorders [2,3].

Classical route of sex steroid biosynthesis in human placenta

Human placenta is formed mainly by decidual and trophoblast cells of maternal and fetal origin, respectively. Trophoblast cells form villous and extravillous structures. Villous trophoblasts, formed by the fusion of mononucleated cytotrophoblast cells (syncytiotrophoblasts) are involved in steroid hormones production for pregnancy maintenance, maternal-fetal transfer of nutrients and gases, and the clearance of fetal waste. Extravillous trophoblasts may be interstitial that invade the decidua and a portion of the myometrium or endovascular that remodel the maternal uterine spiral arteries [4].

In early pregnancy, ovarian luteal cells have an important role in progesterone (P4) production. However, the placenta is the main organ that carries out steroids biosynthesis throughout gestation. Steroid hormones are produced using two enzymes types, the cytochromes P450s (CYPs) and the hydroxysteroid dehydrogenases (HSD), located at the mitochondria and/or endoplasmic reticulum membrane where the oxidations or reductions processes for hormonal biosynthesis take place [1].

As shown in Figure 1, placental steroid biosynthesis requires maternal low-density lipoprotein (LDLs) that are internalized and the cholesterol released is transferred to mitochondria where cytochrome P450 side chain cleavage (CYP11A1) hydroxylates and cuts the cholesterol side chain producing PREG. Then, the 3β-HSD type I (3β-HSDI) changes and modifies PREG for the production of P4. Part of placental PREG is transferred to the fetus where adrenal sulfotransferases produce pregnenolone sulfate (PREGS). Subsequently, PREGS is metabolized by CYP17A1 producing dehydroepiandrosterone sulfate (DHEAS). Then, DHEAS is hydroxylated forming the 16α-OH-DHEAS, which, together with DHEAS, return to the placenta to be used as substrates of 3β-sulfatase, 3β-HSD and 17β-HSD, resulting in the production of androstenedione and testosterone (T), respectively. These two steroids are used for placental production of main estrogens [estrone (E1), estradiol (E2) or estriol (E3)] by aromatase enzyme (CYP19A1) [5]. In the placenta, over 90% of placental E3 derive from fetal 16α-OH-DHEAS, while maternal and fetal DHEAS contributes with equimolar amount of E1 and E2 [6]. In addition, placental E1 and E2 can be hydroxylated through the action of CYP1A1 and CYP3A4 producing catecholestrogens.

Generally, the presence of CYP17A1 involved in placental androgen production has been questioned. However, in 2002, it has been demonstrated placental DHEA synthesis using PREG

CONTACT David Barrera barrera1912@gmail.com Departamento de Biología de la Reproducción, Dr. Carlos Gual Castro. Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, Col. Belisario Domínguez, Sección XVI, México, D.F. 14080, México

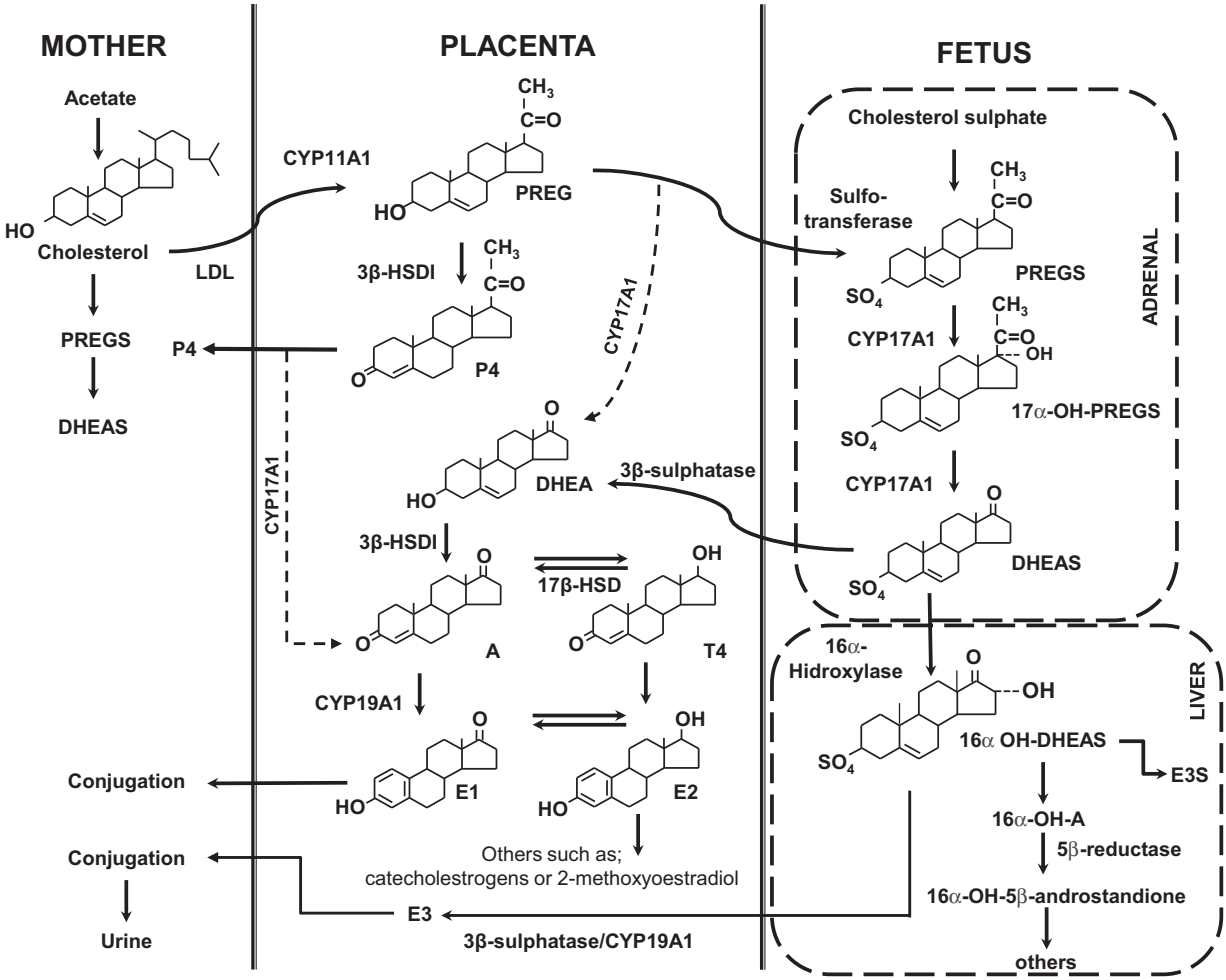

Figure 1. Steroid biosynthesis in maternal, placental and fetal compartments. In this scheme, the presence of CYP17A1 in the human placenta is considered. Enzyme with discontinuous arrow indicates minor expression compared with the others enzymes.

[7]. Subsequently, in 2003, placental gene expression of CYP17A1 has been reported [8]. Moreover, in 2011, both CYP17A1 placental gene expression and enzyme activity have been documented [9]. Interestingly, in 2017 we have demonstrated the presence and evolution of CYP17A1 gene expression through the trophoblast differentiation process using human enriched trophoblastic cell preparations [10]. Nevertheless, it is important to indicate that placental gene expression ratio of CYP19A1/CYP17A1 is in favor of estrogen production during pregnancy avoiding exacerbated androgen production and accumulation. Thus, the efficient bioconversion to estrogens could protect the fetus of adverse effects of androgens during pregnancy. Finally, any excess of steroids are metabolized by steroid-inactivating enzymes such as glucuronyltransferases and sulfotransferases that are involved in the production of more polar steroidal forms that cannot bind to steroid receptors and can pass into the general circulation for their maternal urinary excretion [11]. In fact, the sulfate forms provide both a protective mechanism since they are in general biologically inactive or may be used as a reserve, yielding active steroids if they are locally needed. It has been demonstrated the existence of membrane uptake carriers for sulfated steroids such as the sodium dependent organic anion transporter (SOAT) that is highly expressed in reproductive tissues including human placenta [6]. Moreover, the presence of others candidate carriers for sulfated steroids in the placenta have been described and include the organic anion-transporting peptides OATP1A2, OATP1B3, OATP2B1, OATP3A1, and OATP4A1, the organic anion transporter OAT4 and the organic solute carrier protein OSCP1. The regulation of these sulfated steroid carriers deserve to be further investigated in human placenta.

Synthesis of dihydro- and thetrahydro-derivatives in the maternal and fetal compartments

Alternatively, P4 and T can be converted to 5α or 5β dihydro-derivatives by 5α- and 5β-reductase, respectively. In addition, the 3α or 3β hydroxylation on C3 of these dihydro-compounds by 3α- and 3β-HSD results in the formation of tetrahydro-derivatives 3α,5α; 3β,5α; 3α,5β and 3β,5β. Although the contribution of the placenta in the production of these types of steroids remains unclear, it has been reported that both P4 and T can be biotransformed in dihydro- and tetrahydro-compounds and others derivatives in maternal and fetal tissues.

Indeed, it has been demonstrated that T is transported to fetal liver to produce 5β-reduced androgens such as 5β-dihydrotestosterone (5β-DHT) due to the action of 5β-reductase. Meanwhile, 5α-reduced metabolites such as 5α-DHT, androsterone and epienadrosterone are produced in fetal lung and gastrointestinal tract [12]. These findings showed the conversion of the dihydro and tetrahydro derivatives in the fetal compartment. Besides, in the fetal liver DHEAS may contribute to the formation of 16α-DHEAS or it can be hydroxylated on carbon 7 of the molecule

for the production of 7α and 7β derivatives. In addition, T can be biotransformed into E2 which in turn is converted to estretol (E4) by 15α-hydroxylase in the fetal liver [13,14]. However, the biological significance of these steroids in many cases remains elusive.

