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Capacidad antifibrótica de las secreciones de las células madre derivadas de tejido adiposo equino en un modelo de endometrosis

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Resumen

La endometrosis en yeguas es una enfermedad degenerativa, de gran impacto a nivel productivo. Es una patología multifactorial pero con un común denominador, se presenta en yeguas incapaces de resolver una inflamación post-monta en el endometrio, la cual conlleva a la perpetuación de esta condición y generar un ambiente fibrótico, caracterizado por una excesiva deposición de matriz extracelular, que compromete la arquitectura del tejido y funcionalidad de las glándulas endometriales, lo que a su vez genera un ambiente no favorable para la implantación embrionaria. Diversos tratamientos han sido probados pero con resultados poco favorables, es en este punto donde las terapias regenerativas aparecen como una alternativa. Las células madre presentan una probada actividad regenerativa y además han demostrado también capacidad antifibrótica en otros órganos. Esta acción terapéutica no está basada solo en su interacción directa con el tejido, sino también a partir de una acción paracrina de su secretoma, el cual puede contener diversas moléculas como factores de crecimiento, prostanoides y vesículas extracelulares. A partir de esto, se plantea la siguiente hipótesis: Las secreciones de las células madre derivadas de tejido adiposo equino presentan actividad antifibrótica en un modelo de endometrosis. Para ello, primero se buscó establecer un modelo in vitro de endometrosis en células endometriales estromales, evaluándose el efecto de citoquinas proinflamatorias (IL1-β, IL-6, TNF-α) y TGF-β en células estromales provenientes de diferentes etapas del estro (Folicular y Luteal media). En la fase folicular, las citoquinas en combinación con TGF-β potencian la transcripción de genes relacionados a la fibrosis (*CTGF*, *COL1A1*, *COL3A1* y *TIMP1/MMP9*) a partir un aumento de la fosforilación y posterior translocación a nivel nuclear del factor de transcripción SMAD2, aumento de expresión de miARNs profibróticos (mir-17, mir-21 y mir-433) en las células y en las vesículas extracelulares. Por otro lado, en la fase luteal media, se observó un aumento de la síntesis de PGE_2 (prostanoide con actividad antifibrótica) y a su vez un aumento de miARNs antifibróticos (mir-26a, mir-29b, mir-29c, mir-145, mir-378 y mir-488), sin observar cambios en la expresión de los miARNs profibróticos. Una vez establecido este modelo in vitro, se procedió a evaluar la actividad antifibrótica de las secreciones de las células madre derivadas de tejido adiposo equino en el modelo de endometrosis anterior. Para ello, se pre-condicionó con TGF-β en 3 tiempos de exposición, 0, 4 y 24 horas. La exposición a TGF-β cambió las propiedades de las células madre, aumentando

significativamente su capacidad de migración y favoreciendo la diferenciación a linaje condrocitario. Sin embargo, se observó una disminución de la diferenciación a linaje osteo y adipogénico con respecto al control (0 horas). Por otro lado, se demostró una notoria contracción de las células en la monocapa, así como la formación de nódulos, evidenciando los cambios a nivel de citoesqueleto y síntesis de matriz extracelular. A nivel molecular, 24 horas de exposición propició un aumento significativo de la expresión de los marcadores génicos de miofibroblastos *αSMA*, *COL1A*1 y *TGF-β*. A las 4 horas se observó en menor grado el aumento de marcadores miofibroblasticos y el alza de la expresión de los genes implicados en la síntesis y función de la PGE_2, *COX2*, *PTGES* y el receptor de la PGE_2, *EP4*. El aumento de secreción de PGE_2 en el medio fue confirmado mediante ELISA. Estos hallazgos permitieron establecer una estrategia potencial del uso de secreciones de las células madre como terapia libre de células en la patología fibrótica endometrial. Con los resultados obtenidos se definió un perfil secretor antifibrótico basado en un preacondicionamiento con TGF-β en las MSC, y se procedió a determinar los posibles blancos asociados a la actividad antifibrótica de las secreciones de dichas células derivadas de tejido adiposo equino en el modelo de endometrosis. Para ello, se analizaron los cambios en la expresión de miARNs relacionados con procesos fibróticos en MSC expuestas a diferentes tiempos de preacondicionamiento, la presencia y expresión de tales miARNs en vesículas extracelulares y los efectos de vesículas extracelulares derivadas de MSC pre-condicionadas, en la reversión de la fibrosis en el modelo *in vitro*. El perfil de expresión de miARNs tanto en las células como en las vesículas extracelulares fue congruente con lo encontrado en el anterior experimento, observándose una regulación a la alza de miARNs antifibróticos a las 4 horas (mir-29c, mir-145 y mir-200) y en contraposición un aumento de miARNs profibróticos a las 24 horas (mir-192,mir-199 y mir-433). Las vesículas extracelulares fueron añadidas por 48 horas al modelo fibrótico estromal y se evaluaron los marcadores génicos relacionados con la fibrosis, observando una baja significativa de los marcadores miofibroblásticos (*αSMA, CTGF, COL1A1*) en las células tratadas con las vesículas extracelulares derivadas de 4 horas de preacondicionamiento. Los cambios génicos globales fueron analizados mediante secuenciación de nueva generación. Los resultados evidenciaron 1526 genes sobreexpresados con respecto a las células estromales fibróticas. Mediante análisis de ontología génica se pudo evidenciar una prevalencia mayor de genes involucrados en la respuesta celular a interferones tipo I. Este mecanismo ha sido observado como antagonista a la acción de TGF-β ya que estimula directamente al represor del complejo

SMAD2/3, SMAD7. Los resultados obtenidos no solo respaldan la acción antifibrótica de las vesículas derivadas del preacondicionamiento de 4 horas, sino que también identifican una diana molecular que podría ser relevante en el tratamiento de la endometrosis y enfermedades relacionadas.

Summary

Endometrosis in mares is a degenerative disease of great impact at the productive level. It is a multifactorial pathology but with a common denominator, it occurs in mares unable to resolve a post-breeding inflammation in the endometrium, which leads to perpetuate this condition and generate a fibrotic environment, characterized by an excessive deposition of extracellular matrix, which compromises the tissue architecture and functionality of the endometrial glands, generating an unfavorable environment for embryo implantation. Several treatments have been tried but with unfavorable results, it is at this point where regenerative therapies appear as an alternative. Stem cells have a proven regenerative activity and have also proven to have antifibrotic activity in other organs. This therapeutic action is not only based on their direct interaction with the tissue but also on the paracrine action of their secretome, which may contain various molecules such as growth factors, prostanoids and extracellular vesicles. From this, the following hypothesis is proposed: The secretions of stem cells derived from equine adipose tissue present anti-fibrotic activity in a model of endometrosis. For this purpose, we first sought to establish an in vitro model of endometrosis in endometrial stromal cells, evaluating the effect of proinflammatory cytokines (IL1-β, IL-6, TNF-α) and TGF-β in stromal cells from different stages of estrus (follicular and mid-luteal). It was obtained that in the follicular phase, cytokines in combination with TGF-β potentiate the transcription of genes related to fibrosis (*CTGF, COL1A1, COL3A1* and *TIMP1/MMP9*) from increased phosphorylation and subsequent nuclear translocation of the transcription factor SMAD2, increased expression of profibrotic miRNA (mir-17, mir-21 and mir-433) in the cells and in the extracellular vesicles. On the other hand, in the mid-luteal phase, we observed an increase in the synthesis of PGE_2 (prostanoid with antifibrotic activity) and in turn an increase in antifibrotic miRNAs (miR-26a, miR-29b, miR-29c, miR-145, miR-378 and mir-488) was observed, without observing changes in the expression of profibrotic miRNAs. Once this in vitro model was established, we proceeded to evaluate the antifibrotic activity of equine adipose tissue-derived stem cell secretions in the previous

endometrosis model. For this purpose, they were preconditioned with TGF-β at 3 exposure times, 0, 4 and 24 hours. Exposure to TGF-β changed the properties of the stem cells, significantly increasing their migration capacity and favoring differentiation to chondrocyte lineage. However, a decrease in differentiation to osteo- and adipogenic lineage was observed concerning the control (0 hours). On the other hand, a notorious contraction of the cells in the monolayer was demonstrated, as well as the formation of nodules, evidencing changes at the cytoskeleton and extracellular matrix synthesis levels. At the molecular level, 24 hours of exposure led to a significant increase in the expression of myofibroblast gene markers *αSMA*, *COL1A1* and *TGF-β*. At 4 hours, the increase in myofibroblast markers and the upregulation of the expression of genes involved in the synthesis and function of *PGE2, COX2, PTGES* and the PGE_2 receptor, *EP4*, were observed to a lesser degree. The increased secretion of PGE_2 in the medium was confirmed by ELISA. These findings allowed establishing a potential strategy of using stem cell secretions as a cell-free therapy in endometrial fibrotic pathology. With the results obtained, an antifibrotic secretory profile was defined based on preconditioning with TGF-β in MSCs, and we proceeded to determine the possible targets associated with the antifibrotic activity of the secretions of these cells derived from equine adipose tissue in the endometrial endometrosis model.

To this end, we analyzed the changes in the expression of miRNAs related to fibrotic processes in MSCs exposed to different pre-conditioning times, the presence and expression of such miRNAs in extracellular vesicles, and the effects of extracellular vesicles derived from pre-conditioned MSCs on the reversal of fibrosis in the in vitro model. The expression profile of miRNAs in both cells and extracellular vesicles was congruent with that found in the previous experiment, observing an up-regulation of anti-fibrotic miRNAs at 4 hours (mir-29c, mir-145 and mir-200) and in contrast an increase of profibrotic miRNAs at 24 hours (mir-192, mir-199 and mir-433). Extracellular vesicles were added for 48 hours to the stromal fibrotic model and fibrosis-related gene markers were assessed, observing a significant downregulation of myofibroblastic markers (*αSMA, CTGF, COL1A1*) in cells treated with extracellular vesicles derived from 4 hours of preconditioning. Global gene changes were analyzed by next-generation sequencing. The results evidenced 1526 overexpressed genes concerning fibrotic stromal cells. Gene ontology analysis revealed a higher prevalence of genes involved in the cellular response to type I interferons. This mechanism has been observed as an antagonist to the action of TGF-β, since it stimulates the direction of the cellular response to type I interferons.

This mechanism has been observed as an antagonist to the action of TGF-β as it directly stimulates the repressor of the SMAD2/3 complex, SMAD7. The results obtained not only support the antifibrotic action of vesicles derived from 4-hour preconditioning, but also identify a molecular target that could be relevant in the treatment of endometrosis and related diseases.

I. Introducción

El endometrio es una de las tres capas que componen el útero, siendo las dos restantes, el miometrio y perimetrio. El endometrio está compuesto por una capa luminal y la lámina propia, la cual a su vez se subdivide en estrato compacto y estrato esponjoso. La capa luminal, es la zona más cercana al lumen y presenta células epiteliales columnares ciliadas. El estrato compacto, presenta gran vascularización y está formado por una población de células estromales, mientras el estrato esponjoso se caracteriza por tener una baja concentración de células estromales pero abundante fluido intersticial. Esta lamina propia transversalmente presenta estructuras glandulares ramificadas o glándulas endometriales (Evans et al., 2007).

En la especie equina, las patologías reproductivas son frecuentes y las relacionadas a la salud del endometrio de la yegua representan un número significativo de los casos (Pasolini et al., 2016), siendo la endometritis una de las más difíciles de manejar, tanto por su tratamiento, como por sus consecuencias.

La endometritis se define como una inflamación del endometrio y es multifactorial y puede ser clasificada en 4 tipos: sexualmente transmitida, producto de una infección crónica, inflamación causada por montas sucesivas y endometritis crónica degenerativa conocida como endometrosis (Troedsson, 1999). Esta última es reconocida como el mayor problema reproductivo al cual se enfrentan los criadores, al ser de naturaleza irreversible y degenerativa.

Normalmente se genera un proceso inflamatorio después de monta o inseminación artificial, cuyo objetivo es eliminar el exceso de espermatozoides o el de bacterias introducidas con la monta o inseminación (Troedsson & Woodward, 2016). La limpieza total del debris inflamatorio no se extiende más de 5 días post deposición del semen, tiempo crítico ya que coincide con la entrada del ovocito fecundado al lumen uterino (Oguri & Tsutsumi, 1972; Betteridge et al., 1982) . Existe un porcentaje de yeguas que no pueden superar esta inflamación convirtiéndola en un proceso persistente y se les denomina “yeguas susceptibles” (Fumuso, et al., 2003). Esta incapacidad pudiera estar asociada a ciertos cambios y defectos anatómicos que interfieren con un correcto drenaje uterino como una falta de contractibilidad miometral, obstrucción de la cérvix y útero pendulante, típicos de una yegua con edad avanzada y varios partos (Leblanc & Causey, 2009). Un papel no menor lo tiene la histomorfometría endometrial, en particular la relación del ancho epitelial y el número de glándulas, con la susceptibilidad, indicando

que además existe una predisposición genética (Herrera et al., 2018). Esta falta de "limpieza rápida" del útero y el establecimiento de una inflamación crónica, pavimenta el camino para el desarrollo de una fibrosis endometrial periglandular (endometrosis), la cual tiene como consecuencia una reducción de la capacidad uterina de nutrir el feto debido a la pérdida de funcionalidad de las glándulas endometriales, lo que aumenta la probabilidad de pérdida gestacional temprana (Oddsdottir et al., 2008; Liepina & Antane, 2010).

Diversos autores señalan que en las yeguas susceptibles, la endometritis genera un ambiente profibrótico mediado por las citoquinas inflamatorias IL1-β, IL-6, TNF-α y TGF-β y que esto gatilla la sobreproducción de proteínas de matriz extracelular (MEC) en la región estromal (Cadario et al., 2002, Fumuso et al., 2003). Aunque esta endometritis puede presentar indicadores clínicos tradicionales, también puede manifestarse de forma subclínica (LeBlanc & Causey, 2009), y el grado de la respuesta inflamatoria influye en la expresión de citoquinas proinflamatorias. Por ejemplo, la IL1-β está significativamente regulada al alza en la endometritis crónica en yeguas susceptibles (Christoffersen et al., 2012), mientras que la IL-6 o el TNF-α predominan en la endometritis crónica subclínica y la sobreexpresión de IL1-β, IL-6 y TNF-α en la endometritis supurativa subaguda (Siemieniuch et al., 2016). Estos procesos inflamatorios ocurren de manera especial en la región estromal, la cual es partícipe activa de la remodelación tisular a través del ciclo estral. Los fibroblastos endometriales, el principal componente células de la región estromal, están regulados por los esteroides ováricos y sus receptores a lo largo del ciclo estral (Hartt et al., 2005; Silva et al., 2014). En particular, los cambios cinéticos que se producen en el útero incluyen la proliferación durante la fase folicular bajo altas concentraciones de estradiol (E2) y el aumento de la producción de metaloproteinasas específicas de matriz (MMP2 y MMP9), lo que es un indicativo de procesos de remodelación activos que se producen durante esta fase (Curry & Osteen, 2003). Por el contrario, bajo una concentración elevada de progesterona (P4) en la fase luteica media, la expresión de las MMP se regula a la baja con un aumento simultáneo de la expresión del inhibidor tisular de las metaloproteinasas (TIMP) y de la síntesis de prostaglandina E_2 (PGE_2), lo que conduce a un entorno antiinflamatorio que refleja la preparación del endometrio para la receptividad embrionaria (Osteen et al., 2005; Niringiyumukiza et al., 2018). Con la inflamación crónica o la endometritis subclínica, la orquestación paracrina se altera: la actividad del TGF-β exacerba la expresión de αSMA (actina muscular lisa alfa) en los fibroblastos endometriales, al

tiempo que reduce la expresión de los receptores ováricos y provoca un mal funcionamiento del sistema prostanoide, un desequilibrio de las MMP y los TIMP y una deposición incontrolada de MEC (Szóstek et al., 2012, 2013). Esta acción está mediada por la unión del TGF-β a sus receptores transmembrana heterodiméricos (ALK1, ALK5) que inducen la fosforilación de los factores de transcripción SMAD2 y SMAD3 y su posterior translocación al núcleo dando lugar a la activación de la mayoría de los genes relacionados con la fibrosis (Walton et al., 2017). TGF- β al estar altamente expresada puede vincularse con otras vías moleculares como la vía Hippo y Wnt-β catenina, las cuales en otros órganos han demostrado ser necesarias para el establecimiento de una fibrosis mediante genes y micro ARNs (Piersma et al., 2015; H.-Y. Zhu et al., 2017).

Se han observado nuevos mecanismos de comunicación del componente estromal uterino con los tejidos adyacentes, incluidas las células inmunitarias (Walton et al., 2017). Esta comunicación intercelular se lleva a cabo mediante vesículas extracelulares (VE), que son pequeñas partículas de 80 a 220 μm de origen heterogéneo compuestas por bicapas lipídicas que contienen cargas de una plétora de moléculas (principalmente miARNs, ARNm y proteínas) capaces de regular células diana a largas distancias (Brigstock, 2021; Xu et al., 2022; Zhu et al., 2023). Se ha demostrado que varios miARNs, tales como mir-17, mir-21, mir-29, mir-192 y mir-199 están implicados en procesos fibróticos en hígado, pulmones y riñones (O'Reilly, 2016). Estos pequeños oligonucleótidos (18-25 nucleótidos) actúan como represores del ARNm de múltiples genes, incluidos los efectores del TGF-β como SMAD2 y SMAD3, la vía de señalización Wnt-β catenina o proteínas clave específicas que limitan la deposición de proteínas MEC como SMAD7 (Ghafouri-Fard et al., 2021).

Toda esta disrupción en el ambiente uterino evita la correcta regeneración del tejido, llevando, como se ha mencionado, a una degeneración gradual del órgano que se ve incrementada al aumentar la edad del animal, el número de inseminaciones y el número de partos (Lane et al., 2016).

Esta patología no presenta un tratamiento adecuado y al ser de naturaleza inflamatoria crónica, su resolución usando tratamientos convencionales no ha mostrado efectividad total (LeBlanc, 2010). Se han usado agentes ecbólicos (Risco et al., 2009), mucolíticos (Gores-Lindholm et al., 2013) e inmunomoduladores (Bucca et al., 2008) para prevenir la instalación de inflamación post-monta; sin embargo, en yeguas con un grado de patología avanzada no se han registrado cambios significativos en el tejido endometrial. Se han probado tratamientos alternativos enfocados en generar un legrado del exceso de

tejido conectivo. Dentro de estos figuran agentes de naturaleza orgánica, incluido infusiones de DMSO y keroseno combinados con curetajes superficiales en el epitelio endometrial (Ley et al., 1989; Crabtree et al., 2012). En ninguno de estos trabajos se logró demostrar diferencias significativas en la tasa de preñez con respecto al control. Finalmente, Griffin & Bennett, 2002, utilizando un proceso de foto ablación lograron retirar los quistes endometriales con un mejoramiento importante en las tasas reproductivas.