Steroids functions during pregnancy

P4 is the main steroid for the maintenance of pregnancy. Indeed, P4 modulates the maternal immune system favoring the tolerance toward fetal allograft. In this regard, it has been demonstrated that P4 increases the biosynthesis of anti-inflammatory cytokines by maternal T lymphocytes [15]. Likewise, P4 favors the decidualization of the endometrium and increases HOXA10 gene expression and nitric oxide synthase activity as well as stimulates endothelial nitric oxide synthesis in the umbilical vein [16,17]. Also, P4 inhibits the activity of matrix-specific metalloproteases, regulating trophoblasts invasion [18]. On the other hand, P4 induces relaxation of human umbilical cord vascular smooth muscle cells as well as, with others progestins, decreases the contractile activity of the nonpregnant and pregnant human myometrium [19,20]. Moreover, it has been demonstrated that P4 decreases the production of prostaglandin E_2 (PGE_2) in the myometrial tissue, inhibits both basal and IL-1β-stimulated PGE_2 production from the first trimester human decidual stromal cells as well as abrogated in this model the effects of IL-1β on the numbers of COX-2 positive cells [21–23]. Furthermore, P4 downregulates *in vitro* the myometrial gap junction density [23], composed of connexin proteins that are involved in coordinated myometrial contractions due, at least in part, to the estrogen induction. In a physiological context, P4 could prevent or delay the onset of labor during pregnancy.

Regarding androgens, they have been considered as precursors of placental estrogen production. Nonetheless, it has been showed that T promotes endothelial cells proliferation [24]. Besides, studies carried out in animal and human tissues showed that DHEA and metabolites 5α- and 5β-reduced inhibit *in vitro* the uterine contractility, participate in cervical remodeling during pregnancy and modulate maternal vasculature [25]. Interestingly, it has been suggested that intracrine biosynthesis of endometrial androgens during decidualization may play a key role in endometrial receptivity offering an alternative for fertility treatment [26].

Concerning estrogens, they stimulate placental angiogenesis and induce connexin 43 and gap junctions formation in the myometrium [18,27]. Interestingly, CYP11A1 is stimulated by estrogen in human trophoblast cells. In nonhuman primates, E2 increases LDL receptors expression [28]. Likewise, estrogens stimulate the secretion of apoB and LRP2 (member of the LDL receptor-related protein family) in trophoblast-like BeWo cells [29]. In addition, it has been suggested that SGK1 activation by E2 suppresses LPS-mediated apoptosis and promotes the anti-inflammatory TH2 responses in decidual stromal cells, thus contributing to a successful pregnancy [30]. Prior to labor, E2 increases both oxytocin and α-adrenergic receptors concentration, modulates the opening of calcium-dependent ion channels and stimulates the expression of prostaglandin E2 and F2α receptors in the uterus to induce the labor.

Steroid enzymatic regulation in the human placenta

Considering the relevance of the steroids, the factors that modify their synthesis has been the aim of several research groups. Indeed, the production of each one of steroids depends largely on precursor availability, tissue-specific steroidogenic enzyme expression as well as regulation and functionality of these enzymes. During pregnancy, steroidogenic enzyme expression and activity undergoes regulation by several factors into the microenvironment as well as temporal and spatial variations.

As shown in Table 1, several factors including exogenous synthetic compounds (pesticides, fungicides or organic compounds) modify both the expression and activity of steroidogenic enzymes and the hormonal production in human placenta [31–54].

It is well-known that the regulation of gene expression of most of these enzymes is dependent on rapid responses mediated by second messengers such as cAMP [55–57]. In fact, human chorionic gonadotropin and luteinizing hormone stimulate the expression of CYP11A1 in JEG-3 cells by an cAMP-dependent pathway [56]. Similarly, cAMP stimulates CYP17A1 gene expression in cultured trophoblast and JEG-3 cells [58]. Likewise, cAMP and phorbol esters increase the expression of 3β-HSDI and 17β-HSD2 in JEG-3 cells [55].

In particular, it has been shown that phosphorylation/dephosphorylation process is an important mechanism for post-translational regulation. In this regard, it has been demonstrated in JEG-3 cells that phosphorylation of aromatase by calcium/calmodulin kinase II results in its degradation and its dephosphorylation by calcineurin preserves its integrity [59]. The existence of similar phosphorylation/dephosphorylation process upon others cytochromes in placenta is unknown and deserves to be further investigated.

On the other hand, in the human placenta, various transcription factors can bind to specific response elements found upstream of exon I .1 of the CYP19A1 gene. Interestingly, it has been recently demonstrated that the promoter-specific expression of CYP19A1 mRNA (derived from promoters I .1, I .4, I .8 or total transcript) and aromatase activity are increased *in vitro* during villous trophoblast syncytialization, with a maximum increase of gene expression and activity at 48 h of primary culture [60]. In addition, it has been shown that both vascular endothelial growth factor (VEGF) and dexamethasone decreased CYP19A1 gene expression without affecting CYP19A1 activity. Thus, changes on gene expression are not always consistent with those observed in the catalytic activity of CYP19A1 [60].

Interestingly, an atypical vitamin D response element has been identified in promoter I .1 of the *CYP19* gene suggesting that this site is responsible for the stimulation of aromatase expression and activity by calcitriol and retinoids in JEG-3 [61]. In fact, it has been reported that calcitriol is a regulator of the enzymes involved in the sex steroids production in human placenta. It has been demonstrated that calcitriol upregulates CYP11A1, CYP19A1 and 3β-HSDI gene expression in short times, which is in line with the ability of calcitriol to stimulate in a dose-dependent manner both P4 and E2 secretion in culture primary of trophoblast [10,34]. Even so, gene expression of steroidogenic enzymes in placental cells are regulated in a time-dependent manner by calcitriol, but like in other studies the expression of these enzymes do not always correlates with protein abundance and/or activity [10,31,60]. Interestingly enough, it has been observed that calcitriol decreases both CYP19A1 gene and protein expression at 24 h of treatment, which could inhibit estrogens production [10]. However, it has been suggested that continuous and increased production of calcitriol during pregnancy could preserve the positive regulation of the expression of this enzyme, this possibility is likely since both calcitriol and estrogen circulating levels increase during healthy pregnancy

Table 1. Regulators of steroidogenic enzymes in the human placenta.

Factor	Target	Effects	Cells	Ref.
P4 and E2	3β-HSDI CYP11A1	Increases mRNA without changes in protein abundance	Cytotrophoblasts	[31]
E2	CYP19A1	Increases mRNA	Syncytiotrophoblasts	[44,59]
T	CYP19A1	Decreases mRNA and E2 production	JEG-3	[41]
Calcitriol	CYP19A1	Increases mRNA and enzyme activity	JEG-3	[61]
		Increases P4 and E2 production	Syncytiotrophoblasts	[34]
	CYP11A1 CYP17A1 CYP19A1 3β-HSDI 17β-HSD3	Increases and/or decreases mRNA and protein time-dependent manner	Cytotrophoblasts and Syncytiotrophoblasts	[10]
hCG and LH	CYP11A1	Increases mRNA	JEG-3	[56]
Prolactin		Increases and decreases P4 and E2 secretion, respectively	placental explants	[53]
IL-1α and IL-1β	CYP19A1	Increases enzyme activity and estrogens production	Cytotrophoblasts	[49]
IL-1β		Increases P4 production	TCL-1	[37]
TNF-α		Decreases P4 and increases E2 production	JEG-3	[38]
		Increases P4 production		[46]
	3β-HSDI CYP19A1	Decreases mRNA	Cytotrophoblasts	[35]
TGF-β1		Decreases P4 and E2 production	JEG-3 and NPC	[33]
	CYP19A1	Decreases mRNA and enzyme activity		
		Decreases enzyme activity	Syncytiotrophoblasts	[54]
Activin	CYP19A1	Increases enzyme activity	Syncytiotrophoblasts	[54]
Insulin and Inositolglycan insulin mediators	3β-HSDI CYP19A1	Increase enzyme activity Decrease enzyme activity	Cytotrophoblasts	[47,50]
IGF-I	CYP11A1 CYP19A1	Increases enzyme activity Decreases enzyme activity	Cytotrophoblasts	[47]
IGF-II	CYP11A1 3β-HSDI CYP19A1	Increases enzyme activity Decreases enzyme activity	Cytotrophoblasts	[48]
VEGF and Dexamethasone	CYP19A1	Decrease mRNA, without changes in enzyme activity	Cytotrophoblasts	[60]
Forskolin and PMA		Increase mRNA		
ATP/Mg^{2+}/Ca^{2+}	CYP19A1	Decrease protein and enzyme activity	JEG-3	[57]
Ca^{2+}	3β-HSDI CYP11A1 17β-HSD1	Increases mRNA	JEG-3	[32]
Hypoxia	CYP19A1	2 % O_2, Decreases enzyme activity and prevent mRNA induction	cytotrophoblasts to syncytiotrophoblast	[74]
		1% O_2, Decreases mRNA and protein	JEG-3	[73]
c-Myc miR-17 miR-19b miR-106a	CYP19A1	Decrease mRNA	Cytotrophoblasts	[36]
Bisphenol A	CYP19A1	Decreases mRNA, protein and enzyme activity	JEG-3	[52]
		Decreases P4 and E2 production	JEG-3 and Syncytiotrophoblasts	[45]
	CYP11A1 CYP19A1	Decreases mRNA		
	CYP19A1	Increases mRNA	Syncytiotrophoblasts	[51]
Alkylphenols	CYP19A1	Inhibit enzyme activity	JEG-3	
Perfluorooctane Sulfonate	CYP19A1	Decreases mRNA and enzyme activity	Syncytiotrophoblasts	[43]
Fungicides	3β-HSDI and/or CYP19A1	Inhibit enzyme activity and P4/E2 production	JEG-3	[44]

hCG: human chorionic gonadotropin; LH: luteinizing hormone; miR: microRNA; ATP: adenosine triphosphate; Mg^{2+}: magnesium; Ca^{2+}: calcium; VEGF: vascular endothelial growth factor; IGFs: insulin-like growth factors; PMA: phorbol myristate acetate.

while the estrogens and vitamin D status has been found deficient in preeclamptic women. In fact, the vitamin D supplementation has been suggested as an alternative therapeutic action in order to reduce the risk of preeclampsia development [62].

Results about regulation of steroid hormones by TNF-α or bisphenol A are conflictive (Table 1). Indeed, stimulatory and inhibitory effects of TNF-α upon P4 production have been observed in cultured JEG-3 cells [38,46]. Similarly, mRNA expression of CYP19A1 has been found stimulated or inhibited by bisphenol A in JEG-3 cells [42,45,52]. These discrepancies may be due to multiple factors such as differences in cell/tissue preparation and techniques or doses used. In addition, cell type used could be implicated in contradictory responses when JEG-3 cells and primary trophoblast cells were compared.

Taken together, the regulation of both gene and protein expression as well as activity of the enzymes results to be complex and the specific mechanisms involved in the regulation of each enzyme in human placenta has been barely studied and deserve to be deeper investigated.

Steroid hormones concentration in normal and pathological pregnancies

Generally, steroidogenesis increased throughout gestation in order to support different physiological demands that guarantee the success of pregnancy. Table 2 contains hormonal

Table 2. Steroids concentration in different biological samples throughout uncomplicated and complicated pregnancies.

Hormone	Pregnancy outcome	Biological samples: Maternal serum	Biological samples: Umbilical cord serum	Biological samples: Placenta	Trimester	Method	Ref.
P4	Uncomplicated	13.00 ± 1.65			1	RIA	[63]
pg/mL		15.81 ± 1.03			2		
		89.93 ± 11.99			3		
T		0.53 ± 0.05			1		
ng/mL		0.27 ± 0.05			2		
		0.70 ± 0.08			3		
E2		176.61 ± 21.87			1		
ng/mL		211.91 ± 42.04			2		
		499.62 ± 87.98			3		
Total T	Uncomplicated	154.5 ± 14.8			3	RIA	[64]
ng/dL	PE	213.6 ± 25.9*					
Free T	Uncomplicated	0.3 ± 0.03					
ng/dL	PE	0.5 ± 0.1*					
E2	Uncomplicated	53.22			3	ELISA	[65]
ng/mL	PE	29.55 ± 11.17*					
P4	Uncomplicated			0.34 ± 0.31	3	IMMULITE	[66]
ng/mL/g tissue	PE			0.16 ± 0.21*			
E2	Uncomplicated			25.9 ± 18.0			
pg/mL/g tissue	PE			14.6 ± 14.2*			
E3	Uncomplicated			67.6 ± 23.1			
ng/mL/g tissue	PE			41.9 ± 23.2*			
P4	Uncomplicated	268.4 (229.0–384.2)	492.0 (385.5–658.9) Female newborns 572.1 (407.2–701.4) Male newborns		3	ELISA	[67]
ng/mL	PCOS	247.6 (117.1–574.1)	405.9 (122.6–593.9) Female newborns 462.8 (445.7–519.0) Male newborns				
E1 ng/mL	Uncomplicated	4.5 (0.9–11.9)	7.8 (4.8–9.9) Female newborns 7.4 (3.6–9.5) Male newborns			RIA	
	PCOS	1.0 (0.9–4.7)	4.1 (2.2–6.8) Female newborns 2.2 (1.7–7.6) Male newborns				
E2 ng/mL	Uncomplicated	15.4 (9.8–21.8)	6.8 (5.2–10.8) Female newborns 6.2 (3.2–7.8) Male newborns			RIA	
	PCOS	14.8 (10.6–25.9)	4.2 (2.8–5.8) Female newborns 3.5 (2.9–6.5) Male newborns				
E3 ng/mL	Uncomplicated	123.0 (58.6–168.0)	484.5 (400.9–616.9) Female newborns 598.6 (536.8–931.0) Male newborns			ELISA	
	PCOS	118.5 (11.4–188.0)	657.0 (531.0–711.0) Female newborns 648.3 (558.7–805.0) Male newborns				
T ng/mL	Uncomplicated	1.2 (0.4–1.8)	0.9 (0.3–1.1) Female newborns 1.3 (0.9–1.5) Male newborns			RIA	
	PCOS	1.8 (1.1–3.1)*	0.8 (0.7–1.5) Female newborns 1.1 (0.9–1.4) Male newborns				
Androstenedione	Uncomplicated	2.4 (1.9–3.3)	9.2 ± 4.1 Female newborns 8.5 (5.6–11.1) Male newborns			RIA	
ng/mL	PCOS	3.7 (2.9–5.8)*	6.2 ± 3.7 Female newborns 6.0 (4.9–7.4) Male newborns				
DHEA ng/mL	Uncomplicated	2.7 (1.9–4.4)	20.6 (17.3–23.1) Female newborns 16.2 (14.5–16.6) Male newborns			RIA	
	PCOS	4.0 (1.9–5.9)	20.4 (18.5–24.5) Female newborns 19.0 (17.2–19.5) Male newborns				
DHEAS mg/dL	Uncomplicated	93.0 (56.7–104.6)	200.1 ± 93.3 Female newborns 195.8 ± 69.7 Male newborns			RIA	
	PCOS	93.5 (68.9–110.9)	160.4 ± 85.6 Female newborns 246.2 ± 80.6 Male newborns				
DHEAS μmol/L	Uncomplicated	1.89 (1.13–2.84)			3	GC-MS or LC-MS	[71]
	PCOS	2.30 (1.38–3.62)					
DHEA nmol/L	Uncomplicated	21.23 (8.74–28.63)					
	PCOS	22.01 (11.72–33.58)					
Androstenedione	Uncomplicated	8.03 (5.37–12.04)					
nmol/L	PCOS	10.85 (7.57–17.21)*					
T nmol/L	Uncomplicated	2.85 (1.74–4.23)					
	PCOS	3.89 (2.71–7.11)*					
DHT nmol/L	Uncomplicated	0.48 (0.27–0.68)					
	PCOS	0.58 (0.45–0.93)*					
E1 nmol/L	Uncomplicated	24.41 (16.94–39.46)					
	PCOS	40.39 (23.86–58.84)					
E2 nmol/L	Uncomplicated	79.09 (55.83–105.91)					
	PCOS	50.57 (19.26–84.39)*					
P4 ng/mL	Uncomplicated	51.70 (42.98–66.24)			2	GC-MS	[70]
	PE	57.88 (42.99–76.33)					
	SGA	53.77 (46.51–69.70)					
E1 ng/mL	Uncomplicated	5.16 (3.00–7.15)					
	PE	3.41 (1.67–4.54)*					
	SGA	5.68 (3.46–7.16)					

(*continued*)

Table 2. Continued.

Hormone	Pregnancy outcome	Biological samples		Placenta	Trimester	Method	Ref.
		Maternal serum	Umbilical cord serum				
E2 ng/mL	Uncomplicated	6.08 (4.37–7.75)					
	PE	4.97 (3.39–5.95)*					
	SGA						
6.56 (5.00–7.39)							
E3 ng/mL	Uncomplicated	2.28 (1.36–3.43)					
	PE	1.48 (0.69–2.19)					
	SGA	1.39 (0.86–2.20)*					
T ng/mL	Uncomplicated	0.32 (0.16–0.44)					
	PE	0.42 (0.22–0.83)					
	SGA	0.24 (0.18–0.35)					
Androstenedione ng/mL	Uncomplicated	0.49 (0.36–0.80)					
	PE	0.86 (0.39–1.12)					
	SGA	0.60 (0.44–1.07)					
DHEAS ng/mL	Uncomplicated	1633.30 (1142.1–2222.0)					
	PE	1139.80 (503.80–1943.10)					
	SGA	1433 (1057.8–1957.4)					
DHEA ng/mL	Uncomplicated	1.32 (0.96–1.61)					
	PE	1.00 (0.81–2.19)					
	SGA	1.35 (1.07–1.84)					

The values are expressed as the mean ± SD or as the median (interquartile range). *$p < .05$ between uncomplicated and complicated pregnancies. PE: preeclampsia; PCOS: polycystic ovarian syndrome; SGA: small for gestational age; RIA: radioimmunoassay; ELISA: enzyme-linked immunosorbent assay; IMMULITE: competitive chemiluminescent enzyme immunoassay solid phase; GC-MS: gas chromatography coupled to mass spectrometry; LC-MS: liquid chromatography coupled to MS.

concentrations in different biological samples at different trimester of gestation in normal and pathological pregnancies [63–67].

It is well-known that endocrine disorders are associated with adverse pregnancy outcomes where either deficient or exacerbated concentration of hormones alters the body's physiology that are associated with different pathologies such as infertility, polycystic ovary syndrome (PCOS), miscarriage, preeclampsia (PE), gestational diabetes mellitus (GDM) and preterm birth among others [25]. In particular, exacerbated androgens production is correlated with PCOS, PE, GDM and preterm birth [67–70]. In contrast, estradiol concentration has been found lower in PE and PCOS [71,72]. It has been suggested that this disorder is due to decreased placental aromatase expression or less enzymatic activity as compared to healthy pregnancy [67,73].