Ante esta situación, se han propuesto terapias regenerativas, tales como el uso de las células madre mesenquimales (MSC; por sus siglas en inglés) o de sus secreciones (Cequier et al., 2021). Las MSC se han convertido en un tratamiento de vanguardia en un amplio abanico de patologías humanas y equinas, aplicándose principalmente en patologías mediadas por inflamación crónica o enfermedades degenerativas (Kornicka et al., 2019; Hoang et al., 2022). Una de las patologías que podría beneficiarse de la capacidad terapéutica de las MSC es la endometrosis.

Estas células han demostrado actividad antifibrótica en otros órganos y modelos biológicos (Li & Wang, 2022). Su acción se basa en sus capacidades inmunomodulatorias, angiogénicas, proliferativas y anti apoptóticas que permiten regular la homeostasis de estos tejidos (Cao et al., 2020). Sin embargo, los informes sobre el tratamiento con MSC en la endometrosis muestran una inconsistencia en sus resultados que puede deberse a las diferentes fuentes de MSC que no sólo presentan diferencias en la huella molecular sino también en las propiedades moleculares. Además, el microambiente de la fibrosis es aberrante en cuanto a la señalización de TGF-β y las citoquinas pro inflamatorias y podría afectar a las MSC exógenas, induciendo la transcripción de genes relacionados con la fibrosis.

Al inicio de la era de las terapias regenerativas con MSC se hipotetizó que la actividad regenerativa de éstas estaba mediada a través de la migración a la zona dañada y posterior anidamiento y diferenciación a células residentes (Fu et al., 2019). Actualmente, nuevas evidencias apuntan a mecanismos paracrinos como responsables de la actividad terapéutica, incluyendo factores paracrinos con actividad inmunomoduladora y actividad antifibrótica como la PGE_2 (Baraniak & McDevitt, 2010; Bouffi et al., 2010).

PGE_2 ejerce su rol fibroprotector a través de la unión a sus receptores transmembránicos EP2 y EP4, los cuales aumentan la expresión de cAMP intracelular y gatillan la señalización de PKA, teniendo como resultado la inhibición de la proliferación celular ,migración y deposición de MEC de miofibroblastos, al antagonizar la vía TGF- β/SMAD

(Li et al., 2021). Diferentes reportes han demostrado su acción terapéutica, modulando negativamente la fibrogénesis en pulmón, isquemia hepática, riñón y queloides dérmicos. Dentro de los componentes terapéuticos del secretoma de las células mesenquimales destacan las vesículas extracelulares, por presentar un amplio espectro en su contenido, que abarca factores de crecimiento, lípidos, proteínas y ARNs no codificantes como los miRNA. Algunos de estos pequeños oligonucleótidos han presentado una documentada actividad antifibrótica, al poder acoplarse a las regiones 3' UTR de componentes de la vía de señalización de TGF-β o vías accesorias como Hippo y Wnt-β catenina (Kou et al., 2022). Dentro de estos miRNA antifibróticos destacan las isoformas de mir-29, las cuales tienen afinidad por genes que codifican para diferentes proteínas del MEC, entre ellas fibronectina y laminina (Harmanci et al., 2017).Otro miRNA de actividad antifibrótica potente es mir-214, el cual es un represor del factor de crecimiento conectivo (CTGF) (Chen et al., 2014). Mir-448 también ha demostrado indirectamente una actividad antifibrótica, al participar en la inhibición de la proliferación de miofibroblastos, promoviendo su apoptosis (Xu et al., 2020). Estudios de Capomaccio et al., 2019 y Navarrete et al., 2020 mediante secuenciación de nueva generación han demostrado la presencia de estos miRNA como cargo de las VE de MSC derivadas de tejido adiposo equino.

Se han diseñado diferentes estrategias para incrementar la potencia inmunomoduladora de las MSC e impulsar un patrón secretómico específico, siendo la más utilizada el preacondicionamiento con citoquinas y factores de crecimiento (Sarsenova et al., 2022) incluyendo TGF-β en el caso fibrótico. Esta alternativa explora el preacondicionamiento de las MSC con moléculas específicas que impulsen un fenotipo más acorde con la patología a tratar. Esta "activación" ha reportado un aumento de la capacidad inmunosupresora e inmunomoduladora de las MSC y de su secretoma. Anteriormente, Ghosh et al., 2021 informaron de una mejora en la funcionalidad de las MSC derivadas de médula ósea pre-condicionadas con TGF-β en un modelo de heridas crónica, mientras que Salehipour Bavarsad et al., 2022, mostraron que el TGF-β modula el secretoma de las MSC derivadas de gelatina de Wharton a un secretoma enriquecido en propiedades antifibróticas, capaz de revertir las células esteladas hepáticas activadas.

Debido a lo discutido con anterioridad, en este trabajo se evaluó cómo las secreciones de células madre mesenquimales derivadas de tejido adiposo impactaron sobre los mecanismos de fibrosis en un modelo in vitro de fibrosis endometrial. Para ello se formuló, la siguiente hipótesis general:

II. Hipótesis

Las secreciones de las células madre derivadas de tejido adiposo equino presentan actividad antifibrótica en un modelo in vitro de fibrosis estromal endometrial.

III. Objetivo

iii.a Objetivo General

Evaluar la capacidad antifibrótica de las secreciones de las células madre derivadas de tejido adiposo equino en un modelo in vitro de fibrosis estromal endometrial.

iii.b Objetivos Específicos

1.Establecer un modelo in vitro de fibrosis estromal endometrial.
2.Probar la actividad antifibrótica de las secreciones de las células madre derivadas de tejido adiposo equino en el modelo de endometrosis del objetivo anterior.
3. Determinar los posibles blancos asociados a la actividad antifibrótica de las secreciones de las células madres derivadas de tejido adiposo equino en el modelo de endometrosis.

IV. Cuerpo del texto

A continuación el texto se dividirá en capítulos, los cuales están basados en cada objetivo específico propuesto. Los capítulos constarán del artículo publicado o enviado, el cual con sus resultados y discusiones responde a los objetivos trazados. Estos artículos se encuentran en la sección de Anexos.
1.Mare stromal endometrial cells differentially modulate inflammation depending on oestrus cycle status: an in vitro study. doi: 10.3389/fvets.2023.1271240. PMID: 37869492; PMCID: PMC10587403. Front Vet Sci. 2023 Oct 6;10:1271240.
2. Short preconditioning with TGFb of equine adipose tissue-derived mesenchymal stem cells predisposes towards an anti-fibrotic secretory phenotype: a possible tool for treatment of endometrosis in mares **(Enviado a Theriogenology)**
3. Transforming growth factor β-1 modulates the anti-fibrotic secretome of horse adipose mesenchymal stem cells via micro-RNAs enclosed in extracellular vesicles: Implications for the treatment of endometrosis (**Aceptado con revisiones menores en Veterinary Quarterly**)

V. Capítulo I, Establecimiento de un modelo in vitro de fibrosis estromal endometrial

Hipótesis

"El estado del ciclo estral del cual provienen las células estromales endometriales modula el efecto profibrótico de las citoquinas proinflamatorias y del TGF-β"

Resumen

La modulación de la inflamación es fundamental para la homeostasis uterina. Se evaluó el efecto del ciclo estral en la expresión de marcadores pro inflamatorios y antiinflamatorios en un modelo celular de fibrosis inducida. Células estromales de endometrio de yegua aisladas de la fase folicular o de la fase lútea media se estimularon durante 24 horas, con 10 ng/mL de TGF-β solo o en combinación con IL1-β, IL-6 o TNF-α (10 ng/mL cada uno) o un coctel que incluía a todos los factores. Las células control, de ambos orígenes (fase folicular o luteal) no fueron estimuladas. La expresión de ARN mensajeros y microARNs se analizó mediante PCR cuantitativo con reverso transcripción y en tiempo real (RT-qPCR). Las células de la fase folicular que fueron estimuladas con citoquinas proinflamatorias mostraron una mayor expresión de genes relacionados con el colágeno (*CTGF, COL1A1, COL3A1* y *TIMP1*) y de genes marcadores mesenquimales (*SLUG, VIM, CDH2 y CDH11*) con respecto al grupo control; $p < 0,05$. Las células expuestas durante la fase luteal sobreexpresaron genes asociados con la deposición de matriz extracelular, el procesamiento y la síntesis de prostaglandina (prostaglandina E_2 sintasa), así como *MMP2, MMP9, PGR, TIMP2* y *PTGES*; $p < 0,05$. Hubo una notable regulación al alza de miARNs profibróticos (mir-17, mir-21 y mir-433) en la fase folicular cuando las células fueron expuestas a TGF-β + IL1-β, TGF-β + IL-6 o a TGF-β + IL1-β + IL-6 + TNF-α. Por el contrario, en las células de la fase luteal, los tratamientos no aumentaron la expresión de los mismos miARNs. Contrariamente, los miARNs antifibróticos (mir-26a, mir-29b, mir-29c, mir-145, mir-378 y mir-488) no aumentaron con los tratamientos en la fase folicular, sino que se sobreexpresaron en las células de la fase luteal, observándose la mayor sobreexpresión en los grupos que recibieron el tratamiento TGF-β + IL1-β + IL-6 + TNF-α. Estos microARN también se analizaron en las vesículas extracelulares secretadas por las células. Se observó una tendencia similar a la observada con los microARN celulares, en la que los microARN antifibróticos estaban regulados a la baja en la fase folicular, mientras que se observaron microARN

profibróticos notablemente elevados en las vesículas extracelulares procedentes de la fase folicular. Las citoquinas proinflamatorias pueden amplificar la señal del TGF-β en la fase folicular, lo que da lugar a una regulación al alza significativa de los genes relacionados con la matriz extracelular, un desequilibrio en las metaloproteinasas, una regulación a la baja de los receptores de estrógenos y una regulación al alza de los factores profibróticos. Por el contrario, en la fase luteal, se observa una función protectora mediada principalmente por un aumento de los microARN antifibróticos, una disminución de la fosforilación de SMAD2 y una menor expresión de genes relacionados con la fibrosis. De modo general se puede concluir que en la fase luteal hay una marcada actividad antifibrótica en contraste con la actividad profibrótica de las células estromales endometriales foliculares.

VI. Capitulo II, Actividad antifibrótica de las secreciones de las células madre derivadas de tejido adiposo equino en el modelo in vitro de fibrosis estromal endometrial

Hipótesis

"El tiempo de preacondicionamiento de las células madre mesenquimales con TGF-β modula su fenotipo y secretoma antifibrótico"

Resumen

El factor de crecimiento transformante beta (TGF-β) es crucial para el establecimiento y la progresión de la fibrosis en la endometrosis en yeguas. En este trabajo se desarrolló un enfoque basado en terapias regenerativas para el tratamiento potencial de la endometrosis, mediante el uso de células madre mesenquimales (MSC). Para ello, es crucial comprender el efecto del TGF-β sobre las MSC exógenas. Se aislaron y caracterizaron MSC adiposas equinas de seis donantes, se hicieron pooles de dichas células y se expusieron a 10 ng/mL de TGF-β durante 0, 4 y 24 horas, tras lo cual, se estudió en las células la proliferación, migración, diferenciación mesodérmica, expresión de ARNms relacionados con la fibrosis y la secreción de prostaglandina E_2. A las 24h de exposición al TGF-β, se produjo un aumento progresivo de la contracción de la monocapa que dio lugar a estructuras nodulares, mientras que la viabilidad celular no varió. La exposición a TGF-β impidió la diferenciación adipogénica y osteogénica tras 4h de tratamiento, lo que fue más marcado a las 24h, demostrado por una disminución en la tinción con Oil Red y Rojo de Alizarina respectivamente, así como en el descenso significativo ($p<0,05$) de la expresión de genes reguladores clave de los procesos de diferenciación (*PPARG* para la adipogénica y *RUNX2* para la osteogénica). TGF-β incrementó la diferenciación condrogénica, como lo demuestra el aumento del tamaño de la micromasa resultante y la intensidad de la tinción con azul Alcián, así como una significativa regulación al alza de la expresión de *SOX9* ($p<0,05$) a las 4h, que alcanzó un pico máximo a las 24h ($p<0,01$), lo que indica una regulación al alza de la síntesis de glicosaminoglicanos. El preacondicionamiento de las MSC con TGF-β condujo a un aumento significativo ($p<0,05$) de la expresión de los marcadores génicos de miofibroblastos *αSMA*, *COL1A1* y *TGF-β* a las 24h de exposición, mientras que la expresión de *COL3A1* no cambió respecto al control pero registró una disminución

significativa en comparación con las 4h ($p<0,05$). El TGF-β también afectó a la expresión de genes implicados en la síntesis y función de la PGE_2, *COX2*, *PTGES* y el receptor de la PGE_2, *EP4*, todos ellos fueron significativamente regulados al alza a las 4h ($p<0,05$). Las células expuestas a TGF-β mostraron un aumento significativo de la secreción de PGE_2 a las 4h en comparación con las células no tratadas ($p<0,05$), mientras que a las 24h, los valores de PGE_2 disminuyeron significativamente en comparación con las células control ($p<0,05$). El preacondicionamiento de las MSC durante 4 horas propició un fenotipo secretor antifibrótico, mientras que un período más largo (24 horas), por el contrario, condujo a un fenotipo profibrótico. Estos resultados permiten proponer el preacondicionamiento con TGF-β durante 4 horas de las MSC exógenas, para conducirlas hacia un fenotipo antifibrótico útil en terapias celulares y libres de células para la endometrosis equina

VII. Capitulo III, Determinación de los posibles blancos asociados a la actividad antifibrótica de las secreciones de las células madres derivadas de tejido adiposo equino en el modelo in vitro de fibrosis estromal endometrial

Hipótesis

"El preacondicionamiento de las células madre mesenquimales con TGF-β por 4 horas enriquece su secretoma con vesículas extracelulares cargadas de miRNAs antifibróticos y este secretoma es capaz de reducir las características fibróticas en un modelo *in vitro*"

Resumen

La endometrosis de la yegua es la principal patología asociada a la baja fertilidad de éstas y se caracteriza por una inflamación persistente, señalización de TGF-β y como consecuencia, la deposición de matriz extracelular que compromete las glándulas endometriales. Los productos basados en células madre mesenquimales como las vesículas extracelulares han ganado atención debido a sus efectos reguladores ejercidos por la carga de miARNs. En este trabajo se evaluó el efecto del preacondicionamiento de las células madre mesenquimales adiposas equinas con TGF-β durante dos períodos de tiempo sobre las propiedades antifibróticas del contenido de las vesículas extracelulares secretadas. Se aislaron células madre mesenquimales derivadas de tejido adiposo de 6 caballos sanos y se expusieron a TGF-β durante 0, 4 y 24 horas. Se midió la expresión de miARNs antifibróticos (mir-19, mir-29b/c, mir-145, mir-200, mir-486) y profibróticos (mir-17,mir-192,mir-199,mir214,mir-433) en las células tratadas.

Adicionalmente, se evaluaron los miARNs en la carga de vesículas extracelulares secretadas. Las VE resultantes fueron añadidas a células endometriales estromales previamente inducidas a un estado fibrótico y co-cultivadas durante 48h (ver capítulo 1 de esta tesis). La expresión de genes anti/profibróticos y se evaluó en dichas células mediante RT-qPCR en tiempo real y secuenciación de nueva generación, de igual modo se analizó por PCR la expresión de miARNs involucrados en fibrosis. El preacondicionamiento de las MSC con TGF-β durante 4h enriqueció los miARNs antifibróticos (mir-29c, mir-145 y mir-200) tanto en células, como en VE. Por el contrario, el preacondicionamiento de las células durante 24 horas condujo a un fenotipo profibrótico que sobre expresaba mir-192 y mir-433. El tratamiento con las VE derivadas

de MSC preacondicionadas por 4 horas, disminuyo significativamente la expresión de los ARN mensajeros relacionados a fibrosis (*αSMA*, *CTGF*, *COL1A1*, *COL3A1*) en el modelo de células endometriales estromales y los resultados de secuenciación indican que tienen un impacto en la activación de la respuesta de interferones tipo I, el cual es un antagonista de la vía TGF-β. Este hallazgo podría tener implicaciones para el desarrollo de un protocolo basado en VE para tratar la fibrosis endometrial en yeguas.

VIII. Discusión General

En el capítulo I ,de acuerdo a lo propuesto en el primer objetivo específico, se estableció un modelo in vitro de fibrosis estromal endometrial. Se exploró la interacción de las diferentes citoquinas proinflamatorias (IL1-β, IL-6, TNF-α) con la citoquina profibrótica (TGF-β) en fibroblastos endometriales provenientes de diferentes fases del ciclo estral (folicular y luteal media) y los cambios en señalización paracrina mediada por las vesículas extracelulares.

Se determinó que las citoquinas proinflamatorias podrían amplificar la señal de TGF-β en la fase folicular, conduciendo a un ambiente profibrótico, mientras que durante la fase luteal, hay un papel protector mediado esencialmente por la presencia de prostaglandina E_2, que favorece la regulación al alza de miARNs de actividad antifibrótica. Estos hallazgos podrían ayudar a comprender la conexión entre el mecanismo inflamatorio en yeguas susceptibles y el establecimiento de la endometrosis.

En la endometritis crónica, existe una señalización continua de citoquinas proinflamatorias de la vía NF-kB, lo que constituye un mal funcionamiento del sistema inmunitario innato (Rebordão et al., 2014; Zdrojkowski et al., 2023) profundamente influido por la fase del ciclo estral y la expresión alterada de receptores hormonales en glándulas fibróticas periféricas (Aupperle et al., 2010; Trundell, 2022). Las células estromales endometriales desempeñan un papel clave en la regulación local de la homeostasis de la matriz extracelular, juegan además un papel activo en la vigilancia inmunitaria, actuando como centinelas, que generan mediadores inflamatorios en respuesta a retos biológicos, y la intensidad de esta respuesta se ve muy afectada por la ciclicidad hormonal (Wira et al., 2015; Cavagnero & Gallo, 2022). Para explorar la respuesta fibrótica en diferentes fases del ciclo estral, se simularon las fases folicular y luteal media, añadiendo E2 y P4 a niveles similares a las condiciones fisiológicas (Nelson et al., 1985; Lapko et al., 2017). Tras desafiar al modelo celular con citoquinas proinflamatorias, se evaluó la expresión de genes y miARNs relacionados con el fenotipo

miofibroblástico y la regulación de la MEC, así como los miARNs contenidos en las VEs liberadas por estas células. Por último, se estudió el estado de fosforilación de la proteína SMAD2, factor de transcripción clave en la vía metabólica de señalización de TGF-β, para comprender mejor su interacción en este entorno.