The first stage of preeclampsia development is associated with decreased utero-placental blood flow, which diminishes the input of oxygen into the placental vasculature. Interestingly, using hypoxic conditions in culture primary of trophoblast and JEG-3 cell line as *in vitro* model and pregnant rabbits as *in vivo* model of placental ischemia, it has been demonstrated that hypoxia downregulates CYP19A1 expression and activity. These results suggested that hypoxia is a factor responsible, at least in part, of down regulation of CYP19A1 placental expression and could be involved in the androgens/estrogens ratio detected in maternal serum of preeclamptic pregnancies [73,74]. In addition, it has been demonstrated that T reduces uterine blood flow, spiral artery elongation and placental oxygenation in pregnant rats [75], which result in fetal growth restriction, events that are usually seen in PE, suggesting that higher T could be a mediator of increased vascular resistance and placental insufficiency during pregnancy. In turn, high circulating levels of T in PE could lead to a marked suppression in estrogen production [41]. In fact, it has been demonstrated that T stimulates miR-22 expression, which inhibits ERα expression resulting in decreased E2 production in human placenta [41].

On the other hand, T inhibits and stimulates significantly gene expression of the others cytochromes such as enzymes involved in the synthesis and degradation of calcitriol; CYP27B1 and CYP24A1, respectively, which results in decreased bioavailability of this important secosteroid during pregnancy [76], like seen in PE [77]. Furthermore, it has been suggested that exacerbated production of maternal androgens downregulates placental amino acid transporters expression which could be related with decreased fetal protein synthesis [78]. In addition to T, others factors (environment factors and endocrine or immunologic disruptors, Table 1) could potentially affect placental steroidogenesis and function in each one of these pregnancy complications, which deserves more investigation.

Hormonal therapies for gestational health

Overtime, some natural or synthetic derivatives of steroids have been used to prevent or reduce infertility, recurrent abortions and/or preterm deliveries.

Regarding the prevention of miscarriages, the results of a randomized, double-blind, placebo-controlled and international multicenter study have demonstrated that vaginal micronized P4 administration in early pregnancy did not result in significant decrease in unexplained recurrent miscarriages as compared with placebo [79]. Using progestogen treatment, the results obtained are conflictive. Indeed, it has been postulated in a double-blind and randomized controlled trial that treatment with dydrogesterone in the first trimester of pregnancy will significantly reduce

the risk of miscarriage in women with threatened miscarriage [80]. In addition, the results of systemic reviews have shown that pregnant women treated with progestogen have a significant reduction in the risk of miscarriage [81–83]. However, these positive effects of progestogen have not been observed in other studies. Indeed, progestogen treatment has been evaluated in a meta-analysis of 14 trials including 2158 women and the authors of this review concluded that regardless of gravity and number of previous miscarriages, progestogen treatment, independently of route of administration (oral, intramuscular, vaginal), did not reduce significantly the risk of miscarriage as compared with placebo or no treatment [84]. Thus, for the prevention of miscarriage, the use of P4 seems to be definitely discarded and dydrogesterone treatment deserves to be further investigated.

In relation to preterm birth, it has been demonstrated that intramuscular administration of P4 is associated with a reduction in the risk of preterm birth and low newborn birthweight [85]. Furthermore, it has been demonstrated that women with a singleton gestation and prior the risk of spontaneous preterm birth, daily vaginal administration of P4 since 16 weeks of gestation, is associated with significantly lower rates of the risk of spontaneous preterm birth < 34 weeks [83]. However, this beneficial effect was not observed in women without previous history of preterm delivery after vaginal administration of natural P4. Therefore, P4 treatment have been questioned since in some cases it was not effective to prevent adverse outcomes of pregnancy including miscarriages, suggesting that hormonal treatment type, route of administration and clinical history are important factors to be considered in the choice of therapy. Indeed, it has been demonstrated in patients with clinical history of recurrent preterm births, that once-weekly intramuscular treatment of 17α-hydroxyprogesterone caproate (17α-HPC) during the second trimester of gestation decreases significantly the incidence of preterm deliveries [86]. However, in a recent and interesting study it has been shown that vaginal P4 treatment in combination with tocolytic agents such as indomethacin and bacterial vaginosis therapy significantly benefits pregnancy outcomes in women with short cervix ($\leq$25 mm) compared with cerclage [87]. In addition, the results of this study showed clearly that vaginal P4 treatment is more effective as compared with dydrogesterone, 17OHP and micronized oral P4 treatments and with cervical cerclage therapy [87]. These results support the observations of another study in which it has been demonstrated that vaginal P4 is suitable for women with singleton pregnancy, short cervix and without previous preterm deliveries [88]. The authors of this study indicated that vaginal P4 treatment does not have any benefit in case of twin pregnancy and when the risk of preterm delivery and preterm fetal membranes rupture are expected [88].

Conclusions

The results obtained from the present review highlight the importance of steroid hormones production and functions during pregnancy. In addition to its primary role in estrogen synthesis, placenta contributes with local androgen production, which could explain high levels of this hormone in several pregnancy disorders such as preeclampsia, gestational diabetes Mellitus and polycystic ovary syndrome. Although preeclampsia is associated with hormonal steroid levels alterations, no information is available about clinical intervention for their correction. However, hormonal treatments for the prevention of miscarriages and preterm deliveries are accepted for clinical uses.

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Review

Vitamin D and Inflammatory Cytokines in Healthy and Preeclamptic Pregnancies

David Barrera, Lorenza Díaz, Nancy Noyola-Martínez and Ali Halhali *

Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, Tlalpan, México D.F. 14000, Mexico; E-Mails: barrera1912@gmail.com (D.B.); lorenzadiaz@gmail.com (L.D.); biolirio_12@hotmail.com (N.N.-M.)

* Author to whom correspondence should be addressed; E-Mail: ali.halhalib@quetzal.innsz.mx; Tel.: +52-55-5487-0900 (ext. 2417).

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Abstract: Preeclampsia is a pregnancy disease characterized by hypertension and proteinuria. Among several disorders, the imbalance of inflammatory cytokines and the alteration of vitamin D metabolism have been reported in preeclampsia. The effects of calcitriol upon inflammatory cytokines has been demonstrated. In healthy pregnant women there is a shift toward a Th2 cytokine profile, which is necessary for an adequate pregnancy outcome. As compared with normal pregnancy, high pro-inflammatory and low anti-inflammatory cytokine levels have been observed in preeclamptic women. Preeclampsia has been associated with low calcitriol levels and vitamin D deficiency is correlated with a higher risk of the development of this disease. It has been demonstrated that placenta is a source as well as the target of calcitriol and cytokines and placental dysfunction has been associated with preeclampsia. Therefore, the present manuscript includes a review about serum calcitriol levels in non-pregnant, pregnant, and preeclamptic women as well as a review on the fetoplacental vitamin D metabolism in healthy and preeclamptic pregnancies. In addition, circulating and fetoplacental inflammatory cytokines in healthy and preeclamptic pregnancies are reviewed. Finally, the effects of calcitriol upon placental pro-inflammatory cytokines are also explored. In conclusion, maternal and placental calcitriol levels are low in preeclampsia which may explain, at least in part, high pro-inflammatory cytokine levels in this disease.

Keywords: vitamin D; inflammatory cytokines; pregnancy; preeclampsia

1. Introduction

Preeclampsia is a pregnancy disease characterized by hypertension and proteinuria [1]. Among several disorders, the alteration of vitamin D metabolism and the imbalance of inflammatory cytokines have been reported in preeclampsia [2–10]. In addition to its calcium effects, calcitriol, the active form of vitamin D, exerts non-calcemic actions [11]. A relationship between calcitriol and inflammatory cytokines has been demonstrated [12–15]. Since preeclampsia is associated with placental dysfunction, the present review includes the findings of several studies related to calcitriol and cytokines in healthy and preeclamptic pregnancies.

2. Calcitriol in Non-Pregnant and Pregnant Women

In non-pregnant women, calcitriol (1,25-dihydroxivitamin D) is synthesized in the kidney from calcidiol (25-hydroxivitamin D) through the action of the 25-hydroxivitamin D-1alpha-hydroxylase (CYP27B1). Calcidiol is the product of the hydroxylation in the liver of cholecalciferol (vitamin D_3 from an animal source) and ergocalciferol (vitamin D_2 from a vegetal/fungi source). This hydroxylation is catalyzed by the vitamin D-25-hydroxylase (CYP27A1, CYP2R1, CYP2J2, or CYP3A4). It is important to indicate that the human body receives the major amount of vitamin D from cutaneous synthesis of cholecalciferol, whose precursor is 7-dehydrocholesterol [11]. Indeed, it has been reported [16] that skin produces cholecalciferol photochemically from its precursor, which is present in the epidermis, by the action of sunlight or artificial ultraviolet light that converts the 7-dehydrocholesterol to previtamin D, which is in turn thermally isomerized to vitamin D. Renal calcitriol synthesis is tightly adjusted to calcium and phosphate needs [11]. In addition to these two nutrients, renal calcitriol production is regulated by parathyroid hormone (PTH) [17]. When circulating calcitriol levels are high, the excess of this secosteroid is catabolized to inactive products by the 25-hydroxivitamin D-24-hydroxilase (CYP24A1) [11]. Vitamin D receptor (VDR) is largely distributed in the body and mediates calcitriol effects [11,16]. In addition to its calcemic effects, it has been demonstrated that this hormone is involved in non-calcemic actions. Indeed, the role of vitamin D has been described in different conditions including immune and autoimmune diseases [18]. During pregnancy, the role of calcitriol in the regulation of the immune function at the maternal-fetal interface has been also reviewed [19].

During the last trimester of pregnancy, most of the skeletal fetal bone mineralization takes place [20]. At this time period, maternal calcium requirements increase. Interestingly, maternal circulating calcitriol levels increase in parallel fashion, resulting, at least in part, in higher calcium intestinal absorption that covers the maternal and fetal needs of this nutrient [21]. As previously reported, the maternal calcitriol level rises during the first trimester and then plateaus until term [20,22]. However, in our and other longitudinal studies [23,24], it has been shown that maternal calcitriol concentrations keep increasing in the second and third trimesters of pregnancy. Overall, these observations may suggest that increased calcitriol in the first trimester could be related mainly to the non-calcemic effects of this hormone, and its increase in the second part of pregnancy could explain, at least in part, higher calcium intestinal absorption according to the fetal requirements of this nutrient. Interestingly, Hollis *et al.* [25] have shown a significant association between circulating levels of calcitriol and calcidiol in pregnant women who received 400, 2000, or 4000 international units (IU) vitamin D/day from 12–16 weeks of

gestation until delivery. However, this association has been observed only with calcidiol concentrations under 40 ng/mL. Indeed, increased calcidiol levels above this concentration have not been associated with significantly higher calcitriol. On the other hand, a calcidiol concentration of 40 ng/mL has been considered by the authors of this study to be required to reach an optimum concentration of calcitriol [25], while calcidiol levels under 40 ng/mL were considered vitamin D insufficient or deficient. When observed, this association indicated that calcitriol production is calcidiol-dependent during pregnancy. In contrast, an inverse association between calcidiol and calcitriol are observed in non-pregnant subjects with vitamin D deficiency and insufficiency [26]. Therefore, calcitriol levels are differentially controlled in pregnant and non-pregnant women in insufficient and deficient conditions. In non-vitamin D supplemented pregnant women, maternal calcitriol concentrations increase without significant changes in serum calcidiol levels [27].

Increased calcitriol levels during pregnancy could not be attributed only to a higher circulating PTH concentration since we have shown a significant increase in plasma calcitriol levels in both thyroparathyroidectomized and control pregnant rats, indicating that increased plasma calcitriol levels in pregnancy are PTH-independent [28]. Similarly, in pregnant women, the increase of this secosteroid has not been associated with PTH [23,29]. In addition, serum calcitriol levels increased two- to three-fold in pregnant women with pseudohypoparathyroidism while serum PTH was decreased by about 50% in these patients [30]. It is possible that insulin-like growth factor I (IGF-I) and parathyroid hormone-related peptide (PTHrP) could be potential stimulators of calcitriol synthesis during pregnancy. Indeed, both maternal circulating IGF-I and PTHrP increase during pregnancy [23,24] and correlation studies have shown significant associations between calcitriol and IGF-I [24] and PTHrP [23]. Interestingly, IGF-I has been suggested as an additional regulator of vitamin D metabolism since this growth factor stimulates calcitriol synthesis in mouse kidney [31] and human placenta [32]. Regarding PTHrP, it has been observed that administration of this peptide to mice was associated with increased serum calcitriol levels [33] and renal CYP27B1 activity [34]. In healthy human volunteers, PTHrP infusion resulted in an increase of not only calcitriol [35,36] but also IGF-I levels [35]. These findings suggest that IGF-I and PTHrP may have an important role in vitamin D metabolism during pregnancy which deserves to be further investigated.

3. Calcitriol in Preeclamptic Pregnancy

3.1. Association between Calcitriol and Hypocalciuria in Preeclampsia

Several alterations of calcium metabolism have been observed in preeclampsia, and the most common of them is hypocalciuria, which has even been suggested as an early predictor of the development of the disease [37,38]. The pathophysiological mechanisms involved in low urinary calcium excretion are still unclear. Thus, several studies have been done in order to investigate if hypocalciuria results from an alteration of vitamin D metabolism. Taufield *et al.* [38] were the first researchers who observed that preeclamptic patients have significantly lower 24-hour urinary total calcium excretion as compared with normotensive pregnant women, without significant differences in circulating calcitriol levels between the groups [38]. However, results from our studies [3–5,39] and other laboratories [2,6] showed that maternal serum levels of calcitriol are lower in preeclampsia. In our study [3], urinary

total calcium excretion correlated significantly and positively with maternal serum calcitriol levels in both normotensive pregnant women and preeclamptic patients. In another study, we have shown that fractional urinary calcium excretion is significantly lower in preeclamptic women than in normotensive pregnant women [4]. Circulating calcitriol levels correlated significantly and positively with both total and fractional urinary calcium excretion in normotensive pregnant women [4]. In the preeclamptic group, serum calcitriol levels have been significantly associated with only total urinary calcium excretion [4]. Our finding of a positive association between renal calcium excretion and serum calcitriol suggests that low calcitriol levels in preeclampsia may contribute to a complex mechanism leading to hypocalciuria.

3.2. Alteration of Vitamin D Metabolism in Preeclampsia

The observation of abnormal vitamin D metabolism in preeclampsia has been reported by August *et al.* [2], who demonstrated that maternal calcitriol levels were significantly lower in preeclamptic women compared to normotensive pregnant women. In the same year, Seely *et al.* [6] have shown a similar observation since calcitriol levels were significantly lower in preeclamptic women than in normotensive pregnant controls without significant changes in calcidiol concentrations between groups. In our laboratory, we have determined circulating levels of calcitriol in preeclamptic and normotensive pregnant women and our data were in accordance with those described above since the preeclamptic group had significantly lower levels of this secoesteroid [3–5,39]. Decreased maternal calcitriol levels in preeclampsia have been observed at the moment of the diagnosis of the disease in the third trimester of pregnancy. However, in a longitudinal study, no significant changes in circulating calcitriol levels were observed between normotensive pregnant women and those who later developed preeclampsia [24]. Nevertheless, decreased maternal calcitriol levels may be observed early in pregnant women with a risk of preeclampsia development and who present vitamin D insufficiency or deficiency [25].

The cause of decreased calcitriol levels in preeclampsia is still unknown since classical inhibitors such as increased serum calcium or phosphate levels are not found in preeclamptic patients [3–6,38,40,41] and stimulators such as circulating PTH are not significantly different as compared with healthy pregnant women [3–5,38,40,41]. However, circulating levels of IGF-I have been found lower in preeclamptic women [3–5,39,42–49]. Furthermore, low placental IGF-I expression [47,50] and levels [39] have been found in preeclampsia. As shown for IGF-I, maternal circulating PTHrP levels have been found lower in preeclamptic women [51,52]. Therefore, low IGF-I and PTHrP may explain, at least in part, the decreased calcitriol levels observed in preeclampsia. However, additional studies are needed to ascertain this assumption.

Calcidiol, considered the vitamin D status marker, has been also studied in preeclampsia. Maternal serum calcidiol levels have been found to be lower in pregnant women who subsequently developed preeclampsia and in preeclamptic women with a diagnosis of early-onset severe preeclampsia [51–69]. However, other studies have not a found significant association between calcidiol levels and preeclampsia [70–73]. Since vitamin D status is influenced by sunlight exposure [74], Lechtermann *et al.* [75] studied the influence of the season upon maternal vitamin D status and they found that preeclamptic women have significantly lower serum calcidiol levels only in summer, while their calcitriol concentrations were significantly lower only in winter. It is noteworthy that the

concentration of these two vitamin D metabolites have not been found significantly different in winter or summer, respectively, between healthy and preeclamptic pregnancies. It is possible that these findings may explain the controversy about the existence of an association between calcidiol concentration and preeclampsia development. As mentioned above, low calcitriol in maternal serum from preeclamptic women has been demonstrated in several studies. Therefore, the observation of a significant decrease in circulating levels of this secosteroid only in winter deserves to be confirmed by future investigations. On the other hand, several studies have found that vitamin D deficiency or insufficiency may represent a risk factor for preeclampsia development. Therefore, vitamin D supplementation during pregnancy has been suggested as a beneficial strategy to reduce the incidence of different diseases including preeclampsia [57,76,77]. The aim of vitamin D supplementation is to reach a circulating calcidiol level of 30 ng/mL or more without exceeding 150 ng/mL in order to avoid the risk of toxicity. This strategy is of importance for pregnant women with a vitamin D status diagnosed as insufficient or deficient (below 30 ng/mL and 20 ng/mL, respectively). However, Hollis *et al.* [25] estimated that circulating calcidiol levels of 40 ng/mL are required to optimize calcitriol production during pregnancy. In addition, calcium supplementation may be recommended for pregnant women with a dietary calcium intake below the requirement since several studies have demonstrated that this nutrient significantly reduces the risk of preeclampsia development [78].