En la fase folicular, se produjo un aumento de la expresión de SLUG independiente de la(s) citoquina(s) inflamatoria(s) utilizada(s). *SLUG* es clave para el establecimiento de la senescencia de los fibroblastos y la secreción de citoquinas proinflamatorias (IL1-β, IL-6 y TNF-α) (Coppé et al., 2010). También regula la transición epitelio-miofibroblasto y suprime la proteína proapoptótica *PUMA* (Zilfou et al., 2005; Medici et al., 2008). Además, *SLUG* se une a la caja E del receptor de colágeno tipo I, potenciando así la síntesis de MEC (Medici et al., 2008).

En la fase folicular, no hubo diferencias en la expresión de genes marcadores de miofibroblastos (*αSMA, VIM, CDH2 y CDH11*) ni de *COL3A1* cuando se utilizó un cóctel de citoquinas, en comparación con TGF-β solo. Por el contrario, la expresión de *SLUG*, CTGF y COL1A1 se potenció por la combinación TGF-β + IL-6 y TGF-β + IL1-β + IL-6 + TNF-α. Esto sugiere que la presencia de IL-6 en el cóctel de inducción favorece el depósito de MEC. Jasiński et al., 2021 y Li et al., 2022 encontraron una asociación significativa entre la vía NF-kB y un tipo destructivo de endometrosis en yeguas, caracterizado por una elevada expresión de IL-6 en la fase folicular. En esta investigación, la presencia de IL-6 también indujo la regulación al alza de *MMP9* y una mayor relación *MMP9:TIMP1*, que a su vez promueve la deposición exacerbada de MEC. Se habían notificado resultados similares en un modelo in vitro de fibrosis endometrial de yegua (Amaral et al., 2020), así como en macrófagos de pacientes con linfoma no Hodgkin maligno (Kossakowska et al., 1999; Kothari et al., 2014). La *MMP9* también tiene actividad contra el colágeno de tipo III, el colágeno típico que se encuentra en el endometrio sano; sin embargo, no puede degradar el colágeno de tipo I que está presente en la endometrosis destructiva (Chakrabarti & Patel, 2005). La *MMP9* posee una amplia actividad proteolítica y tiene afinidad por el colágeno tipo IV, el constituyente más abundante de la membrana basal, y su degradación es clave en la progresión de la fibrosis pulmonar y hepática (Veidal et al., 2011).

La otra metaloproteasa implicada en el recambio de la MEC es la *MMP2*, una gelatinasa con una gran capacidad para escindir la elastina y la fibra de colágeno de tipo I, pero con una débil actividad proteolítica frente al colágeno de tipo III (Lauer-Fields et al., 2002). En nuestro trabajo se demostró la regulación a la baja de la *MMP2* en la fase folicular en

presencia de citoquinas proinflamatorias, que pueden ser responsables de la sobreexpresión y acumulación de colágeno de tipo I. Nuestros resultados sugieren que la regulación a la baja de la *MMP2* es necesaria para facilitar la progresión de la fibrosis. Otros investigadores como Onozuka et al., 2011 y Radbill et al., 2011 demostraron resultados similares en el modelo de fibrosis hepática murina.

Szóstek-Mioduchowska et al., 2019 informaron de un aumento de la expresión de los inhibidores de las MMP en presencia de TGF-β en los fibroblastos del endometrio. De forma similar, nuestro modelo mostró una regulación al alza de *TIMP1* y *TIMP2* en la fase folicular, específicamente en presencia de citoquinas proinflamatorias. En la fase media luteal, la tendencia sólo es observable en *TIMP1* pero no en *TIMP2*, favoreciendo la relación equimolar *MMP2/TIMP2*. Este resultado sugiere una dependencia hormonal en la modulación de la rigidez de la matriz, así como la facilidad de alteración de la red mecánica en la fase folicular y la importancia de la regulación a la baja de *MMP2* para favorecer un escenario fibrótico.

Se ha demostrado que la desregulación de la señalización hormonal favorece la progresión de la endometrosis (Galvão et al., 2013; Buczkowska, et al., 2014;). Los receptores de estrógenos, *ESR1*, *ESR2* y de progesterona (*PGR*), se expresan normalmente en el estroma del endometrio de yeguas sanas, mientras que en las zonas fibróticas se produce una dramática regulación a la baja (Hoffmann et al., 2009; Lunelli et al., 2013). En nuestros resultados se observaron resultados similares: en la fase folicular, se produjo una regulación a la baja en ambos receptores de estradiol, *ESR1* y *ESR2*, en comparación con los del control. El TGF-β provoca un descenso de la expresión sin interacción observable con las interleuquinas. El efecto de los estrógenos se ejerce a través de receptores intracelulares, y diferentes informes han destacado el papel antiinflamatorio de la actividad de los receptores de estrógenos en enfermedades inflamatorias crónicas (Wise et al., 2009; McCarthy & Raval, 2020). Por ejemplo, en tejidos no reproductivos, las interacciones del 17β-estradiol con el ESR1 puede inhibir la inflamación bloqueando el tráfico de NF-kb al núcleo mediante la activación de la vía PI3K/AKT (Monteiro et al., 2014). En nuestro modelo, la regulación a la baja de *ESR1* y *ESR2* se produjo únicamente en presencia de TGF-β. Esta actividad represora de TGF-β con el receptor de estrógenos tipo 1 se ha observado en células epiteliales bronquiales de fibrosis pulmonar idiopática y en líneas celulares de cáncer de mama (Fridriksdottir et al., 2015; Smith et al., 2018). En el presente estudio, el receptor de prostaglandina mostró la misma tendencia decreciente con todos los tratamientos.

En general, a nivel del ARNm, se observó una regulación al alza de genes profibróticos en la fase folicular, en comparación con la fase luteal media. Como tal, es tentador especular que este efecto está mediado por la represión de los receptores de estrógenos bajo la influencia del TGF-β, lo que permite la libre acción de la vía NF-kb.

La fase luteal media registra un pico de P4 y altos niveles de PGE_2 que ejercen una actividad no sólo luteoprotectora sino también antifibrótica (Dackor et al., 2011; K. Li et al., 2021). Se encontró una alta expresión de ARNm de *PTGES* en la fase luteal media, pero no en la folicular. Estos hallazgos concuerdan con los de otros autores que demostraron que una combinación de P4 y niveles bajos de E2 en las células estromales inducía niveles elevados de ARNm de *PTGES* y también de *PGE_2* (Wise et al., 2009, Monteiro et al., 2014). Por el contrario, las citoquinas proinflamatorias favorecen la expresión aberrante de receptores hormonales y la regulación a la baja de la PGE_2 en la fase folicular.

En esta investigación, se encontró un patrón antifibrótico de expresión génica para las células estromales del endometrio en la fase luteal media, con una menor expresión de *COL1A1*, *CTGF y MMP2* y una mayor expresión de *TIMP1* y *COL3A1* en comparación con las de la fase folicular. Estos resultados concuerdan con los de Szóstek-Mioduchowska et al., 2020, y muy probablemente estén relacionados con el efecto antifibrótico de la PGE_2. La diana descendente inmediata del TGF-β son las proteínas SMAD2/3, que se fosforilan al interaccionar con el TGF-β. En nuestra investigación, se observó una fosforilación significativamente menor de SMAD2 en las células de la fase luteal media en comparación con las de la fase folicular. Este efecto está mediado por la adición de P4, de forma dependiente con la concentración en células epiteliales pulmonares A549 previamente tratadas con TGF-β (Kunzmann et al., 2018), en consonancia con nuestros propios hallazgos. Además, la P4 genera una respuesta antiinflamatoria bajo estímulos patogénicos, al aumentar la IL10 y disminuir la secreción de IL1-β, TNF-α e IL-6 en explantes placentarios expuestos a lipopolisacáridos, antes de la estimulación con P4 (Garcia-Ruíz et al., 2015). Esta acción se ejerce a través del receptor nuclear P4 y de receptores unidos a la membrana (PR) que inhiben la activación de la vía NF-kb (Kalkhoven et al., 1996; Fedotcheva et al., 2022).

Recientemente, se demostró que los miARN son una vía alternativa para regular el delicado eje inflamación/fibrosis, en particular los que actúan sobre las vías TGF-β y NF-kb (Vettori, 2012;Das & Rao, 2022). En este trabajo se evaluó la expresión de un conjunto de miARNs relacionados con la fibrosis en nuestro modelo celular. Los resultados

demostraron que en la fase folicular se producía un aumento de la expresión de mir-17, mir-21 y mir-433, todos ellos con conocida acción profibrótica (Chung & Lan, 2015; H. Kang, 2017). Mientras tanto, en la fase luteal media, se sobreexpresaban los miARNs antifibróticos 29a, b y c, así como mir-145. En ambos casos, la expresión se intensificó cuando el TGF-β se combinó con citoquinas proinflamatorias en comparación con la expresión con TGF-β solo. Este perfil de miARN es congruente con los perfiles profibrótico y antifibrótico de los ARNm en las fases folicular y luteal media, respectivamente, como se ha comentado anteriormente. Estudios previos han demostrado que mir17 y mir21 están directamente implicados en la progresión profibrótica en diferentes líneas celulares y modelos murinos mediante la inhibición de SMAD7. En estos trabajo, también se observó la activación indirecta de la vía NF-kb, lo que sugeriría la importancia de la regulación de miARN en la prolongación de la inflamación favoreciendo un proceso fibrótico (Yu et al., 2015; Zhou et al., 2018; Liu et al., 2021).

El rol antifibrótico de los miARN en la fase luteal se atribuye principalmente a la generación de PGE_2 y a su influencia directa sobre los miofibroblastos y la vía NF-kb en células endometriales (Borthwick et al., 2013). Sin embargo, en otros tipos de células, como las células del músculo liso aórtico (Han et al., 2018) y los hepatocitos (Liu et al., 2019), se ha demostrado de manera sólida que el mir-29 actúa como un regulador clave de la fibrosis tisular, principalmente haciendo diana en los factores de transcripción y los promotores de los genes que controlan la expresión de *COL1A1*, *TGF-β*, *αSMA* y fibrilina para prevenir la acumulación excesiva de matriz extracelular y restablecer la sensibilidad a la apoptosis en miofibroblastos a través de la señalización del ligando FAS (Wang et al., 2012; Matsushima & Ishiyama, 2016; Fan et al., 2020). Por otro lado, mir-145 es directamente mediado por P4/PGR, lo que actúa como un inhibidor de la proliferación de células endometriales epiteliales (Yuan et al., 2019).

Otro miARN altamente expresado en la fase luteal media es el mir-378. Este miARN se encuentra en el primer intrón del gen *PPARGC1-β*, un coactivador de *PPARG*. La activación de *PPARG* tiene un impacto negativo en la síntesis de TGF-β / COL1A1 en el tejido fibrótico (Ghosh, 2021; Kökény et al., 2021). Además, el mir-378 ha demostrado tener actividad antifibrótica al inhibir la vía MAPK/ERK en la fibrosis miocárdica (Yuan et al., 2018; Liu et al., 2019). Asimismo, mir-348 actúa como represor de PGR y ER (Eichner et al., 2010; Toms et al., 2015), lo que podría explicar la reducción de los receptores de estrógeno observada en nuestros resultados. Por último, el mir-488 también mostró una regulación al alza en la fase lútea media en presencia de IL1-β, IL-6, TNF-α

y TGF-β. Un estudio realizado por Liu et al., 2020 demostró la acción antiinflamatoria en el útero bovino al inhibir la producción de ROS, así como la vía AKT/NF-kb. Además, Qiu et al., 2023 observaron que este miARN tiene un efecto antifibrótico en las células estrelladas hepáticas al inhibir el TET 3, lo que resulta en la inhibición de la vía TGF-β/SMAD2.

Las vesículas extracelulares han cobrado importancia recientemente como actores en el proceso de fibrosis al transportar miARNs que promueven la transición epitelio-mesenquimal en las células vecinas (Fujita et al., 2018; Martín-Taboada et al., 2022). En este trabajo se estudió las VE secretadas por las células estromales endometriales como posibles herramientas para el tratamiento de la fibrosis endometrial. En las células de la fase folicular tratadas con TGF-β + IL1-β + IL-6 + TNF-α, se observó un aumento en la secreción de VE en comparación con las células de la fase lútea media. Trabajos previos han establecido una relación entre el aumento de las VE liberadas por el tejido lesionado y el estímulo proinflamatorio (Yang et al., 2018). En este escenario, los VE provenientes de las células alteradas actúan como amplificadores de señales y modificadores de la respuesta innata inmunitaria (Hezel et al., 2017; Javeed et al., 2021).

Entre sus acciones, aumentan el reclutamiento de neutrófilos debido a su contenido de quimiocinas, posiblemente allanando el camino para la liberación masiva de trampas extracelulares de neutrófilos, característica de la mayoría de las enfermedades fibróticas (Useckaite et al., 2020). Del mismo modo, las VE secretadas por el tejido inflamado contribuyen a la polarización de los macrófagos M1, estableciendo un circuito de interacción con el tejido lesionado y prolongando la inflamación (Lv et al., 2020). Las señales inflamatorias no solo modifican la carga de VE liberados sino también su tamaño y distribución, lo que refleja la liberación de subpoblaciones que probablemente estén enriquecidas con citoquinas inflamatorias, como observaron Yang et al., 2018 y Hosseinkhani et al., 2020. Además, el contenido de miARN de dichas VE se correlacionó con los miARN encontrados en las células. En las EVs de células en la fase folicular, hubo una regulación positiva de los miARN profibróticos, mir-21 y mir-17, mientras que en las células de la fase luteal, los miARN antifibróticos, mir-29b y mir-29c, fueron regulados positivamente. Otros investigadores han informado de la congruencia de las cargas de vesículas extracelulares con el entorno celular (Raposo & Stoorvogel, 2013; Maas et al., 2017).

No se encontraron resultados similares en la búsqueda bibliográfica realizada, por lo que aparentemente, este es el primer informe que muestra una respuesta diferente de los

fibroblastos endometriales de yegua en condiciones inflamatorias, marcada por la presencia de citoquinas proinflamatorias y TGF-β durante la fase de estral. La figura 1 muestra un resumen grafico de lo obtenido en el capítulo I.

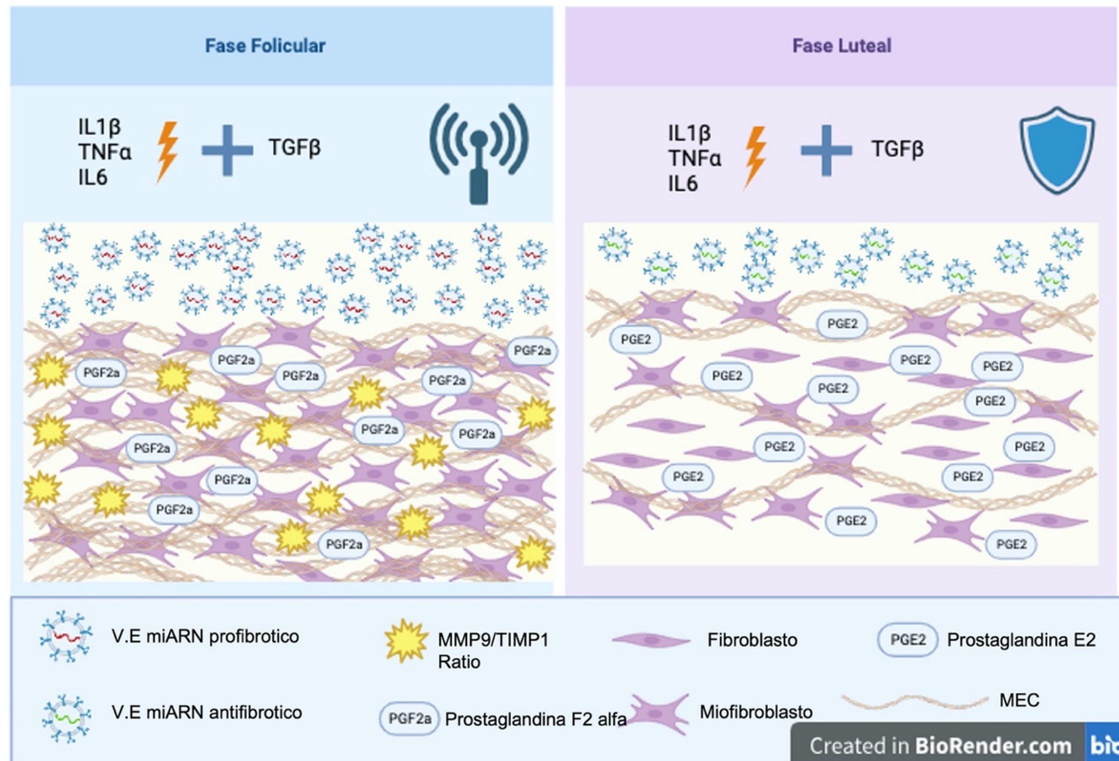


Figura 1.Modelo propuesto de la acción de la citoquinas proinflamatorias y TGF-β en la fase folicular y luteal equinas. En la fase folicular se observa una amplificación de la señal proinflamatoria mediada por un aumento de la producción de PGF2α, y la proporción MMP9/TIMP1, resultando en una mayor síntesis de matriz extracelular y liberación de VE con contenido profibroótico. Por otro lado, en la fase luteal se genera un efecto protectivo, mediado por liberación de PGE_2 y de VE con cargo antifibrótico.

De acuerdo a lo establecido en el objetivo específico II, se propuso evaluar la actividad antifibrótica de las secreciones de las células madre derivadas de tejido adiposo equino en el modelo in vitro de fibrosis estromal endometrial descrito anteriormente. En este trabajo se estudiaron los cambios del precondicionamiento de TGF-β en (i) las propiedades biológicas asociadas al fenotipo de las células madre mesenquimales, (ii) los genes relacionados con la fibrosis, (iii) la secreción de PGE_2, (iv) la expresión de proteínas en respuesta a la señalización de TGF-β.

El propósito de este estudio fue investigar el impacto del preacondicionamiento con TGF-β sobre las propiedades de las eAT-MSC para su uso como herramienta terapéutica

antifibrótica en la endometrosis de yegua. Nuestros resultados indican una orientación hacia un fenotipo antifibrótico utilizando 4 horas de preacondicionamiento con TGF-β, que se traduce en un aumento de las secreciones potencialmente antifibróticas de PGE_2, mientras que 24 horas de preacondicionamiento conllevan a un fenotipo caracterizado por un aumento de transcritos relacionado con la fibrosis.