3.3. Fetoplacental Synthesis of Calcitriol in Pregnancy

Calcitriol synthesis has been demonstrated in decidua and placenta, tissues of maternal and fetal origin, respectively. Indeed, human decidual cells have been considered an extrarenal source of calcitriol since it has been reported that the presence of 6 nM of tritiated calcidiol was readily hydroxylated, yielding a compound that was able to bind to a specific rachitic chick receptor and had a mass spectrum identical to and comigrated with authentic calcitriol [79]. On the other hand, the data of the study done by Weisman *et al.* [80] showed that vitamin D and calcidiol cross the placenta and enter the fetus. In addition, the presence of calcitriol has been observed in the maternal serum of nephrectomized pregnant rats and the authors have suggested that the fetal portion of the fetoplacental unit is the most likely site of calcitriol production [80]. Alternately, the study of Gray *et al.* [81] done on vitamin D deficient pregnant and non-pregnant rats demonstrated that nephrectomy prevented the conversion of calcidiol to calcitriol in non-pregnant rats, while reducing but not abolishing the formation of calcitriol from its precursor in pregnant rats. In another study, Weisman *et al.* [82] have demonstrated for the first time that human placenta is able to synthesize calcitriol, which indicates the extrarenal synthesis of this hormone in pregnant women. In addition, it has been demonstrated that human placenta expresses the VDR [24,83], reinforcing the plausibility of calcitriol effects in this organ. Thus, the researchers interested in this area used human placenta as a biological model for the study of vitamin D metabolism and also as a target of calcitriol. In human placenta, Zerwekh and Breslau [84] demonstrated that CYP27B1 activity was significant in trophoblasts. Hollis *et al.* [85] have shown calcitriol synthesis not only in mitochondria but also in microsomes isolated from human trophoblasts and suggested that placental production of this secosteroid was not dependent on the presence and activity of CYP27B1, therefore postulating the possibility that calcitriol synthesis in this tissue may result from the insertion of oxygen at carbon 1 of calcidiol by a free radical chemistry mechanism. However, in human syncytiotrophoblast

cell cultures, we have demonstrated that the presence of cycloheximide significantly inhibited the stimulatory effect of IGF-I upon calcitriol synthesis, which indicated the participation of a protein enzyme in the biotransformation of calcidiol into calcitriol [32]. Later, we identified a CYP27B1 gene transcription product in cultures of human syncytiotrophoblast cells [86]. To date, several researchers have consistently observed calcitriol synthesis in human placenta [87,88]. Overall, both decidua and trophoblasts contribute to calcitriol production during pregnancy.

3.4. Placental Vitamin D Metabolism in Normotensive and Preeclamptic Pregnancies

Although low calcitriol concentrations have been found in maternal and umbilical cord compartments from preeclamptic women as compared with normotensive pregnant women [2–6,39], the results of human placental CYP27B1 expression are controversial, depending on the biological material used. Indeed, when CYP27B1 has been studied in syncytiotrophoblast cells in culture, our laboratory has demonstrated low gene expression and activity of this enzyme in preeclampsia [89]. In addition, we have demonstrated that calcitriol was significantly lower in preeclampsia in human placental homogenates obtained from cotyledons [39]. In contrast, in whole human placental tissues, the expression of CYP27B1 mRNA [90] and protein [91] have been found higher in preeclampsia. Decreased placental calcitriol levels seen in preeclampsia may be due to the higher degradation of this secosteroid as a consequence of increased CYP24A1 expression that has been found in preeclampsia [91]. High CYP24A1 expression could be explained by the stimulatory effect of tumor necrosis factor (TNF)-α upon this catabolic enzyme as observed in human cultured trophoblast cells [92,93]. However, it has been observed that the promoter of CYP24A1 is methylated in normal human placenta, a process resulting in the decreased expression of the enzyme, and therefore preventing calcitriol degradation [94]. This observation deserves to be investigated in placentas obtained from preeclamptic women in order to establish if the promoter of CYP24A1 is differentially methylated in this disease in which the milieu is different to that observed in normal pregnancy. In summary, further investigations are needed in order to know if the placental calcitriol clearance is altered in preeclampsia. However, the finding of decreased placental calcitriol synthesis seen in preeclampsia [39,89] may be associated with several alterations of placental functions. Indeed, it has been demonstrated that calcitriol regulated placental human chorionic gonadotropin expression [95], placental steroid hormone secretion [96], placental calbindins expression [97], and placental invasion, as indicated by the increased secretion of matrix prometaloproteinases 2 and 9 by human extravillous trophoblasts [98]. In addition, calcitriol promoted vascular endothelial growth factor and the antioxidant CuZn-superoxide dismutase expression in endothelial cells [99], and reversed the adverse effects of preeclampsia serum or conditioned medium from hypoxic placenta on endothelial colony-forming cells' capillary tube formation and migration [100]. Furthermore, calcitriol has been considered a regulator of placental inflammatory cytokines [93,101,102] (Figure 1) and cytokines have been able to regulate placental CYP27B1 and CYP24A1 expression [92].

It has been recognized that preeclampsia development occurs in two stages [103]. An early preclinical stage I occurs during the first half of pregnancy, in which placental invasion and endothelial function are altered, and stage II occurs where the clinical signs such as hypertension and proteinuria are diagnosed after 20 weeks of pregnancy and where dysfunctional maternal endothelium and maternal systemic

It has been recognized that preeclampsia development occurs in two stages [103]. An early preclinical stage I occurs during the first half of pregnancy, in which placental invasion and endothelial function are altered, and stage II occurs where the clinical signs such as hypertension and proteinuria are diagnosed after 20 weeks of pregnancy and where dysfunctional maternal endothelium and maternal systemic inflammation occur [103]. Tamblyin *et al.* [19] have published an interesting review related to the importance of the immunological role of vitamin D at the materno-fetal interface, particularly in the decidua, for fetal-maternal immune tolerance at phase I of preeclampsia and for the prevention of adverse events during the course of the whole pregnancy. However, the role of placenta as a source and target of cytokines has been also documented [104]. Therefore, the remaining review will be focused on circulating cytokine levels as well as fetoplacental cytokine expression in healthy and preeclamptic pregnancies. In addition, the effects of calcitriol upon cytokines in human placenta are also reviewed.

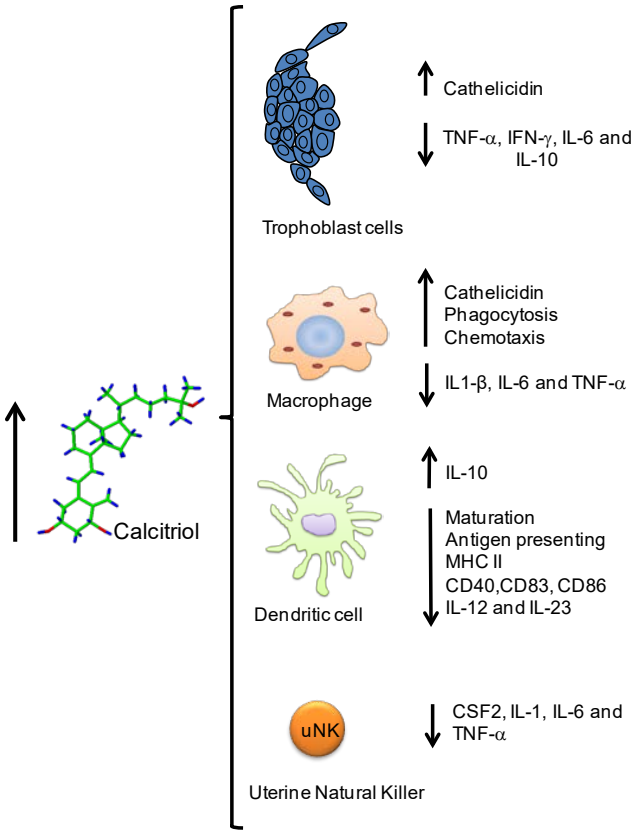

Figure 1. Calcitriol effects upon the immune system during pregnancy. Calcitriol regulates several components of the immune system at the systemic and fetoplacental compartments both in immune cells (macrophage, dendritic, uterine natural killer, and lymphocytic) and non-immune cells (trophoblast) leading changes toward a Th2 profile.

4. Circulating Cytokines in Healthy and Preeclamptic Pregnancies

Pregnancy is a unique condition where an organism with a different antigenic profile coexists peacefully while developing inside the mother in a tolerogenic environment until birth. The survival of the "fetal allograft" in mammalian pregnancies has long been considered a paradox (fetal antigens are recognized as foreign by the mother, yet the fetus continues to grow unrejected); however, it is much more complicated than just the simple nurturing and acceptance of an allograft [105]. Indeed, maintenance of pregnancy also implies a complex communication network between fetal and maternal tissues aimed to prevent excessive invasion of the maternal uterine wall while providing the fetus a quiescent environment. All these processes are undertaken by different types of immune maternal and fetal cells such as uterine natural killer cells (NK), dendritic cells, T cells, and macrophages, all of

which are of particular importance at the feto-maternal interface. Placental cells such as trophoblast and decidual cells also contribute to this process. In order to sustain pregnancy by regulating the processes described above, immune and placental cells produce different molecules including growth factors and hormones as well as cytokines and chemokines. In particular, the cytokine profile during pregnancy has been a matter of several studies, given that its deregulation may endanger pregnancy, as seen in inflammatory pathologies such as preeclampsia (Table 1). During pregnancy, the placenta, a temporal organ of fetal origin, governs many of the immune features of the mother. Therefore, any alteration of placental regulatory factors may also abnormally systemically impact the mother's immune profile.

Table 1. Inflammatory cytokines in preeclampsia.

Cytokines in Preeclamptic Pregnancies	References
Genetic polymorphisms of TNF-α and IL-1 result in increased levels of these pro-inflammatory cytokines and are associated with preeclampsia.	[106]
Serum and placental levels of pro-inflammatory IL-18 are increased in preeclampsia.	[107]
The ratios IL-2/IL-10 and TNF-α/IL-10 in maternal serum are higher in preeclampsia than in normal pregnancy.	[108]
Pro-inflammatory cytokines IL-6 and IL-8 are increased in maternal serum from women with preeclampsia as compared with normal pregnancy.	[109]
Serum levels of pro-inflammatory IL-15 and IL-16 are significantly higher in preeclampsia than in normal pregnancy.	[110]
Gene expression of anti-inflammatory IL-4 is low in preeclampsia.	[111]
In preeclampsia, the pro-inflammatory TNF-α and IL-6 and C-reactive protein are higher compared to normal pregnancy.	[112]

The T helper (Th) 1 and Th2 lymphocytes are distinguished by their opposite pattern of cytokine production. Th1 cells preferentially produce interferon-γ (IFN-γ), IL-2, and TNF. The potent inflammatory cytokine TNF-α, which is involved in systemic inflammation and acute phase reaction, is primarily produced by activated macrophages, but also may be produced by Th1 cells, NK cells, and placental cells. On the other hand, Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. It is noteworthy that the potent immunosuppressive cytokine IL-10 may also be produced by Th1 cells, while IL-6 may act both as a pro-inflammatory and as an anti-inflammatory cytokine. Other important immunomodulatory cytokines are the members of the IL-1 family such as IL-1α and IL-1β, which possess strong pro-inflammatory effects, and transforming growth factor β (TGF-β), which mainly exerts immunosuppressive effects by blocking lymphocyte activation.

Several researchers have studied the variation of serum cytokine levels as pregnancy progresses. A longitudinal study including 45 normal pregnant women found that basal and stimulated levels of IFN-γ, TNF-α, IL-1β, and IL-6 decreased throughout gestation, while lipopolysaccharide-stimulated IL-10 expression increased as pregnancy progressed [113]. Nonetheless, decreased serum IL-10 in normal pregnancy compared to healthy non-pregnant women has also been described [114]. Other studies have

analyzed the cytokine secretion profile of *in vitro* activated peripheral blood lymphocytes obtained from normal term pregnancies, finding that Th2 dominance is a feature of a successful pregnancy [115]. Saito *et al.* [116], by using flow cytometry, were the first to show that in normal pregnancy the percentage of Th1 cells was significantly lower in the third trimester, and the ratio of Th1:Th2 was also significantly lower in the second and third trimester, compared to non-pregnant subjects. In contrast, the percentage of Th1 cells and the ratio of Th1:Th2 in preeclampsia were significantly higher than in normal third-trimester pregnant women. Accordingly, the percentage of Th2 cells in preeclampsia was significantly lower than in the third trimester of normal pregnancy [116]. Similarly, Szarka *et al.* [114], by using multiplex suspension array, found that with the exception of serum IL-1β and TGFβ1, the levels of all inflammatory cytokines tested differed significantly among normal and preeclamptic pregnant women *versus* non-pregnant women. In particular, normal pregnancy was found to be characterized by a shift towards Th2-type immunity and the inhibition of cytotoxic Th1 immune responses, while preeclampsia was associated with an overall pro-inflammatory systemic environment. The authors attributed the Th2-type immunity in normal pregnancy to the relative abundance of circulating IL-18 over IL-12p70 and the relative deficiency of the bioactive IL-12p70 in relation to IL-12p40. Meanwhile, serum IL-12p70 levels were significantly higher in preeclamptic women as compared to healthy pregnant women [114].

The predominance of the type 2 immune response during normal pregnancy is understandable given the need of a pro-tolerogenic environment. Nevertheless, Holtan *et al.* [117] have recently suggested that the description of pregnancy in terms of type 2 immune responses may be an oversimplification, both in the overall description of key cytokines as well as in the temporal dynamics. This postulate was based on their analysis of the levels of different cytokines using a multiplex bead-based assay on serum from a small group of pregnant women from the first trimester through parturition. They found that as pregnancy progresses, a maternal shift away from a type 2-biased immune response and towards an inflammatory/counter-regulatory response was observed. In particular, this research group found that IL-1β, IL-6, IL-8, IL-12p70, IL-13, and IL-15 increased as pregnancy advanced. Similarly, Christian *et al.* [118] found a tendency for increased IL-6 levels across pregnancy and a significant rise in TNF-α from early and middle to later normal pregnancy. In accordance, Mor *et al.* [119] proposed to change the paradigm of pregnancy from an overall immunosuppressed state to a new one that takes into account fetal-maternal immune communication as well as the immunological response of the mother to microorganisms. In fact, in several studies, normal pregnancy has been found to be associated with an overall mild increase in both Th1 and Th2 cytokines [120–126].

On the other hand, even though preeclampsia pathophysiology remains far from clear, in general, the association of placental-derived factors such as inflammatory cytokines with the preeclampsia phenotype has been widely demonstrated and is commonly associated with the genesis of this complex syndrome. As mentioned above, the studies by Saito *et al.* [116] and Szarka *et al.* [114] consistently showed an overall pro-inflammatory systemic environment with increased Th1 cytokines. These observations have been supported by many other studies; for example, Conrad *et al.* [127] found that the median concentrations of plasma TNF-α and IL-6 were increased two-fold and three-fold in preeclamptic women compared to normal third-trimester pregnant women, respectively [127]. However, no differences in plasma IL-1β and IL-10 were found between groups [127]. Likewise, preeclamptic women in an Iranian

study showed significantly higher serum levels of TNF-α and IL-15 but lower IL-10 levels in comparison with normotensive pregnant women [128]. Moreover, two recent systematic reviews and meta-analyses found maternal serum TNF-α, IL-6, and IL-10 concentrations all significantly higher in preeclamptic patients *versus* controls [129,130]. These findings might be explained in part by the fact that the three cytokines TNF-α, IL-6, and IL-10, which are normally down-regulated by calcitriol at the placental level [93,101], could be over-expressed due to the decreased placental production of calcitriol seen in placentas obtained from preeclamptic women [39,89].

In particular, IL-6 and TNF-α are important players contributing to the endothelial dysfunction observed in preeclampsia. Other authors have also found these two cytokines as well as TNF-α soluble receptors significantly higher in the serum of preeclamptic patients, compared with age- and gestation-matched controls in the third trimester of pregnancy [125,131–133].

5. Fetoplacental Cytokines in Healthy and Preeclamptic Pregnancies

In pregnancy, the immune system is not completely Th1 or Th2, but an active and cautiously modulated system that allows fetal development while carefully fighting infectious threats. This is especially important at the implantation site. For example, during implantation, high amounts of pro-inflammatory cytokines such as IL-6, IL-15, and TNF-α, as well as several chemokines, are detected [134]. This early state of controlled inflammation at the feto-maternal interface is needed to promote adequate trophoblast invasion [135,136] (Figure 2). TNF-α was reported to play a role for adequate trophoblast growth and invasion of maternal spiral arteries, and also in limiting excessive trophoblastic infiltration [137]. TNF-α is one of several decidua/trophoblast/immune cell-derived factors with the capacity to inhibit extravillous trophoblast invasion. Other placental cytokines involved in this process are TGF-β1, 2, and 3, which inhibit trophoblast invasion by a mechanism dependent on reduced protease activity, and interferon (INF)-γ, which works in concert with TNF-α [138,139].

Significant amounts of TNF-α, IL-6, and IL-10 have been detected in the normal maternal decidua and chorionic villi of human placenta, but IFN-γ is scarcely detected [140]. Mor *et al.* [119] clearly explain how inflammation is required during both implantation and parturition. Indeed, during implantation, the trophoblast actively struggles to invade and prevail by damaging the maternal vascular and endometrial tissue, resulting in the recruiting and activation of immune cells. Then, the second trimester of pregnancy takes place with a peaceful immune profile (anti-inflammatory), allowing the fetus to grow. Finally, for parturition to occur, the reactivation of inflammation must take place (Figure 3) [119].

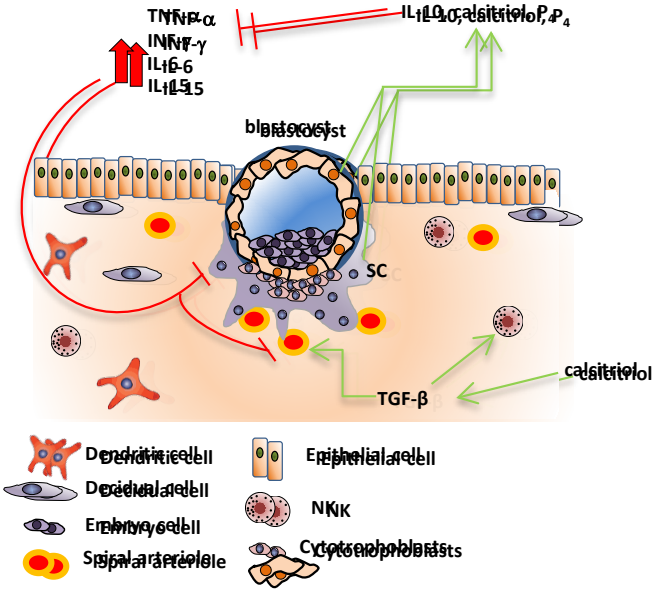

Figure 2. Cytokine profile during implantation. An early state of controlled inflammation at the feto-maternal interface is needed to promote adequate trophoblast invasion. TNF-α controls trophoblast growth and invasion of maternal spiral arteries, limiting excessive trophoblastic penetration. Another placental cytokine involved in this process is TGF-β, which interacts with its receptor endoglin in blood vessels and controls trophoblast invasion/penetration. TGF-β produced by macrophages interacts with NK cells, making them accept trophoblasts while avoiding them to kill fetal cells. In order to prevent excessive inflammation that could result in the rejection of the fetal allograft, calcitriol, IL-10, and progesterone (P_4) produced by decidual cells, trophoblasts, and syncytiotrophoblasts act as anti-inflammatory factors modulating the immunological milieu. Calcitriol and IL-10 may also be produced by immune cells present in the feto-maternal interface. Calcitriol is also known to induce TGF-β.