TGF-β ejerce una regulación dependiente del contexto y participa en diversos procesos relacionados con la cicatrización de heridas, incluida la proliferación y migración celular hacia el lugar lesionado. Un mal funcionamiento de la señalización conduce a un proceso de fibrosis. Como la mayoría de las moléculas pleiotrópicas, todas las células expresan sus receptores (TGFbRI y TGFbRII) (Heldin & Moustakas, 2016). Tras la activación del receptor, se fosforilan proteínas específicas, SMAD2 ySMAD3 (Hata & Chen, 2016). Este complejo fosforilado se transloca al núcleo y se asocia a factores de transcripción específicos, activando un gen diana relacionado con la síntesis de MEC, la respuesta inmunitaria, la proliferación celular y la quimiotaxis (Verrecchia & Mauviel, 2002).

En tejido fibrótico, TGF-β favorece la proliferación del fibroblasto induciendo la expresión de FGF-2 y la fosforilación de p38 MAPK (Strand et al., 2014) y por el contrario, en MSC hematopoyéticas y células endoteliales induce quiescencia vía represión de c-Myc (Larsson et al., 2003; Zhang et al., 2017). Nuestros resultados no demuestran inducción de la proliferación, lo mismo que fue observado por Elsafadi et al., 2018, quienes trabajan con MSC derivadas de médula ósea. Esto podría explicarse debido al origen pericítico (endotelio vascular) de las MSC en diferentes estadios de diferenciación (Lin et al., 2008).

El ensayo transwell reveló que el TGF-β es un potente regulador de las propiedades de migración de las eAT-MSC, con una acción progresiva a lo largo del tiempo. Esta característica se observó como acción canónica en el reclutamiento de células estromales y MSC de médula ósea al lugar lesionado a través de la vía SDF1/CXR4 y la regulación al alza de genes con funciones pro migratorias (Liu et al., 2019; Nam et al., 2020). A las 24 horas se observó la formación de nódulos en la monocapa, estos nódulos son consecuencia de la migración celular y la producción de matriz extracelular enriquecida en colágeno I y fibronectina (Xu et al., 2007).

La capacidad de diferenciación de las MSC se regula mediante la interacción con el entorno tisular, factores específicos de señalización y señales mecánicas (Almalki & Agrawal, 2016). El preacondicionamiento con TGF-β disminuye la diferenciación adipogénica a las 4 horas y la suprime a las 24 horas, evidenciado por la baja tinción de

Oil red y una caída en la expresión de PPARG. TGF-β inhibe potentemente la diferenciación adipogénica mediante la asociación de SMAD2/3 con C/EBPβ, lo que provoca la supresión de la expresión de PPARG (Choy & Derynck, 2003).

El factor de transcripción relacionado con Runt (Runx2) es el gen maestro de la diferenciación osteogénica y promueve la diferenciación preosteoblástica de las MSC (Xu et al., 2015). En este trabajo se observó una disminución constante de la expresión de *RUNX2* y de la diferenciación osteogénica a lo largo de los tiempos de exposición. TGF-β actúa como represor a través de un mecanismo complejo, reclutando histona deacetilasa (HDCA) 4 y 5, mediante la interacción con el complejo SMAD3/RUNX2, lo que resulta en la represión transcripcional y la regulación a la baja de la osteocalcina y otros genes de linaje osteogénico (Kang et al., 2005).

Por otro lado, se ha descrito que el TGF-β promueve la diferenciación condrogénica de las MSC a través de la fosforilación de SMAD3 y la interacción con el complejo *SOX9/CPB/p300* (Grafe et al., 2018), lo que concuerda con el aumento de la expresión de *SOX9* y la síntesis de glicoaminoglicanos en diferentes momentos del preacondicionamiento.

Nuestros resultados muestran una regulación al alza de *αSMA*, *TGF-β* y *COL1A1* a las 24 horas de tratamiento, lo que evidencia un fenotipo de miofibroblasto. Anteriormente, Marriott et al., 2014 informaron de que las MSC de pulmón tratadas in vitro con TGF-β cambian su transcriptoma global, el cual es muy similar al de los miofibroblastos aislados del modelo de pulmón de ratón.

Otro aspecto de la fibrosis endometrial en la yegua es la pérdida del equilibrio del sistema de prostaglandinas, ya que el endometrio requiere un estrecho control de la expresión de PG porque influye en la constante remodelación del tejido a lo largo de las fases estrales (Schöniger & Schoon, 2020). En la fibrosis pulmonar idiopática y la endometrosis equina, la señalización de PGF2α facilita la proliferación de miofibroblastos y la deposición de colágeno (Oga et al., 2009; Szóstek-Mioduchowska et al., 2020), mientras que la PGE_2 actúa como agente anti-fibrótico al funcionar como antagonista del receptor de TGF-β (Li et al., 2021). Esta observación concuerda con el patrón de la expresión dinámica de TGF-β, que apenas se detecta a las 4 horas, coincidente con el valor más alto de expresión de PGE_2. En nuestros experimentos el sistema de prostaglandinas se ve afectado en los diferentes tiempos evaluados. A las 4 horas se registra la expresión de *COX2*, que se acompaña de un aumento de *PTGES* y de un incremento de la expresión de su receptor *EP4* (E- type prostanoids 4 receptor). Por otro lado, la expresión de PGF2α sintasa

(*AKR1B1*) se ve favorecida a las 24 horas, y se observa una disminución de *EP4*. En MSC murinas tratadas con 1ng/ml de TGF-β por 24 horas, se ha descrito un incremento de PGE_2, el mecanismo propuesto es a través de la interacción del complejo SMAD2/3 con el promotor de *COX2* que desencadena la reducción de la producción autocrina de PGE_2 a través de *EP4* (Rodríguez-Barbero et al., 2006; Lynch et al., 2020). Esto concuerda con nuestra observación a nivel de ARNm y detección por ELISA de PGE_2. En ambos casos disminuyó la presencia del prostanoide o su ARNm a las 24 horas.

Este mecanismo también se ve respaldado por nuestros hallazgos a nivel proteico, en donde se observó la fosforilación progresiva de SMAD2 con el tiempo y la fosforilación de SMAD3, lo que resulta en una mayor estabilización del complejo SMAD2/3, que es coherente con el aumento del fenotipo de miofibroblasto observado en las MSC. La vía del TGF-β interactúa con la de Wnt/β-Catenina promoviendo la fibrosis (Piersma et al., 2015). La presencia de SMAD3 fosforilada estabiliza la acumulación de β-Catenina (Cheon et al., 2006) y en las MSC de médula ósea se confirmó la translocación nuclear de β-Catenina dependiente de SMAD3, lo que incrementa la diferenciación osteogénica y la proliferación (Jian et al., 2006), mientras que su inhibición provoca una regulación a la baja de la αSMA y la deposición de MEC en las MSC de médula ósea derivadas de pacientes con neoplasia mioproliferativa (Vukotić et al., 2022).

El TGF-β es una citoquina con una acción pleiotrópica y sus efectos dependen de la intensidad y la dosis, en este estudio se examinó el efecto del preacondicionamiento con TGF-β en tiempos de exposición cortos (4 horas) o prolongados (24 horas). Nuestros resultados mostraron que la migración de las MSC se ve favorecida a las 24 horas y su capacidad de diferenciación muestra un perfil hacia el linaje condrogénico, que podría utilizarse para lesiones articulares. Sin embargo, nuestros resultados más importantes están relacionados con el cambio progresivo que sufren las MSC a lo largo del tiempo de exposición al TGF-β, mostrando un aumento significativo de genes relacionados con la fibrosis y una disminución de factores antifibróticos, como la secreción de PGE_2 a las 24 horas. Lo contrario se observó a las 4 horas, que genera un perfil de ARNm anti-fibrótico. Teniendo en cuenta nuestros resultados, se puede sugerir el uso del preacondicionamiento con TGF-β a las 4 horas para terapias tanto celulares como libres de células dirigidas a enfermedades fibróticas como la endometrosis en yeguas. En la figura siguiente se propone un esquema de acción de TGF-β en función del tiempo de exposición de las MSC al mismo.

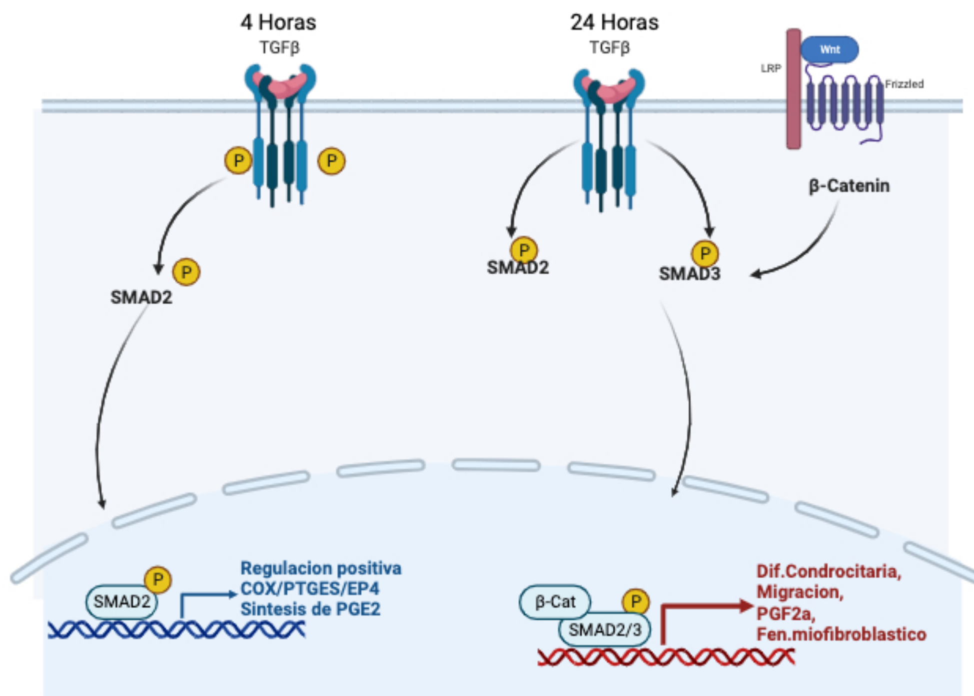


Figura 2. Representación esquemática del efecto de TGF-β en el tiempo. A las 4 horas se produce la fosforilación del factor de transcripción SMAD2, que genera la regulación positiva de COX2, aumentando la la síntesis de PGE_2. A las 24 horas, la fosforilación de SMAD2 y SMAD3 junto a la translocación de β-Catenina estimula el fenotipo miofibroblastoide y síntesis de PGF2a.

Bajo la perspectiva de que la matriz extracelular se encuentra con sobre deposición de componentes y factores profibróticos como TGF-β, la factibilidad de anidamiento de células en un ambiente fibrótico se vería disminuida (Park et al., 2011).
Ante esta desventaja, el uso del secretoma de las MSC se avizora como una solución, el aprovechamiento de la capacidad paracrina de sus componentes ha sido demostrado en diferentes condiciones patológicas, que incluyen regeneración del sistema nervioso central, tejido hepático, cardiaco y muscular; (Kim et al., 2009; Cantaluppi et al., 2012; Mitchell et al., 2019).
Estas células no solo interactúan con su ambiente utilizando los factores solubles como quimoquinas, citoquinas, NOS, prostanoides y factores de crecimiento sino también factores encapsulados en vesículas extracelulares.Su carga incluye una amplia gama de moléculas con función paracrina reguladora probada, como microARNs y otros tipos de ARN pequeños. Clasificados por biogénesis, se describen tres tipos de VE, los exosomas (40-100 nm), las micro vesículas (100-1000 nm) y los cuerpos apoptóticos (1000-5000

nm). Las VE han demostrado poseer o inducir propiedades terapéuticas *in vitro* e *in vivo*, tales como angiogénesis, promoción de la neurogénesis, acción anti-inflamatoria e inhibición de la fibrosis (Fujita et al., 2018; Vilaça-Faria et al., 2019; Yin et al., 2019). Recientemente, en MSCs equinas de tejido adiposo (eAT-MSC), se confirmó la presencia de VE y se secuenció la carga de ARNs (Capomaccio et al., 2019).

La capacidad antifibrótica de estas vesículas extracelulares y sus cargos ha sido demostrada ampliamente (Lv et al., 2022). La MSC pueden atenuar la fibrosis hepática a través de la supresión de los linfocitos TH17 y este mismo efecto ha sido logrado en modelos de ratones, suministrando las vesículas extracelulares de forma periférica (Milosavljevic et al., 2018). En otro modelo murino, en donde se indujo fibrosis hepática a partir de CCL4 , las vesículas extracelulares fueron capaces de reducir la inflamación e inhibir la deposición de colágeno, esto último inhibiendo la vía TGF-β/SMAD 2 (Li et al., 2013). El mismo mecanismo fue observado por (Fang et al., 2016), pero además demostró inhibición en la conversión a miofibroblasto. Otro ejemplo de la acción de estas VE, es la capacidad de suprimir la actividad de los miofibroblastos a partir de la activación de la vía Hippo/TAZ con un consecuente bloqueo de las señales de TGF-β (H. Zhu et al., 2019). Estas propiedades pudieran ser atribuibles a la acción que tienen los miARNs que son parte fundamental del cargo de las vesículas extracelulares. Los siguientes miARNs están descritos como antifibróticos: miR-21, miR23a, miR-125b y miR-145 y se han encontrado en vesículas extracelulares de células madre de origen umbilical.

El perfil del secretoma y el cargo de las vesículas extracelulares de las células madre dependerá del estímulo al cual esté sometida la célula, generando un abanico de posibilidades para su manipulación en enfermedades. Con los resultados previos obtenidos en el capítulo 2, en el cual se definió un perfil secretor antifibrótico basado en un preacondicionamiento con TGF-β de las MSC, se procedió a determinar los posibles blancos asociados a la actividad antifibrótica de las secreciones de las células madres derivadas de tejido adiposo equino en el modelo in vitro de fibrosis estromal endometrial (objetivo 3).

Para ello se analizaron los cambios en la expresión de miARNs relacionados con procesos fibróticos en MSC expuestas a diferentes tiempos de preacondicionamiento con TGF-β, la presencia y expresión de tales miARNs en vesículas extracelulares y los efectos de vesículas extracelulares derivadas de MSC pre-condicionadas, en la reversión de la fibrosis en un modelo in vitro de células estromales de endometrio fibrótico (logradas em el objetivo 1).

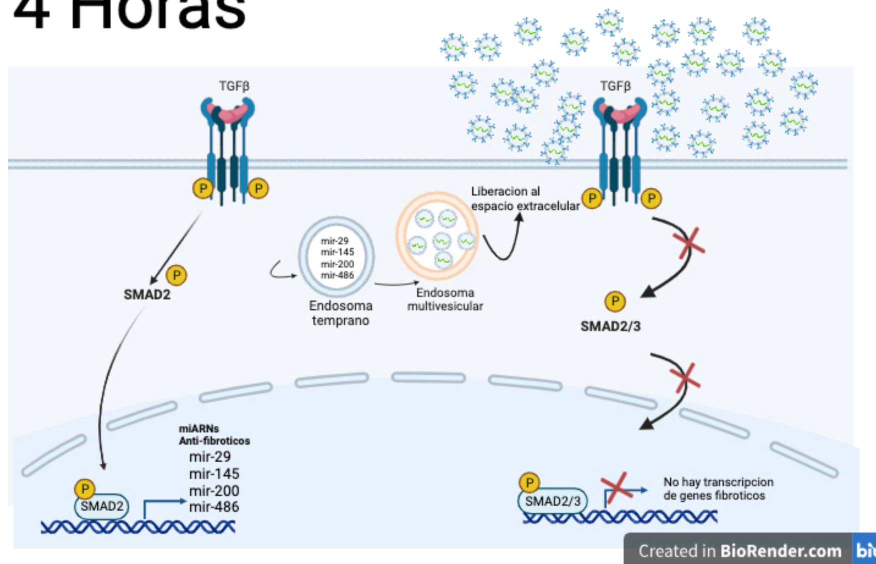
El tratamiento de AT-MSC con TGF-β por 4 horas produjo un notable aumento de miARNs antifibróticos en las VE secretadas por dichas células. Por el contrario, si las células se exponen durante más tiempo (24 horas), se produce un cambio hacia un patrón de ARNm y miARN relacionado con la fibrosis. Estos resultados se corroboraron al exponer células estromales endometriales a VE de AT-MSC pre-condicionadas, lo que provocó una disminución de la expresión de los principales marcadores de fibrosis.

En los tejidos sanos, la sobreproducción de componentes de la matriz extracelular y la aparición de fibrosis están limitadas por miARN antifibróticos, como la familia mir29, que se dirige a 16 genes relacionados con la MEC (Cushing et al. 2011). También se ha observado la regulación al alza de la expresión de mir-29 en macrófagos con un fenotipo polarizado anti inflamatorio (Domingo-González et al. 2015), a través de la inhibición de H4K20me3 (Lyu et al. 2018). Otros miARNs, como mir145 han mostrado una regulación a nivel de los receptores de TGF-β como TGFBRII y por consecuencia una inhibición en la translocación de SMAD3 (Megiorni et al. 2013). La regulación a la baja de este miARN es una característica común en varios tipos de fibrosis. El microARN, mir-200 juega un papel crucial regulando la expresión de *ZEB1/αSMA* (Yang et al. 2012) y mir486 reprime directamente a SMAD2 (Douvris et al. 2022). En este trabajo, estos miARNs fueron regulados al alza a las 4h y se observó disminución de su expresión a las 24h, lo que sugiere una retroalimentación negativa de TGF-β sobre sus efectores para amortiguar el fenotipo miofibroblasto de AT-MSC. Melling et al., 2018, observó este mismo fenómeno en la conversión de fibroblastos a miofibroblastos asociados a un ambiente tumoral, proponiendo una regulación basada en un bucle de retroalimentación negativa similar.

En los tejidos fibróticos, la diferenciación hacia el fenotipo miofibroblasto va acompañada de cambios en los niveles de miARN, que se dirigen a genes que controlan la progresión de la enfermedad (Fu et al., 2021). En nuestro trabajo, a las 24 horas se produjo un aumento de los miARN profibróticos mir-17, mir-192, mir-199 y mir-433. El micro ARN, mir-17 tiene como diana a SMAD7, que regula negativamente la vía TGF-β/SMAD2/3 y está relacionado con la fibrosis quística y renal (Oglesby et al. 2015; Fu et al. 2021). A su vez, la inhibición de SMAD7 conduce a un aumento de la expresión de mir-192 (Chung et al. 2010), que actúa directamente sobre el represor ZEB2-E-box de *COL1A2* (Kato et al. 2007). Altos niveles de expresión de mir-199 fueron observados y estos están relacionados con la fibrosis pulmonar inducida por bleomicina en ovejas y ratones. La diferenciación de miofibroblastos en la fibrosis submucosa oral humana con

incremento de *COL1A1* y *COL3A1* (Yuan et al. 2019; Perera et al. 2021) y la desregulación de mir-433 resultan en la inhibición de *AZIN1*, un regulador de la producción de MEC con la subsiguiente amplificación de la vía de señalización de TGF-β (Li et al. 2013).

En el gráfico 3 se plantea la hipótesis de este trabajo sobre la regulación dinámica de TGF-β en las células madre mesenquimales derivadas de tejido adiposo y el cambio en el patrón del contenido de las vesículas extracelulares.



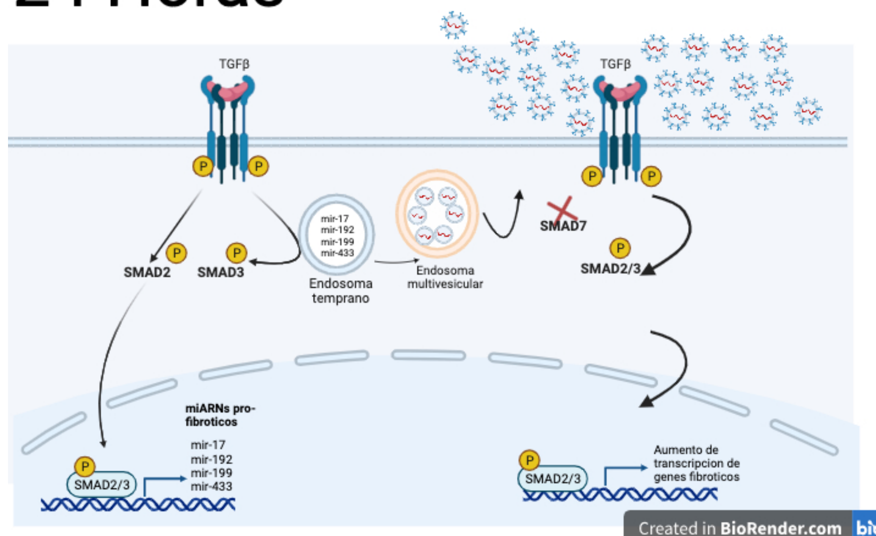


Figura 3. Regulación dinámica de TGF-β en las células madre mesenquimales derivadas de tejido adiposo y cambio en el patrón del contenido de las vesículas extracelulares. A las 4 horas la fosforilación de SMAD2 permite un aumento en la transcripción de los

miARNs antifibróticos, los cuales se cargan en las VE y al ser liberados al espacio extracelular autoregulan negativamente los receptores de TGF-β. Contrariamente, al fosforilarse SMAD3, se estabiliza el complejo SMAD2/3 y permite una transcripción de miARN profibróticos que reprimen al represor del receptor de TGF-β.

En los experimentos de esta tesis, las células estromales endometriales expuestas a VE liberadas por AT-MSC pretratadas con TGF-β durante 4h, redujeron la expresión de genes fibróticos como *αSMA, CTGF* y *COL1A1*, notablemente VE procedentes de l tiempo cero experimental (niveles basales, de células no expuestas a TGF-β), conducen a un fenotipo similar de genes fibróticos. Otros informes apoyan las propiedades antifibróticas del secretoma de AT-MSC y destacan la acción clave de los miARN para revertir el fenotipo de miofibroblasto (Li et al. 2021; Liu et al. 2023). En el trabajo de Basalova et al., 2020, las AT-MSC humanas inhibieron significativamente la expresión de SMA en los miofibroblastos mediante la transferencia de mir-29 antifibrótico dentro de la VE secretada. En la fibrosis endometrial, el mir-145 encerrado en VE generó una mejora en la enfermedad en humanos (Zhou et al. 2023). Previamente nuestro grupo demostró la sobreexpresión de mir-199 profibrótico en AT-MSC equinas (Navarrete et al., 2020), p.or lo tanto, las MSC podrían promover o combatir la fibrosis dependiendo de sus condiciones de cultivo o nicho. *In vitro*, esto puede lograrse mediante un ajuste fino del preacondicionamiento con TGF-β de las AT-MSC como en los experimentos descritos anteriormente. A las 4h de preacondicionamiento con TGF-β, hay una carga antifibrótica de miARNs en las VE, mientras que se observa lo contrario si las células son tratadas durante 24h. Estos resultados son congruentes con (Salehipour-Bavarsad et al. 2022), quienes observaron una reducción del nivel de *αSMA* y *COL1A1* y una inhibición de la proteína fosforilada del complejo SMAD2/3 en células estelladas hepáticas activadas utilizando VE derivadas de MSC de gelatina de Wharton humanas pretratadas con TGF-β.

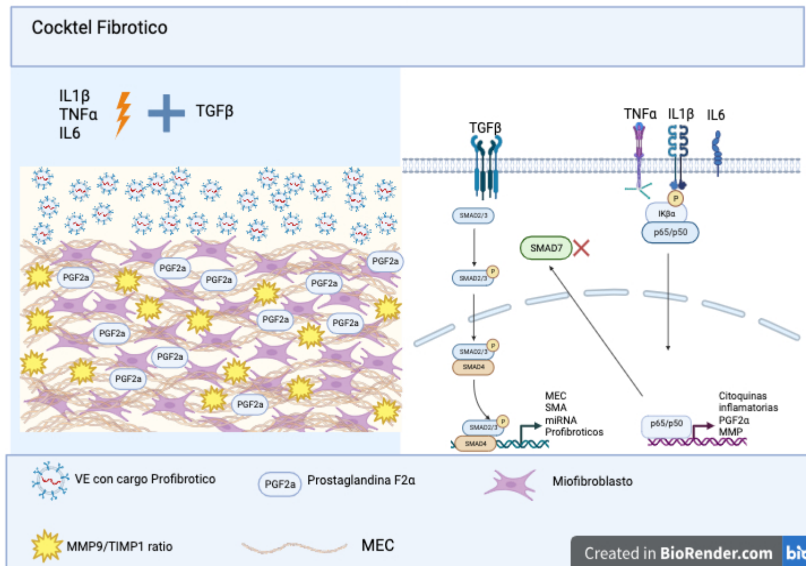
En esta investigación, la secuenciación de última generación (NGS) reveló un patrón transcriptómico diferente en las células endometriales estromales inducidas con cóctel fibrótico en comparación con el control.

Los genes diferencialmente expresados (DEG) en estas células estaban relacionados con la estimulación del TGF-β, la organización de la MEC y la regulación de la respuesta inflamatoria. DE relevancia es la alta expresión de MMP13, una proteína proteolítica relacionada con la progresión de patologías de inflamación crónica y POSTN, una

proteína de la MEC relacionada con el estímulo de TGF-β, que están implicadas en la inducción de la producción de colágeno tipo I y la diferenciación de miofibroblastos en la fibrosis pulmonar (Guo et al. 2021; Alzobaidi et al. 2022). Otros genes relevantes en la fibrosis sobreexpresados en la condición inducida fueron *CCN2*, *ACTA2*, *COL1A2* y *COL3A1*. Estos resultados validan nuestro modelo de fibrosis endometrial caracterizado por inflamación crónica y deposición de MEC en células estromales (Trundell 2022). Cuando estas células se expusieron a VE procedentes de AT-MSC pretratadas con TGF-β, se produjo una regulación al alza de varios genes relacionados con el interferón I. Los interferones de tipo I son estimuladores de genes antivirales, actúan como reguladores de la respuesta inmunitaria y podrían antagonizar la acción del TGF-β (Dhanani et al. 1994; Okada et al. 2018).

La activación del gen TL3, un inductor específico de interferones de tipo I, mitiga el fenotipo profibrótico de los miofibroblastos de la piel a través de la señalización autocrina del interferón, con la interrupción de la fosforilación de SMAD2/3 y la regulación al alza de su inhibidor, SMAD 7 (Fang et al. 2013). Un mecanismo similar se observa en la interferencia de interferón-gamma con la respuesta de TGF-β a través de JAK/STAT en células estelladas hepáticas, lo que lleva a la reducción de la expresión de αSMA y COL1A1 a través de la inhibición del complejo SMAD3/4 y la sobre activación de SMAD 7 (Weng et al. 2007). Así mismo, el fracaso en el control de la inflamación en enfermedades fibróticas se ha relacionado con el eje COX2/IFN-lambda, que está controlado por mir29, a través de su diana directa del gen ADN-metiltransferasa 3A, un inhibidor de COX2 (Fang et al. 2012). En consecuencia, se encontró un aumento de la expresión de mir29 en el grupo tratado con VE por 4h y una alta expresión de SMAD7 estableciendo un posible mecanismo de regulación antifibrótica causada por esta carga de miARN en las vesículas.

En la figura 4 se visualizan los cambios producidos por las vesículas extracelulares derivadas de MSC precondicionadas con 4 horas de TGF-β en el modelo de endometrosis in vitro.



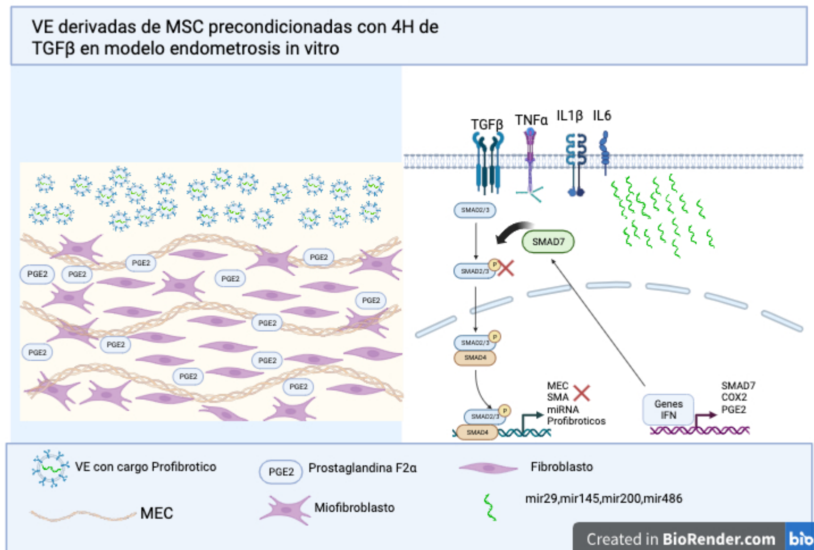


Figura 4. Efecto de las VE derivadas de MSC pre-condicionadas sobre el modelo de endometrosis in vitro. Se observa en el modelo el aporte profibrótico de las citoquinas proinflamatorias que tiene como consecuencia la inhibición del represor SMAD7 y por consiguiente generan el aumento del fenotipo miofibroblasto y síntesis de MEC. Ante la exposición de VE, se genera un bloqueo del complejo SMAD2/3 por parte de genes ligados a IFN y un aumento de síntesis de PGE2 disminuyendo el fenotipo miofibroblástico.

IX. Conclusiones Generales

1. Se obtuvo un modelo in vitro de fibrosis estromal endometrial, a partir de la exposición de fibroblastos endometriales a citoquinas proinflamatorias(IL1-β, IL-6, TNF-α) y TGF-β.

2. Las citoquinas proinflamatorias (IL1-β, IL-6, TNF-α) en combinación con TGF-β, aumentan significativamente la expresión de los genes y miARNs relacionados a la fibrosis en células estromales endometriales.

3. Existe un efecto hormonal sobre el modelo in vitro de fibrosis estromal endometrial: durante la fase folicular, se promueve el efecto de las citoquinas proinflamatorias y TGF-β, en cambio en la fase luteal, se observa un efecto protector caracterizado por una alza en miARNs antifibróticos y síntesis de PGE_2

4. TGF-β ejerce un efecto sobre las MSC, dependiente del tiempo de exposición: el preacondicionamiento por 4 horas con TGF-β, resulta en un fenotipo antifibrótico, caracterizado por un aumento en la síntesis de PGE_2 y producción de miARNs antifibróticos tanto a nivel celular como a nivel de VE de 4 horas.

5. Las VE derivadas de MSC pre-condicionadas con TGF-β por 4 horas inhiben el fenotipo profibrótico en nuestro modelo, activando genes relacionados a IFN tipo I y reduciendo la expresión de genes relacionados a la fibrosis.

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XI. Anexo

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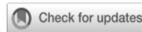

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Mare stromal endometrial cells differentially modulate inflammation depending on oestrus cycle status: an *in vitro* study

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The modulation of inflammation is pivotal for uterine homeostasis. Here we evaluated the effect of the oestrus cycle on the expression of pro-inflammatory and anti-inflammatory markers in a cellular model of induced fibrosis. Mare endometrial stromal cells isolated from follicular or mid-luteal phase were primed with 10 ng/mL of TGFβ alone or in combination with either IL1β, IL6, or TNFα (10 ng/mL each) or all together for 24 h. Control cells were not primed. Messenger and miRNA expression were analyzed using real-time quantitative PCR (RT-qPCR). Cells in the follicular phase primed with pro-inflammatory cytokines showed higher expression of collagen-related genes (*CTGF, COL1A1, COL3A1*, and *TIMP1*) and mesenchymal marker (*SLUG, VIM, CDH2*, and *CDH11*) genes; $p < 0.05$. Cells primed during the mid-luteal overexpressed genes associated with extracellular matrix, processing, and prostaglandin E synthase (*MMP2, MMP9, PGR, TIMP2*, and *PTGES*; $p < 0.05$). There was a notable upregulation of pro-fibrotic miRNAs (miR17, miR21, and miR433) in the follicular phase when the cells were exposed to TGFβ + IL1β, TGFβ + IL6 or TGFβ + IL1β + IL6 + TNFα. Conversely, in cells from the mid-luteal phase, the treatments either did not or diminished the expression of the same miRNAs. On the contrary, the anti-fibrotic miRNAs (miR26a, miR29b, miR29c, miR145, miR378, and mir488) were not upregulated with treatments in the follicular phase. Rather, they were overexpressed in cells from the mid-luteal phase, with the highest regulation observed in TGFβ + IL1β + IL6 + TNFα treatment groups. These miRNAs were also analyzed in the extracellular vesicles secreted by the cells. A similar trend as seen with cellular miRNAs was noted, where anti-fibrotic miRNAs were downregulated in the follicular phase, while notably elevated pro-fibrotic miRNAs were observed in extracellular vesicles originating from the follicular phase. Pro-inflammatory cytokines may amplify the TGFβ signal in the follicular phase resulting in significant upregulation of extracellular matrix-related genes, an imbalance in the metalloproteinases, downregulation of estrogen receptors, and upregulation of pro-fibrotic factors. Conversely, in the luteal phase, there is a protective role mediated primarily through an increase in anti-fibrotic miRNAs, a decrease in SMAD2 phosphorylation, and reduced expression of fibrosis-related genes.

KEYWORDS

endometrosis, endometrium stromal cells, fibrosis-related genes, pro-fibrotic miRNA, anti-fibrotic miRNA, extracellular vesicles, TGFβ signaling pathway

1. Introduction

Transient breeding-induced endometritis (TBIE) is a physiological event in mares characterized by local inflammation of the superficial layer of the uterus involving the infiltration of neutrophils and an increase in the expression of genes associated with the innate immune response (1). Typically, TBIE tends to resolve within 48 h following mating or insemination, leading to a full resolution of the inflammatory process (2). TBIE starts with the recognition of damage-associated molecular patterns (DAMPS) related to seminal plasma components or pathogen-associated molecular patterns (PAMPS) that trigger an acute inflammatory response driven by the activity of the NF-kb pathway with IL1β, IL6, and TNFα as principal cytokines and by the rapid response of the innate immune effector cells. Neutrophils release extracellular traps that favor the control of pathogens as well as the posterior production of prostaglandin F2 alpha ($PGF_{2\alpha}$) by macrophages to stimulate myometrial contraction to clear the cellular debris. Soon afterward, anti-inflammatory cytokines such as IL10, IL22, and IL1RN increase and allow for the correct repair of the tissue (3). However, 15% of all mares are unable to suppress this inflammation; therefore, they develop persistent breeding-induced endometritis (PBIE), which leads to the prolonged presence of polymorphonuclear cells, uterine fluid accumulation, and persistence of inflammatory cytokines that modify endometrial receptivity (4, 5).

While PBIE may present traditional clinical indicators, it can also manifest in a subclinical manner (6), and the degree of the inflammatory response influences the expression of pro-inflammatory cytokines. For instance, IL1β is significantly upregulated in PBIE in susceptible mares (7), while IL6 or TNFα are predominant in chronic subclinical endometritis and overexpression of IL1β, IL6, and TNFα is observed in subacute suppurative endometritis (8).

Following the inflammatory stimulus, a remodelling phase starts with an increase in TGFβ released by macrophages. TGFβ is a cytokine with a dual role: suppression of the innate immune system and activation of fibroblast conversion to myofibroblast. The latter are the principal effectors in healing, as the myofibroblasts synthesize extracellular matrix components (ECM) (9). If inflammation persists, the continuous expression of TGFβ helps to maintain the activity of myofibroblasts, which leads to excessive deposition of ECM and alteration in the architecture of the organ (increased stiffness, reducing the functionality of the glands), leading to an unfavorable uterine environment (10). This, in turn, compromises the fertility of the mare, and Kenney and Doig (11) coined this pathology as endometriosis, classifying it depending on the level of damage. Hoffman et al. (12) defined endometriosis as destructive or non-destructive periglandular and stromal fibrosis with varying degrees of metabolic activity.

In mares, endometrial fibroblasts are regulated by ovarian steroids and their receptors throughout the oestrus cycle (13, 14). Notably, the kinetic changes that occur in the uterus include proliferation during the follicular phase under high concentrations of estradiol (E2) and increased production of specific matrix metalloproteinases, MMP2 and MMP9, indicating the active remodeling processes occurring during this phase (15). In contrast, under a high concentration of progesterone (P4) in the mid-luteal phase, the expression of MMPs is downregulated with a simultaneous increase in tissue inhibitor of metalloproteinases (TIMPs) expression and that of prostaglandin E2 (PGE_2) synthesis, leading to an anti-inflammatory environment that reflects the preparedness of the endometrium for embryo receptivity (16, 17). With chronic inflammation as in TBIE or subclinical endometritis, the paracrine orchestration is disturbed: TGFβ activity exacerbates α-SMA (alpha-smooth muscle actin) expression in endometrial fibroblasts while reducing the expression of ovarian receptors and provoking a malfunction in the prostanoid system, an imbalance in MMPs and TIMPs and uncontrolled ECM deposition (18, 19). This downstream action is mediated by the binding of TGFβ to its heterodimer transmembrane receptors which induce phosphorylation of the transcription factors SMAD2 and SMAD3 and their subsequent translocation to the nucleus resulting in the activation of most fibrosis-related genes (20).