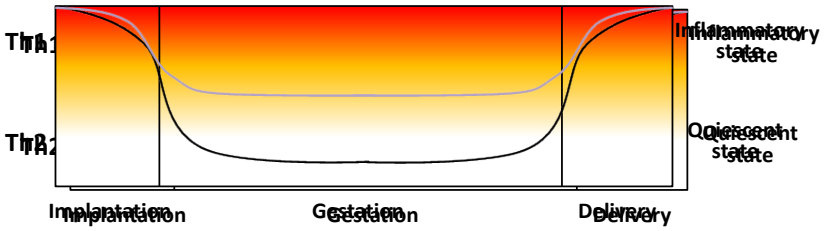

Figure 3. Schematic representation of the cytokine profile from implantation to parturition. The line in black represents a normal pregnancy, while the line in grey represents one complicated with preeclampsia. During implantation, high amounts of pro-inflammatory cytokines such as IL-6, IL-15, and TNF-α are needed so that trophoblasts can invade the maternal vascular and endometrial tissue, which results in the recruiting and activation of immune cells. Then, pregnancy continues with a peaceful immune profile, allowing the fetus to grow. Finally, for parturition to occur, reactivation of inflammation must take place. In preeclampsia, an overall pro-inflammatory systemic environment with increased Th1 cytokines has been observed in maternal serum, amniotic fluid, and umbilical serum.

However, it is also well recognized that exacerbated/perpetuated inflammation may lead to pregnancy complications such as spontaneous abortion, preterm labor, preterm rupture of membranes, and preeclampsia. Placental-derived inflammatory factors in abnormal proportions have been associated with the pathology of preeclampsia. For instance, amniotic fluid and umbilical serum TNF-α levels were found significantly higher in severe preeclamptic pregnancies than in normotensive controls [132,141]. Accordingly, placental TNF-α concentrations and mRNA expression are also increased [142]. On the other hand, IL-37 and IL-18BP, which are considered anti-inflammatory cytokines, have been found significantly up-regulated in placentas obtained from preeclamptic women compared to those from normal pregnancies [143]. This finding has been suggested to be a compensatory placental mechanism to prevent inflammatory damage to the mother [143].

Interestingly, TNF-α and IL-6 expression has been shown to be significantly higher in umbilical arteries and veins from preeclamptic women than controls [144]. Moreover, flow cytometry analysis of lymphocyte subsets isolated from third-trimester decidua showed an increased percentage of the CD3−/CD56+ CD16+, CD8+/CD28+, and a decreased percentage of CD3+, CD19+, CD4+/CD45RA+ lymphocytes in the samples of women with preeclampsia compared to controls. Meanwhile, the profile of secreted cytokines showed significantly higher IFN-γ and lower IL-6, IL-12, and IL-10 secretion [145]. As previously postulated by Sargent *et al.* [146], high INF-γ in placentas obtained from preeclamptic women raises some questions, given that normal placentas barely express this cytokine [146,147], which is a product of NK and T cells critical for fighting viral infections.

In a recent large-scale proteomic analysis, Wang and colleagues performed a comparative proteome profile of human placentas from normal and preeclamptic pregnancies using liquid chromatography-tandem mass spectrometry [148]. The data revealed that 243 peptides were significantly and differentially expressed between normal and preeclamptic placentas. Interestingly, one of the top three networks identified that differentially expressed proteins were those involved in immune system processes. In fact, 21 immunoregulatory proteins were found down-regulated in placentas obtained from preeclamptic women, which included interleukin-27-β and TGF-β together with its receptor CD105 (endoglin) [148]. Endoglin, which mediates TGF-β signaling by interacting with TGF-β receptors I and/or II, was down-regulated 3.5-fold compared to normal placentas. This might be highly relevant in the placenta, given that endoglin is strongly expressed in blood vessels. Its important proangiogenic role has been highlighted by studies showing that endoglin null mice die *in utero* as a result of impaired angiogenesis in the yolk sac [149,150]. It is noteworthy that a key pathogenic mechanism of preeclampsia is that the PE placenta shows impaired invasion of the uteroplacental arteries by extravillous trophoblasts, resulting in insufficient vessel remodeling and aberrant vascularization. Moreover, an excess in resident macrophages at the placental bed of preeclamptic women limits trophoblast invasion of spiral arteries through apoptosis mediated by the combined effect of TNF-α secretion and decreased tryptophan [151]. It is of note that polymorphisms in genes encoding for proteins in the TGF-β signaling pathway are associated with the severity of preeclampsia [152]. Therefore, the immune cell population that normally resides at the feto-maternal interface as well as the cytokine profile in this area are important for pregnancy outcome. This was clearly portrayed by Co *et al.* [153] who have shown that fetal trophoblasts are well-tolerated by the decidual NK cells through the macrophage-dependent production of TGF-β. If macrophages are lacking or an anti-TGF-β1

neutralizing antibody is used to deplete TGF-β1 from the equation, NK cells will be enabled to kill trophoblasts by lysing them. Therefore, the fact that in placentas obtained from preeclamptic patients, the TGF-β signaling pathway is down-regulated [148], together with the observations that in these placentas, the population of macrophage cells is increased [151], raise the possibility of a reasonable immune rejection of the placenta by altered decidual cells in preeclampsia. Notably, the early dominant immune population in the feto-maternal interface is based on NK cells and macrophages, while T and B lymphocytes are rather rare, highlighting the importance of the innate response type of cells during placentation [154]. Primarily, the NK cells' cytokine repertoire includes TNF-α, granulocyte–macrophage colony stimulating factor (CM-CSF), colony-stimulating factor 1 (CSF-1), IL-12, IL-15, IL-18, and especially IFN-γ [146], while that of macrophages includes TGF-β as well as IL-1, TNF-α, and IL-6.

Thus, it is clear that deficient placentation is detrimental for the overall pregnancy process. Indeed, it has been demonstrated that poor stromal cell proliferation/differentiation, reflected as reduced decidualization, may lead to preeclampsia or other pathological outcomes [134]. Another interesting feature of the placentas obtained from preeclamptic women is the observation of a sexually dimorphic level of inflammation. Indeed, in preeclamptic pregnancies, the placentas of male fetuses were associated with a significantly higher expression of inflammation, hypoxia, and apoptosis factors, but a reduced expression of pro-angiogenic markers compared to placentas of female fetuses [155].

6. Effects of Calcitriol upon Inflammatory Cytokines in Human Placenta

It has been documented by Bowen *et al.* [156] that human placenta and extra-placental membranes are able to produce several cytokines, including those involved in inflammatory process. In our laboratory, we have been interested in studying the following pro-inflammatory cytokines IL-6, INF-γ, and TNF-α, as well as the anti-inflammatory cytokine IL-10 in cultured trophoblast cells [93,101,102]. We have chosen these cytokines since they have been the most studied in preeclampsia, as mentioned above. In order to mimic a pro-inflammatory condition, trophoblast cells were treated with TNF-α. TNF-α stimulated IL-6, INF-γ, and its own expression more than three-fold over non-stimulated cells. The presence of calcitriol resulted in a dose-dependent inhibition of the expression of these three cytokines. The co-incubation of TEI-9647, a specific VDR antagonist, prevented the inhibitory effect of calcitriol upon the expression of these cytokines, indicating that this effect was VDR-dependent. Interestingly, the highest calcitriol concentration tested resulted in decreased gene expression with values similar to those observed in control experiments [93]. Using cultured trophoblast cells obtained from preeclamptic women, we have observed that basal gene expression of IL-6 and TNF-α decreased in a time-dependent manner [102]. We have demonstrated that IL-6, INF-γ, and TNF-α were significantly higher in placental cell cultures from preeclamptic women as compared with cells obtained from normotensive pregnant women. Interestingly, calcitriol treatment resulted in a dose-dependent down-regulation of these pro-inflammatory cytokines. It is of note that INF-γ was not detected in the culture media. However, calcitriol treatment resulted in a decreased concentration of IL-6, INF-γ, and TNF-α, both at the mRNA and protein levels [102]. On the other hand, we found that calcitriol inhibited the mRNA expression and protein levels of IL-10 in cultures obtained from normotensive pregnant women. The gene expression of IL-10 has been found significantly lower in placental cells obtained from preeclamptic

women [101]. Interestingly, IL-10 decreases gene expression of β-defensins and cathelicidin, while calcitriol treatment stimulates the expression of these two antimicrobial peptides in cultured trophoblast cells [76]. In this respect, the inhibitory effect of calcitriol upon IL-10 expression could result in a more robust innate immune response in the human placenta.

7. Conclusions

The present review brings out information about vitamin D metabolism in pregnancy and its relation with inflammatory processes in preeclampsia. Indeed, maternal and placental calcitriol levels are low in preeclampsia. This alteration may be related to several dysfunctions at the maternal and placental compartments, including the deregulation of the immune system, which is characterized by high pro-inflammatory cytokine levels in preeclampsia. Since several studies showed that maternal calcidiol levels are low in pregnant women who subsequently developed preeclampsia, vitamin D supplementation has been suggested in order to reduce the incidence of preeclampsia.

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Author Contributions

All the authors contributed in the writing and proof-reading of this review article.

Conflicts of Interest

The authors declare no conflict of interest.

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