The establishment of a fibrotic environment is a very complex process, which in addition to the factors mentioned above, involves the action of micro RNAs (miRNAs), both cellular and also contributed by extracellular vesicles (EVs) of different origins (21, 22). Several miRNAs such as mir192, mir29, mir199, mir21, and mir17 have been shown to be involved in fibrotic processes in the liver, lungs and kidneys (23). The miRNAs act as mRNA repressors of multiple genes including TGFβ effectors like SMAD2/3, SMAD7, WNT signaling pathway, or specific related key proteins to limit the deposition of ECM proteins (24).

Emerging mechanisms of communication of the uterine stromal component with adjacent tissues, including immune cells, have been observed (20, 24). This intercellular communication is carried out by extracellular vesicles, which are small particles from 80 to 220 μm of heterogeneous origin composed of lipid bilayers engulfing cargoes of a plethora of molecules (mainly miRNA, mRNA, and proteins) capable of regulating target cells over long distances (25). Evidence suggests an active participation of EVs in the establishment of different pathologies, including fibrosis. The cargo of EVs can stimulate inflammatory and fibrotic processes or antagonize them (25). This communication system offers the possibility to discover potential biomarkers for several pathological conditions including fibrotic processes such as endometriosis.

In this work, we addressed the following hypothesis: there is a hormonal influence on the fibrotic response induced by the inflammatory environment in stromal cells. To confirm this hypothesis, we simulated the follicular phase and the mid-luteal phase in endometrial fibroblasts based on the respective serum hormone concentration and challenged them with pro-inflammatory cytokines (IL1β, IL6, and TNFα) and TGFβ and evaluated (i) the expression of genes related to fibrosis response, (ii) the miRNA profile of primed cells in the follicular or mid-luteal phase, (iii) the miRNA cargo of EVs, and (iv) the expression of the SMAD2/TGFβ pathway.

2. Materials and methods

The animal study was approved by the Ethics Committee of the Faculty of Veterinary Sciences, University of Concepcion, Chile (CB-10-2019). The study was conducted in accordance with the local legislation and institutional requirements.

2.1. Samples collection and classification

All animals were healthy as determined by official veterinary inspection. The samples were obtained from mares for meat production and collected immediately post-mortem at a local abattoir (Frigosur, Chillan) during the reproductive season (September–January). For a further measure of the basal levels of E2 and P4, 1 mL of blood was withdrawn from the jugular vein into an ethylene-diaminetetraacetic acid (EDTA) tube, before death. The complete uteri, including the ovaries, were transported at 4°C to the laboratory. E2 concentration were determined using horse estradiol ELISA kit (CSBEQ027953HO, CUSABIO, TX, United States), and P4 concentrations were measured using the horse P4 ELISA kit (CSBE13183Hs, CUSABIO). The measure of follicular diameter and the presence of a corpus luteum (CL) was used to discriminate the oestrous cycle phases. Ovaries with follicles over 35 mm in diameter, plasma levels of P4 less than 1 ng/mL, and E2 above 4 pg./mL were from mares considered to be in the follicular phase, while ovaries with follicles smaller than 20 mm and the presence of CL, plasma levels over 2 ng/mL of P4 and E2 less than 2 pg./mL indicated the mare was in the luteal phase. A biopsy of the endometrium at the interhorn region was carefully taken and immersed in 4% buffered formaldehyde for histological analysis based on Kenney and Doig's criteria classification. Only the samples without any sign of endometriosis were selected for the present study.

2.2. Endometrial stromal cell isolation and culture

The surface of the uteri was cleaned with 70% alcohol and sprayed with povidone-iodine. Using a scalpel and surgery scissors, we made a long narrow cut at the interhorn region to expose the endometrial cavity. Using tweezers and scissors a strip was excised from the myometrium and washed three times in PBS containing antibiotic 2X Antibiotic-Antimycotic solution (MT30004 CL, Corning™, NY, United States). The strip was cut into small pieces of around 1 mm with the scalpel and digested with digestion buffer (1 mg/mL collagenase type I, Sigma-Aldrich™, MO, United States), 4 mg/mL dispase-I (D4818, Sigma-Aldrich™ in PBS) for 1 h at 38.5°C with continuous agitation. The cell suspension was filtered using 40 μm cell strainers to remove the remaining undigested tissue and the resulting filtrate was washed with Dulbecco's Modified Eagle Medium (DMEM) high glucose supplemented with Glutamax (10,569,010, Gibco™, NY, United States) and 1x Antibiotic-Antimycotic solution, and centrifuged for 10 min at 500 g. The obtained pellet was resuspended in the culture media (DMEM high glucose supplemented with Glutamax, 1x Antibiotic-Antimycotic solution, and 10% Foetal bovine Serum (FBS) 12,484,028, Gibco™) and seeded in a T75-flask bottle at 38.5°C with a humidified atmosphere of 5% CO_2. Further, the monolayer reached the 90% confluence and was cryopreserved for experimentation.

2.3. The effect of different combinations of pro-inflammatory cytokines in the presence of TGFβ

Depending on the oestrous phase classification, the endometrium stromal cells were pooled and seeded at 38.5°C with a humidified atmosphere of 5% CO_2 in six-well plates in triplicate for each oestrous cycle phase condition at 8×10^6 cells per well in a follicular medium (a culture medium supplemented with 0.5 ng/mL P4 and 30 pg./mL E2) or a mid-luteal medium (a culture medium supplemented with 15 ng/mL P4 and 2 pg./mL de E2) for 24 h. As soon the cells attached to the plate, the medium was changed to the follicular or mid-luteal media with 1% of FBS and the treatments were added:

(1) Control (naive), (2) TGFβ (10 ng/mL), (3) TGFβ + IL1β (10 ng/mL each), (4) TGFβ + IL6 (10 ng/mL each), (5) TGFβ + TNFa (10 ng/mL each), (6) TGFβ + IL1β + IL6 + TNFa (10 ng/mL each) for 24 h.

The monolayers were detached using 0.25% Trypsin–EDTA and the single-cell solutions were split in two: one portion for protein expression of TGFβ /SMAD pathway and the second portion for transcript expression of fibrosis-related mRNA and miRNA. Schematic experimental design is visually represented in Figure 1.

2.4. The effect of different combinations of pro-inflammatory cytokines in the presence of TGFβ on miRNA cargo in extracellular vesicles

Depending on the oestrous cycle phase classification, the endometrium stromal cells were pooled and seeded at 38.5°C with a humidified atmosphere of 5% CO_2 in a 100-mm dish in triplicate for each oestrous condition at 2×10^6 cells per well in the follicular medium (a culture medium supplemented with 0.5 ng/mL P4 and 30 pg./mL E2) or the luteal medium (a culture medium supplemented with 15 ng/mL P4 and 2 pg./mL of E2) for 24 h. As soon as the cells were attached to the plate, the medium was changed to the follicular or the luteal media with 1% of FBS, and as described in 2.3, the cells were subjected to the same treatments for 24 h. Then the monolayers were washed twice with PBS and incubated with culture media with 1% of FBS previously depleted from the EVs using the protocol from Shelke et al. (26) and incubated for 48 h. The medium was then collected and vesicles were isolated, as follows: the medium was briefly centrifugated for 10 min at 500 g and the supernatant was collected and subsequently centrifugated for 30 min at 5,000 g. Then the pellet was discarded and the remaining medium was centrifugated for 1 h at 10,000 g. The resulting supernatant was clarified using an AMICON filter 100 kDa cut-off (UFC9100, Merck™, Germany) and the concentrated fraction was centrifugated at 120,000 g for 18 h. The pellet was resuspended in 50 μL of PBS and one portion was used to quantify the expression of fibrosis-related miRNA and the other portion to validate the EVs.

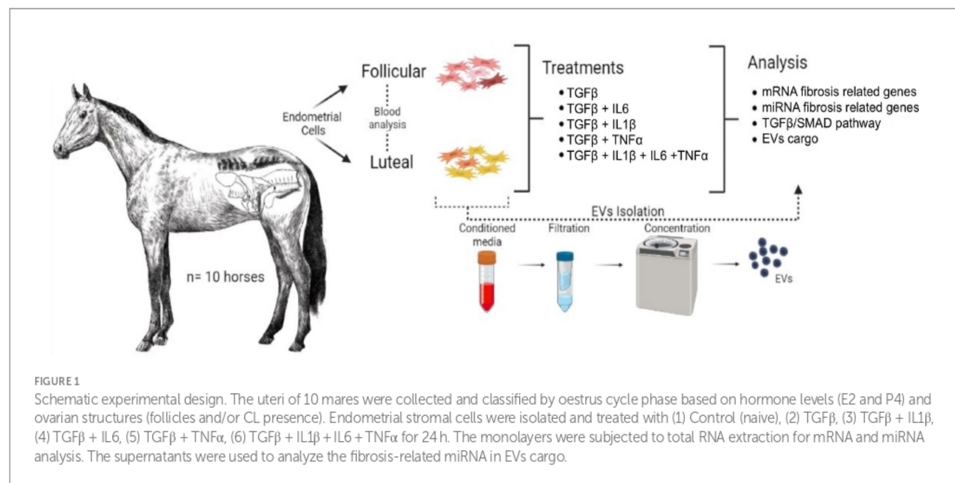

FIGURE 1
Schematic experimental design. The uteri of 10 mares were collected and classified by oestrus cycle phase based on hormone levels (E2 and P4) and ovarian structures (follicles and/or CL presence). Endometrial stromal cells were isolated and treated with (1) Control (naive), (2) TGFβ, (3) TGFβ + IL1β, (4) TGFβ + IL6, (5) TGFβ + TNFα, (6) TGFβ + IL1β + IL6 + TNFα for 24 h. The monolayers were subjected to total RNA extraction for mRNA and miRNA analysis. The supernatants were used to analyze the fibrosis-related miRNA in EVs cargo.

2.5. Protein expression analysis of SMAD2/TGFβ pathway

For protein cell expression, the pellets were lysed using RIPA buffer (NaCl 150 mM, Tris–HCl 10 mM, EDTA 1 mM, Triton X-100 1%, SDS 10%, Sodium deoxycholate 0.1%) supplemented with 1% Protease Inhibitor Cocktail (5,871, Cell Signaling Technology™, MA, United States). The homogenized cells were vortexed and centrifuged for 30 min at 10,000 g, and the resulting supernatants were collected and kept at −80°C until use. The protein concentration was measured using Pierce BCA Protein Assay Kit (23,225, Thermo Scientific™, IL, United States). Approximately 30 μg of protein were dissolved in NuPAGE LDS Sample Buffer 4x (NP0007, Invitrogen™, CA, United States) with 2% of β-mercaptoethanol and heated to 95°C for 10 min and separated in 10% SDS-PAGE. The separated proteins were electroblotted using a semi-dry method onto 0.45 μm PVDF membranes using a Trans-Blot Turbo kit, according to the manufacturer (1,704,270, Biorad™, CA, United States). Furthermore, the membranes were blocked using SuperBlock Blocking Buffer (37,515, Thermo Scientific™) for 1 h at room temperature. They were incubated in primary antibody overnight at 4°C against anti-rabbit phospho-Smad2 (18,338, Cell Signaling Technology™), anti-rabbit Smad2/3 (5,678, Cell Signaling Technology™), and anti-mouse β-actin (sc-47778, Santa Cruz Biotechnology™, TX, United States). After incubating the membranes for 1 h at room temperature with polyclonal anti-rabbit IGG HRP conjugated (7,074, Cell Signaling Technology™) or polyclonal anti-mouse IGG HRP conjugated (7,076, Cell Signaling Technology™), proteins were detected. The membranes were then washed three times with TBS-T buffer (Tris–HCl, Tween 1%) and the signal was detected using Westar Antares ECL substrate (XLS0142, Cyanagen™, Bologna, Italy) in GeneGnome XRQ system (Syngene™, Cambridge, United Kingdom). Band intensities were quantified using ImageJ software and the relative protein expression was calculated according to Heidebrecht et al. (27) using β-actin protein expression as normalizer.

2.6. Gene expression analysis

The total RNA was isolated using E.Z.N.A. Total RNA kit I (R6834-01, OMEGA™, GA, United States) according to the manufacturer's instruction and resuspended in 50 μL of molecular-grade water. The RNA purity was checked using the ratio of 260/280 nm in an Epoch microplate spectrophotometer (Agilent Technologies™, CA, United States). The cDNA was transcribed from 500 ng of RNA using a high-capacity cDNA Reverse Transcription kit (4,368,814, ThermoFisher Scientific™, Vilnius, Lithuania) according to the manufacturer's instructions. For cell miRNA expression, cDNA was synthesized according to the protocol by Balcells et al. (26). Briefly, 500 ng of RNA were incubated with 1 μL of 10x poly (A) polymerase buffer (B0276S, New England Biolabs™, MA, United States), 0.1 mM of ATP, 1 μg of RT primer, 0.1 mM of dNTP mix, 100 units of SuperScript IV reverse transcriptase (18,090,010, ThermoFisher Scientific™) and 1 unit of poly (A) polymerase (M0276S, New England Biolabs™) at 37°C for 30 min and then 52°C for 10 min and an inactivation period of 5 min at 95°C. For EVs miRNA cargo, 20 μL of cell lysis buffer from Cells-to-cDNA kit (AM8723, ThermoFisher Scientific™) and 25 fmol of synthetic spike (cel-mir39, Norgen™, ONT, Canada) was added to 20 μL EVs suspension and heated at 75°C and the cDNA was synthesised as described above.

For the qPCR, 10 μL of total volume reaction was performed in MX3000P (Agilent Technologies™) with 5 μL of KiCqStart SYBRGreen qPCR Ready Mix with Low ROX (KCQS01, Sigma-Aldrich™), 2.5 μL molecular grade water, 0.5 mix of forward and reverse primers at 10 μg and 2 μL of cDNA. Each reaction was performed in triplicate, and the relative gene expression was evaluated using the delta–delta CT method (28) with the set of primers described in Supplementary Table S1. For mRNA expression, *GAPDH* and *B2M* were used as housekeeping genes, while for miRNA expression, *Snord43* was used. The resulting geometric mean of CT was used to normalize the gene expression and the control group of the follicular phase was used as a calibrator. For EVs miRNA cargo,

cel-mir39 expression was used as normaliser. The mRNA primers were designed in-house using AmplifX™ software and the miRNA primers set was designed with miRprimer™ software.

2.7. Extracellular vesicles quantification

The resuspended extracellular vesicles were subjected to nanoparticle tracking analysis using a NanoSight NS300 (Malvern Instruments™, Malvern, United Kingdom) equipped with a 488 nm and sCMOS camera. A depleted medium was used as negative control and EV characteristics were determined at 20 to 100 particles per frame. The samples were diluted 1:100 in depleted medium in 1 mL total volume and loaded in a tuberculin syringe and injected in a continuous flow of up to 5 μL/min into the sample chamber at room temperature (RT) using an automatic syringe pump (Harvard Apparatus™, MA, United States) and the built-in software NTA 3.2 (Malvern Instruments™) were set according to Gerritzen et al. (29) for capture, recording and analysis of the nanoparticles, each sample was performed per triplicate. Graphical analysis showed the size distribution of the nanoparticles per experimental group, and the concentration was reported as particles per millilitre.

2.8. Extracellular vesicles validation

The typical protein surface markers of EVs (CD63, CD9) were evaluated using Western blot. Briefly 20 μL of resuspended EVs were lysed using NuPAGE LDS Sample Buffer 4x (NP0007, Invitrogen™) with 2% of *β*-mercaptoethanol and heated to 95°C for 10 min and separated in 10% SDS-PAGE. The separated proteins were electroblotted onto 0.45 μm PVDF membranes using Trans-Blot Turbo kit (1,704,270, Biorad™). The resulting membranes were blocked using SuperBlock Blocking Buffer (37,515, Thermo Scientific™), for 1 h at room temperature. Membranes were incubated overnight at 4°C with the primary antibody anti-rabbit CD9 (13,174, Cell Signaling Technology™) or anti-mouse CD63 (sc-365604, Santa Cruz Biotechnology™). Secondary antibodies polyclonal anti-rabbit IGG HRP conjugated (7,074, Cell Signaling Technology™) or polyclonal anti-mouse IGG HRP conjugated (7,076, Cell Signaling Technology™) were incubated with the membrane for 1 h at room temperature. The membranes were washed three times with TBS-T buffer (Tris–HCl, Tween 1%) and the signal was detected using Westar Antares ECL substrate (XLS0142, Cyanagen™) using Gene-Gnome XRQ system (Syngene™). For transmission electron microscopy (TEM) visualization, 5 μL of resuspended EVs were mixed with 4% paraformaldehyde in a 1:1 ratio. The mixture was placed on carbon–formvar-coated copper electron microscopy grids for 20 min and washed with PBS. Next, a drop of 1.5% glutaraldehyde was gently applied to the grid; after 5 min, the grid was washed 3 times with molecular grade water, and then a drop of 0.5% uranyl oxalate (Electron Microscopy Sciences, PA, United States) (pH 7.0) was applied for 5 min to facilitate contrast. The grid was dried at room temperature and placed on the TEM stand, where EV images were taken at 40,000× to 80,000× magnification on the Talos™ F200C transmission electron microscope (ThermoScientific™).

2.9. Statistical analysis

Data analyses were performed using Rstudio software and plotted with the ggplot2 package. Data are expressed as mean ± standard deviation and Two-way ANOVA (treatment x oestrus phase) was conducted followed by Tukey's HSD test as a pairwise comparison test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of genes related to fibrosis response

The expression of mesenchymal markers in endometrial stromal cells during the follicular phase is represented in Figure 2. Combined use of TGFβ + IL1β and TGFβ + IL6 produced the highest expression of *SLUG*, Vimentin (*VIM*), and Cadherin genes (*CDH2* and *CDH11*) compared to mid-luteal phase and also to naive (unprimed cells from the follicular phase). In the mid-luteal phase, there were no differences regarding the expression of mesenchymal markers in naive cells for all the analyzed genes, except *SLUG* and *CDH2*, which were upregulated in primed cells (Figure 2).

The analysis of the expression of fibrotic gene markers during the follicular and mid-luteal phases is depicted in Figure 3. There was a significant increase in the expression of α-SMA ($p < 0.05$) in all the treatments compared to that of naive cells. The highest expression levels corresponded to treatments with TGFβ, TGFβ + IL1β, and TGFβ + IL6 in both the follicular and mid-luteal phases. For the CTGF gene, there was a notable reduction in the expression in mid-luteal naive cells compared to that in the follicular naive samples. In the follicular phase samples, higher expression compared to that in the mid-luteal phase was found for all the treatments. However, in the equine endometrial stromal cells from the mid-luteal phase, TGFβ alone yielded the highest expression of the CTGF gene.

The expression of *COL1A1* and *COL3A1* genes was upregulated in the follicular phase in all treatments compared to that of the naive cells, and the highest expression was detected in the TGFβ + IL6 group. In the mid-luteal phase, only in the TGFβ group was there a significant (though discrete) increase in both collagen genes analyzed. The ratio of *COL1A1/COL3A1* expression was higher in the follicular phase, with the highest expression in the TGFβ + IL6 treatment group. No increase in this ratio was detected for the mid-luteal phase (data not shown).

The expression of *MMP2* was downregulated in the follicular phase compared to that of the naive cells, while in the mid-luteal phase, there were no changes in its expression. Conversely, *MMP9* expression was dramatically overexpressed in the follicular phase, whereas again no changes were observed in the mid-luteal phase (Figure 4). There was a steady increase in *TIMP2* expression in the follicular phase, while in the luteal phase, it remained unchanged compared to the naive cells. *TIMP1* was upregulated in both phases, with the most noticeable increase in the cells treated with TGFβ + IL1β (Figure 4). The calculated ratio of equimolar expression of *MMP2*/*TIMP2* confirmed the findings of individual gene analysis and showed an increased activity of *TIMP2* in the follicular phase, while the opposite was found for *MMP9/TIMP1* ratio (Figure 5).

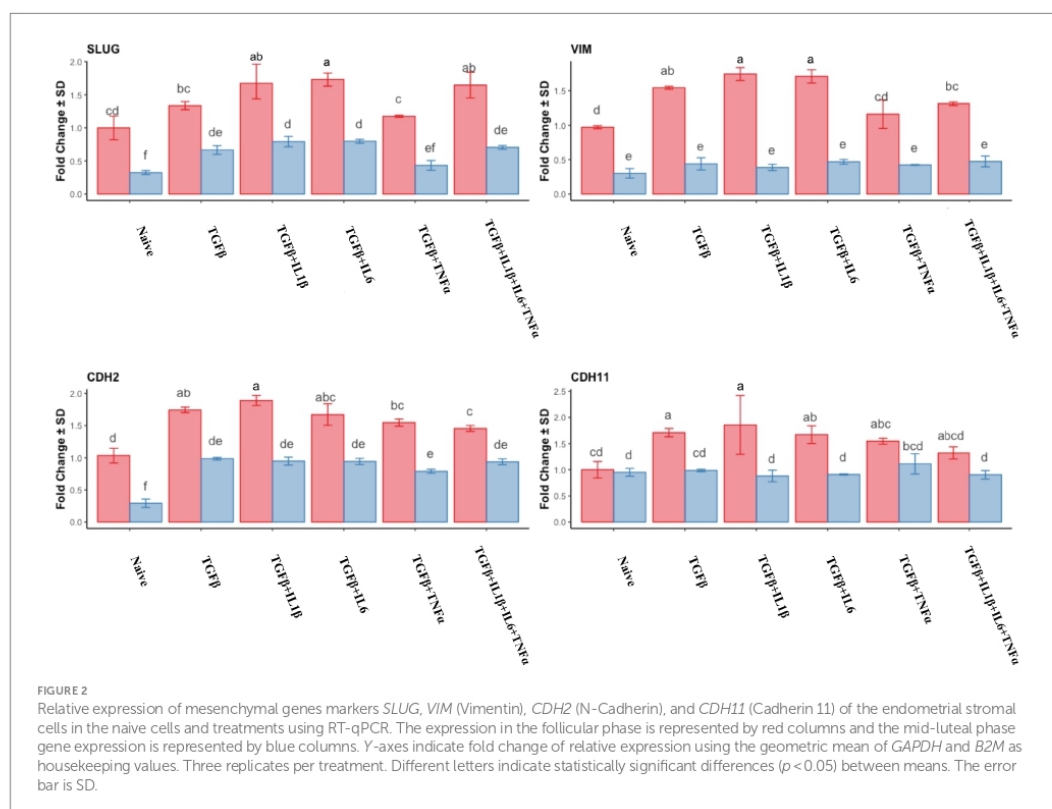

FIGURE 2
Relative expression of mesenchymal genes markers *SLUG*, *VIM* (Vimentin), *CDH2* (N-Cadherin), and *CDH11* (Cadherin 11) of the endometrial stromal cells in the naive cells and treatments using RT-qPCR. The expression in the follicular phase is represented by red columns and the mid-luteal phase gene expression is represented by blue columns. *Y*-axes indicate fold change of relative expression using the geometric mean of *GAPDH* and *B2M* as housekeeping values. Three replicates per treatment. Different letters indicate statistically significant differences ($p < 0.05$) between means. The error bar is SD.

In the follicular phase, all the treatments induced the downregulation of *ESR1* and *ESR2*, with the lowest values observed in the TGFβ + TNFα and TGFβ + IL1β + IL6 + TNFα treatment groups (Figure 6). In the mid-luteal phase, a dramatic downregulation of *ESR1* was observed, with no differences between treatments. The same pattern was observed for *ESR2* expression. In the mid-luteal phase, *PGR* expression was higher in the mid-luteal phase, and the treatments decreased it but did not abolish its expression compared to that in the follicular phase where *PGR* expression was markedly high (Figure 6). The prostaglandin E2 synthase precursor (*PTGES*) gene expression decreased alongside cytokine treatments in the follicular phase, except for the TGFβ + IL1β. Conversely, in the mid-luteal phase, there was an increase in the expression of this gene, particularly in the TGFβ + TNFα group (Figure 6).

In order to have an integrated view of the results discussed above, a heat map was created, which hierarchically clustered the genes expressed in similar amounts (Figure 7). Endometrial stromal cells were primed or not with proinflammatory cytokines, either individually or mixed, during both the follicular or the mid-luteal phase of the oestrous cycle. The resulting fold of expression in the qPCR assays for candidate genes was plotted in a heat map and three clusters were identified: (A) follicular primed, mid-luteal primed (B), and (C) naive (not primed). The cluster A—from cells in the follicular phase primed with proinflammatory cytokines—showed higher expression of collagen-related genes (*CTGF*, *COL1A1*, *COL3A1*, and *TIMP1*) and mesenchymal marker (*SLUG*, *VIM*, *CDH2*, and *CDH11*) genes. In cluster B—composed of primed cells in the mid-luteal phase—the overexpressed genes were associated with extracellular matrix processing and prostaglandin E synthase (*MMP2, MMP9, PGR, TIMP2,* and *PTGES*), while genes expressed in cells not exposed to pro-inflammatory cytokines (cluster C), independently of their oestrous cycle phase, clustered together for higher expression of hormonal receptor markers such as *ESR1* and *ESR2* (in the follicular phase) or PGR in the mid-luteal phase. In addition, the profile of expression of pro-fibrotic gene markers was the lowest in the naive cells.

3.2. MicroRNA profile of primed cells in the follicular or mid-luteal phase

The sets of miRNA were selected based on reported interaction with genes related to fibrosis and confirmed using RNA hybrid software (30). The net free energy is indicated in the Supplementary Table S2. In all cases, the free energy had negative values as expected.

Similar to the mRNA analysis, qPCR was first used to assess the expression of individual miRNA genes, and their expression was compared to that of the untreated (naive cells) cells in the follicular or mid-luteal phase, and primed with the combination of cytokines as above.

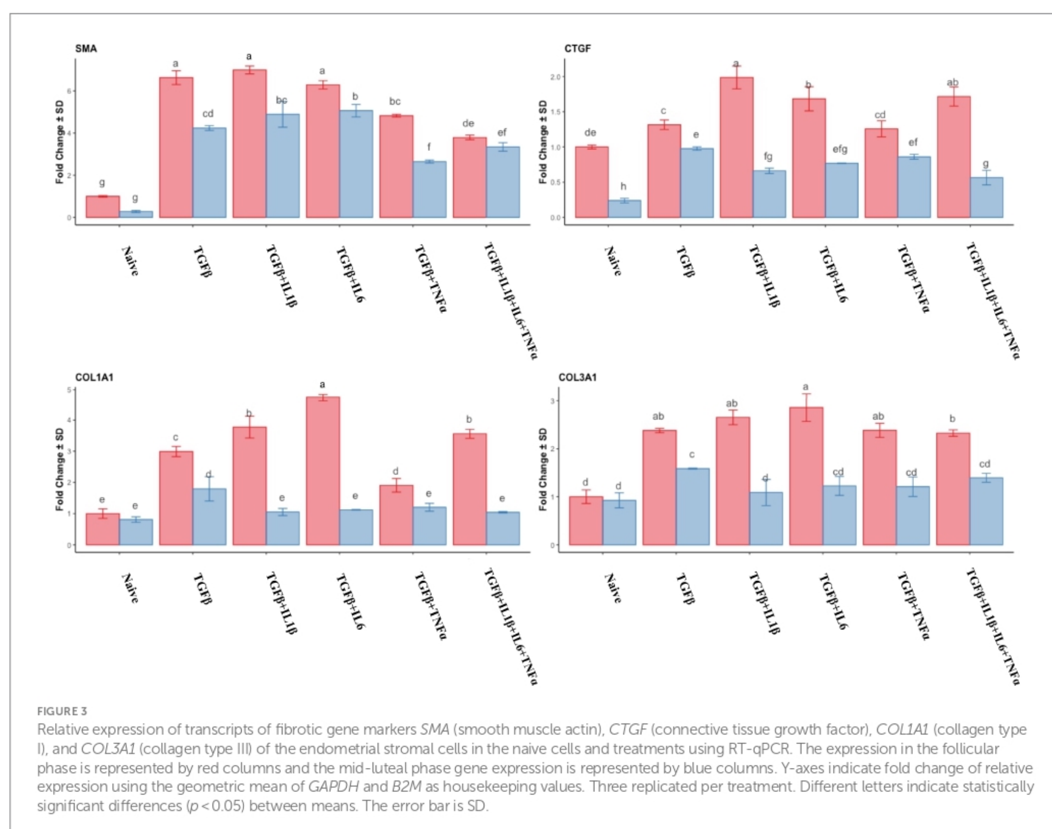

FIGURE 3
Relative expression of transcripts of fibrotic gene markers *SMA* (smooth muscle actin), *CTGF* (connective tissue growth factor), *COL1A1* (collagen type I), and *COL3A1* (collagen type III) of the endometrial stromal cells in the naive cells and treatments using RT-qPCR. The expression in the follicular phase is represented by red columns and the mid-luteal phase gene expression is represented by blue columns. Y-axes indicate fold change of relative expression using the geometric mean of *GAPDH* and *B2M* as housekeeping values. Three replicated per treatment. Different letters indicate statistically significant differences ($p < 0.05$) between means. The error bar is SD.

Furthermore, there was a notable upregulation of pro-fibrotic miRNAs (miR17, miR21, and miR433) in the follicular phase (Figure 8) when the cells were exposed to TGFβ + IL1β, TGFβ + IL6, or TGFβ + IL1β+IL6+TNFα. Conversely, in cells from the mid-luteal phase, the treatments either did not or diminished the expression of the same miRNAs (Figure 8). On the contrary, the anti-fibrotic miRNAs (mir26a, mir29b, mir29c, mir145, mir378, and mir488) were not upregulated with treatments in the follicular phase. Rather, they were overexpressed in cells from the mid-luteal phase, with the highest regulation observed in the TGFβ + IL1β+ IL6+ TNFα treatment group (Figure 9).

The collective analysis of miRNA expression was assessed using the heat map tool. The expression of pro- fibrotic miRNAs was grouped based on the follicular phase, with TGFβ + IL-1β and TGFβ + IL-6 combinations exhibiting significant upregulation compared to cells in their follicular naive state (Figure 10). Anti-fibrotic miRNAs in this phase were notably inhibited in comparison to those in the mid-luteal phase, and these results are in agreement with the clustering of mRNA profile (Figure 7).

3.3. miRNA cargo of extracellular vesicles

We further studied the EVs secreted by cells following the treatment described above, as potential tools for treating endometrial fibrosis. The concentration and size of the isolated vesicles were measured by Nano tracking analysis. Typical exosome surface markers were identified using Western blot for CD9 and CD63 (Figure 11A) and the shape was assessed using transmission electron microscopy (TEM; Figure 11B). Furthermore, the size and concentration of nanoparticles were measured, and the highest values were registered in the presence of proinflammatory cytokines in the follicular phase. The same trend was observed with the concentration values (Figure 11C).

We assayed the miRNA cargo of the isolated EVs via RT-qPCR, and for this purpose, the most expressed anti-fibrotic (mir29b and mir29c) and pro-fibrotic (mir17 and mir21) miRNAs in the previous experiment were selected. The expression of the assayed anti-fibrotic miRNAs was generally lower in the follicular phase compared to that in the mid-luteal phase, with the exception of mir29c in the presence of TGFβ alone, which was higher than that in the mid-luteal phase (Figure 12). Conversely, the pro-fibrotic miRNAs were notably upregulated in the EVs from the follicular phase compared to those in the mid-luteal (Figure 12).

3.4. Expression of the SMAD2/TGFβ pathway

To assess the activity of the TGFβ pathway, we studied the phosphorylation of SMAD2. TGFβ and the follicular phase enhanced

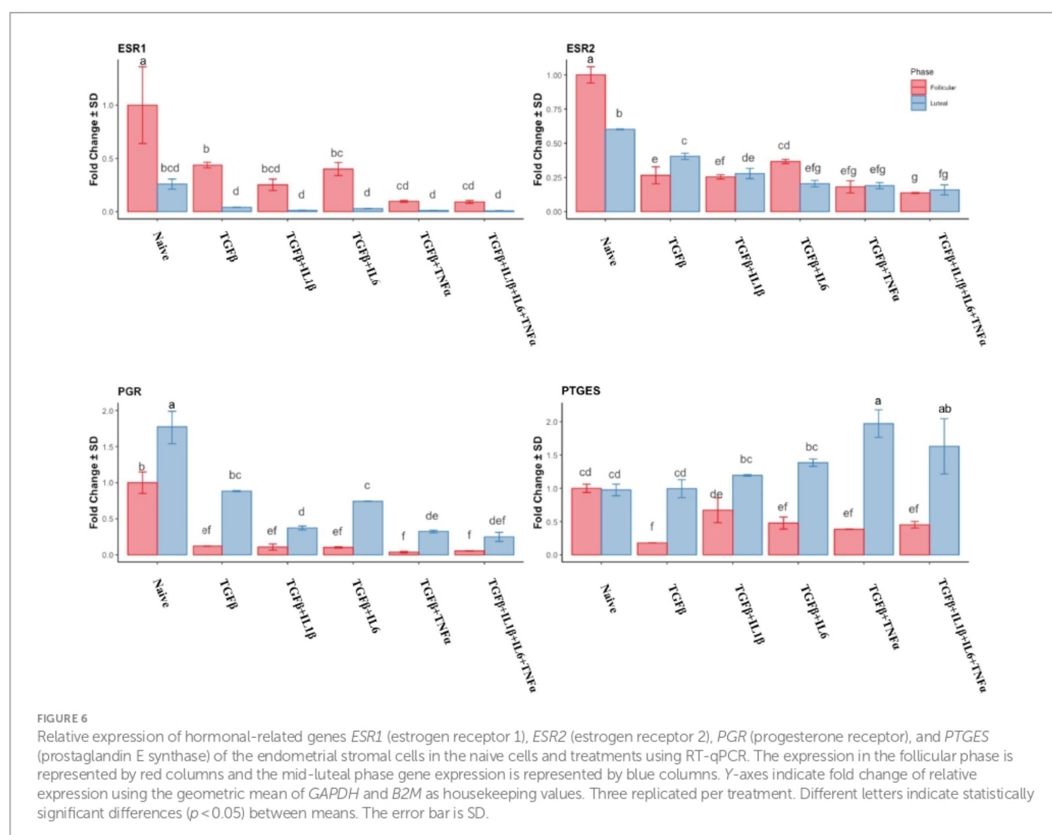

FIGURE 6
Relative expression of hormonal-related genes *ESR1* (estrogen receptor 1), *ESR2* (estrogen receptor 2), *PGR* (progesterone receptor), and *PTGES* (prostaglandin E synthase) of the endometrial stromal cells in the naive cells and treatments using RT-qPCR. The expression in the follicular phase is represented by red columns and the mid-luteal phase gene expression is represented by blue columns. *Y*-axes indicate fold change of relative expression using the geometric mean of *GAPDH* and *B2M* as housekeeping values. Three replicated per treatment. Different letters indicate statistically significant differences ($p < 0.05$) between means. The error bar is SD.

phosphorylation, and this was particularly marked when TGFβ +IL6 was used (Figure 13).

4. Discussion

In this work, we determined that pro-inflammatory cytokines might amplify the signal of TGFβ in the follicular phase, leading to a pro-fibrotic landscape, meanwhile during the mid-luteal phase, there is a protective role mediated essentially by prostaglandin E2, which favors the upregulation of anti-fibrotic miRNAs. These findings might be of help for understanding the connection between the inflammatory mechanism in susceptible mares and the establishment of endometriosis.

There is no clear understanding of the link between endometritis and endometriosis, which seems to be multifactorial. However, the inability of the endometrium to clear out cellular debris or bacteria that accumulate post-breeding has been indicated as an initiator of endometrial inflammation in endometritis, which ultimately leads to chronic inflammation in endometriosis (31, 32).

In this scenario, there is continuous signaling of pro-inflammatory cytokines in the NF-kb pathway, malfunction of the innate immune system (33, 34) deeply influenced by the stage of the oestrous cycle, and the impaired expression of hormone receptors in peripheral fibrotic glands (35, 36). TGFβ is the key molecule in the fibrotic process of the uterus that leads to endometrosis by stimulating the differentiation of gland fibroblasts to myofibroblasts (37). This process is characterized by the expression of pro-fibrotic genes.

Endometrial stromal cells play a key role in regulating the homeostasis of the extracellular matrix locally. They also play an active role in immune surveillance, acting as sentinels, producing inflammatory mediators in response to biological challenges, and the intensity of this response is greatly affected by hormonal cyclicity (38, 39). In order to explore the fibrotic response in different oestrous cycle phases, we simulated the follicular and mid-luteal phases by adding E2 and P4 at levels similar to physiological conditions (40, 41). After challenging the cell model with pro-inflammatory cytokines, we evaluated the expression of genes and miRNAs related to myofibroblast phenotype and ECM regulation, as well as the miRNAs contained in the EVs released by these cells. Finally, the phosphorylation status of protein SMAD2 was studied to better understand their interaction in this environment.

In the follicular phase, there was an increase in *SLUG* expression independent of the inflammatory cytokine(s) used. *SLUG* is key for the establishment of fibroblast senescence and secretion of proinflammatory cytokines (IL1β, IL6, and TNFα) (7, 42). It also

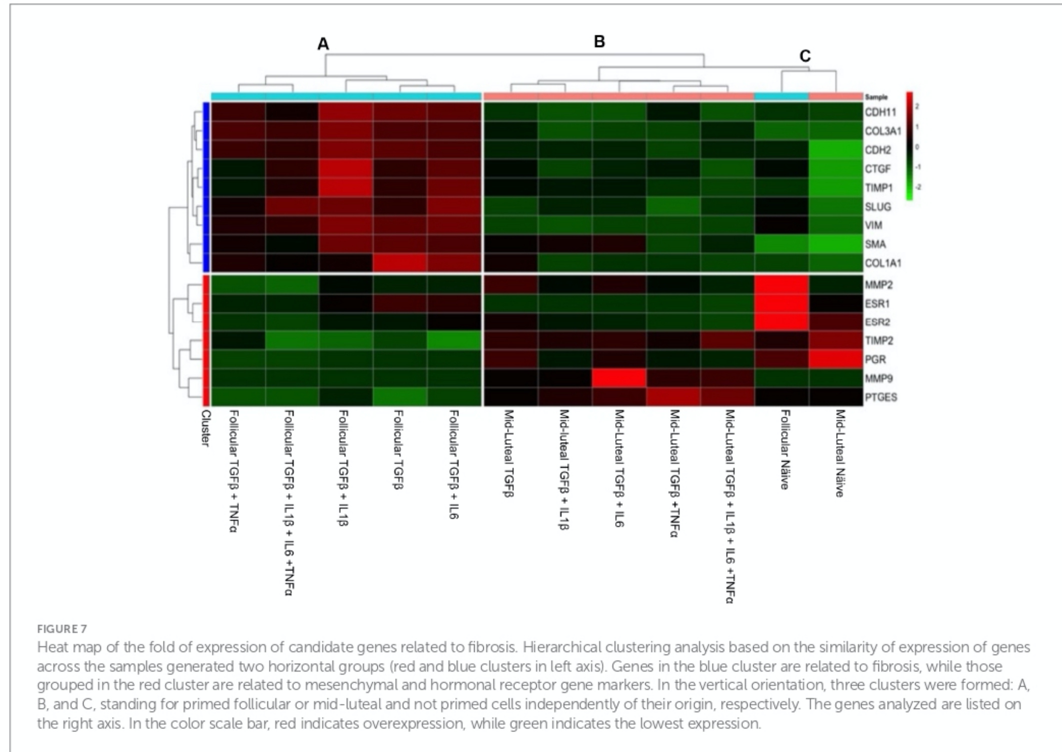

FIGURE 7
Heat map of the fold of expression of candidate genes related to fibrosis. Hierarchical clustering analysis based on the similarity of expression of genes across the samples generated two horizontal groups (red and blue clusters in left axis). Genes in the blue cluster are related to fibrosis, while those grouped in the red cluster are related to mesenchymal and hormonal receptor gene markers. In the vertical orientation, three clusters were formed: A, B, and C, standing for primed follicular or mid-luteal and not primed cells independently of their origin, respectively. The genes analyzed are listed on the right axis. In the color scale bar, red indicates overexpression, while green indicates the lowest expression.

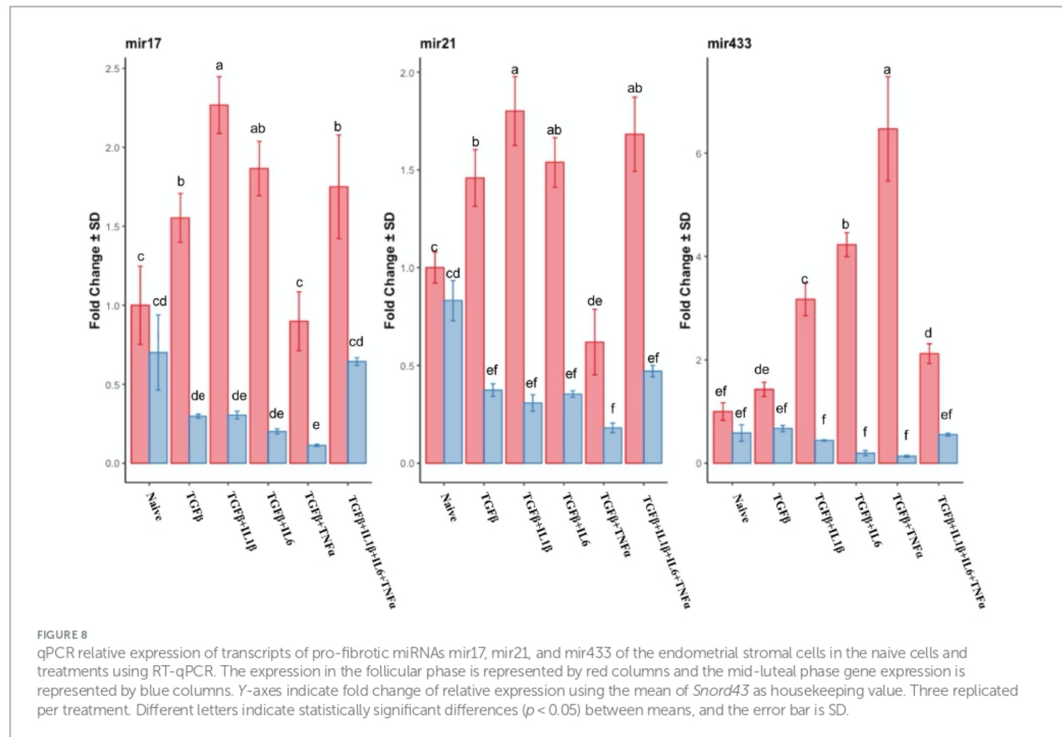

FIGURE 8
qPCR relative expression of transcripts of pro-fibrotic miRNAs mir17, mir21, and mir433 of the endometrial stromal cells in the naive cells and treatments using RT-qPCR. The expression in the follicular phase is represented by red columns and the mid-luteal phase gene expression is represented by blue columns. *Y*-axes indicate fold change of relative expression using the mean of *Snord43* as housekeeping value. Three replicated per treatment. Different letters indicate statistically significant differences ($p < 0.05$) between means, and the error bar is SD.

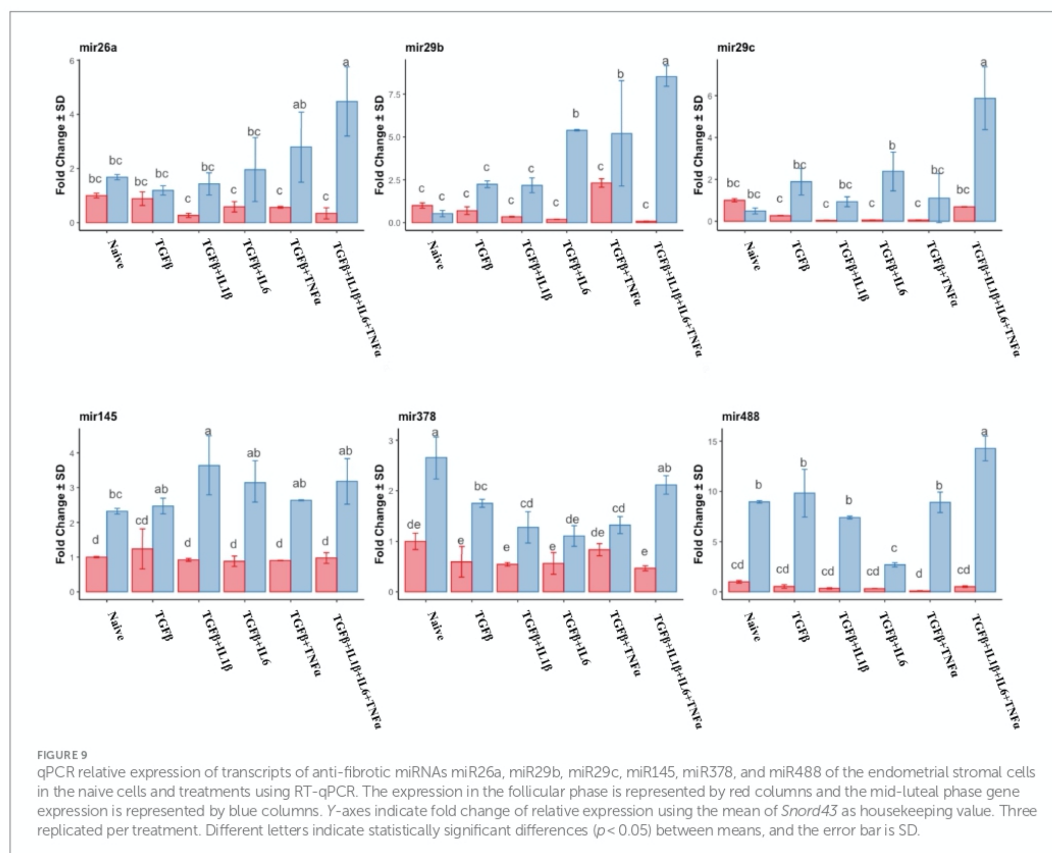

FIGURE 9
qPCR relative expression of transcripts of anti-fibrotic miRNAs miR26a, miR29b, miR29c, miR145, miR378, and miR488 of the endometrial stromal cells in the naive cells and treatments using RT-qPCR. The expression in the follicular phase is represented by red columns and the mid-luteal phase gene expression is represented by blue columns. Y-axes indicate fold change of relative expression using the mean of *Snord43* as housekeeping value. Three replicated per treatment. Different letters indicate statistically significant differences ($p < 0.05$) between means, and the error bar is SD.

regulates epithelial–myofibroblast transition and suppresses the pro-apoptotic protein, *PUMA* (43, 44). In addition, *SLUG* binds to the E-box of collagen type I receptor, thus enhancing ECM synthesis (45).

In the follicular phase, there were no differences in the expression of myofibroblasts marker genes (*SMA*, *VIM*, *CDH2,* and *CDH11*) nor of *COL3A1* when a cocktail of cytokines was used in comparison with TGFβ alone. Conversely, *SLUG*, *CTGF,* and *COL1A1* expression was potentiated by the combination TGFβ + IL6 and TGFβ + IL1β + IL6 + TNFα. This suggests that the presence of IL6 in the induction cocktail favors ECM deposition. Jazinski et al. (46) and Li et al. (47) found a significant association between the NF-kb pathway and a destructive type of endometriosis in mares, characterized by high expression of IL6 in the follicular phase. In this research, the presence of IL6 also induced the upregulation of *MMP9* and a higher *MMP9:TIMP1* ratio, which in turn promotes exacerbated ECM deposition. Similar results had been reported in an *in vitro* model of mare endometrial fibrosis (48) as well as in macrophages among patients with malignant non-Hodgkin's lymphoma (49, 50). *MMP9* also has activity against type III collagen, the typical collagen found in healthy endometrium; however, it cannot degrade collagen type I which is present in destructive endometriosis (51). *MMP9* has a wide proteolytic activity and has an affinity for type IV collagen, the most abundant constituent of basal membrane, and its degradation is key in the progression of lung and liver fibrosis (52).

The other metalloprotease involved in ECM turnover is *MMP2*, a gelatinase with a strong capacity to cleave elastin and collagen type I fibre, but having weak proteolytic activity against type III collagen (53). Here, we showed the downregulation of *MMP2* in the follicular phase in the presence of pro-inflammatory cytokines, which may be responsible for the overexpression and accumulation of collagen type I. Our results suggest that downregulation of *MMP2* is necessary to facilitate the progression of fibrosis, and other researchers like Onosuka et al. (54) and Radbill et al. (55) demonstrated similar results in the murine liver fibrosis model.

Szostek et al. (56) reported an increment in the expression of the inhibitors of MMPs in the presence of TGFβ in endometrium fibroblasts. Similarly, our model showed an upregulation of *TIMP1* and *TIMP2* in the follicular phase, specifically in the presence of pro-inflammatory cytokines. In the mid-luteal phase, the tendency is only observable in *TIMP1* but not in *TIMP2*, favoring the MMP2/*TIMP2* equimolar ratio. This result suggests a hormonal dependency in the modulation of matrix stiffness as well as the ease of disturbance of the mechanical network in the follicular phase and the importance of the downregulation of *MMP2* in favoring a fibrotic scenario. Dysregulation of hormone signaling is known to

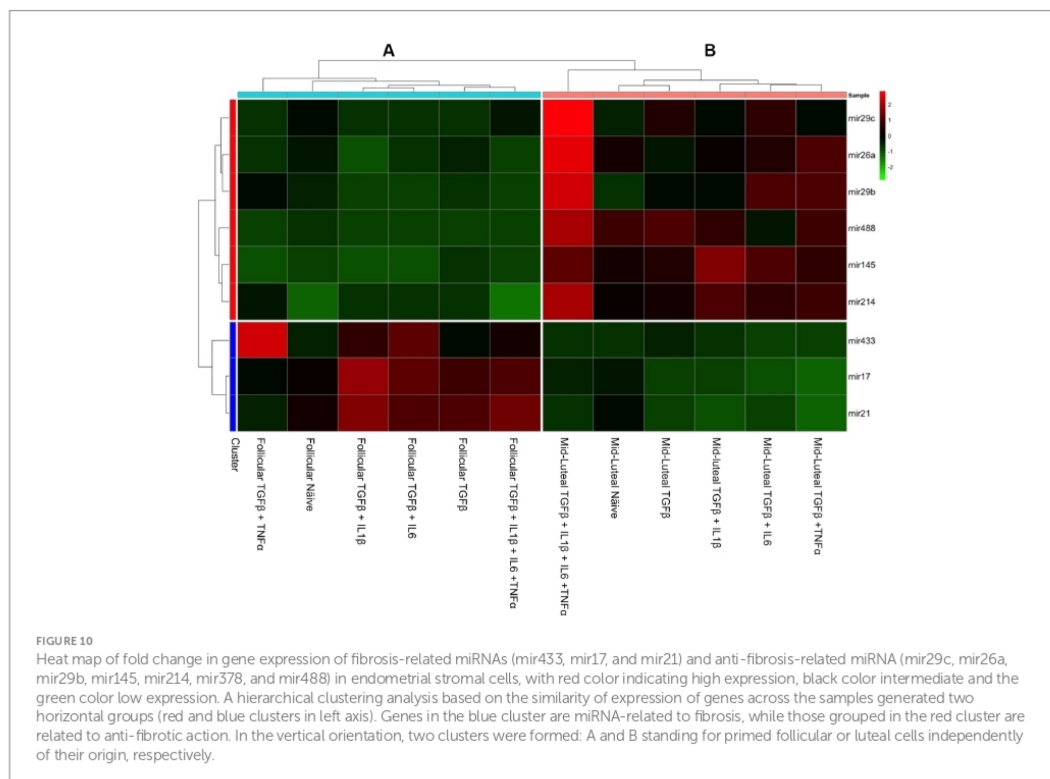

FIGURE 10
Heat map of fold change in gene expression of fibrosis-related miRNAs (mir433, mir17, and mir21) and anti-fibrosis-related miRNA (mir29c, mir26a, mir29b, mir145, mir214, mir378, and mir488) in endometrial stromal cells, with red color indicating high expression, black color intermediate and the green color low expression. A hierarchical clustering analysis based on the similarity of expression of genes across the samples generated two horizontal groups (red and blue clusters in left axis). Genes in the blue cluster are miRNA-related to fibrosis, while those grouped in the red cluster are related to anti-fibrotic action. In the vertical orientation, two clusters were formed: A and B standing for primed follicular or luteal cells independently of their origin, respectively.

favor the progression of endometriosis (57, 58). Oestrogen receptors, *ESR1*, *ESR2,* and *PGR,* are normally expressed in the stroma of healthy mare endometrium, whereas a dramatic downregulation occurs in fibrotic areas (12, 59). We observed a similar effect in our results: in the follicular phase, there was a downregulation in both oestradiol receptors, *ESR1* and *ESR2*, compared to those of the control. TGFβ causes a drop in the expression with no observable interaction with interleukins. The effect of oestrogen is exerted via intracellular receptors, and different reports have highlighted the anti-inflammatory role of oestrogen receptor activity in chronic inflammatory diseases (60, 61). For instance, in non-reproductive tissues, the interactions of 17b-estradiol with *ESR1* can inhibit inflammation by blocking the trafficking of NF-kb into the nucleus through the activation of the PI3K/AKT pathway (62). In our model, the downregulation of *ESR1* and *ESR2* occurred solely in the presence of TGFβ. This repressive activity of TGFβ with the oestrogen receptor type 1 has been observed in bronchial epithelial cells from idiopathic pulmonary fibrosis and breast cancer cell lines (63, 64). In the present study, prostaglandin receptor showed the same decreasing trend with all the treatments.

Overall, at the mRNA level, we found an upregulation of pro-fibrotic genes in the follicular phase, compared to that in the mid-luteal phase. As such, it is tempting to speculate that this effect is mediated by the repression of oestrogen receptors under the influence of TGFβ, which allows for free action of the NF-kb pathway. The mid-luteal phase registers a peak of P4 and high levels of PGE2 that exert not only luteoprotective but also anti-fibrotic activity (65, 66). Here, we found a high expression of *PTGES* mRNA in the mid-luteal but not in the follicular phase. These findings are in agreement with others (61, 62) who provided evidence that a combination of P4 and low levels of E2 in stromal cells induced high mRNA levels of PTGES and also of PGE2. Conversely, pro-inflammatory cytokines favor the aberrant expression of hormonal receptors and PGE2 downregulation in the follicular phase.

In this research, an anti-fibrotic pattern of gene expression was found for endometrium stromal cells in the mid-luteal phase, with a lower expression of *COL1A1, CTGF* and *MMP2* and a higher expression of *TIMP1* and *COL3A1* compared to those in the follicular phase. These results are in agreement with those of Szostek et al. (67), and are most likely related to the anti-fibrotic effect of PGE2. The immediate downstream target of TGFβ is SMAD2/3 proteins, which become phosphorylated upon interaction with the TGFβ. In our research, there was a significantly lower phosphorylation of SMAD2/3 in cells in the mid-luteal phase compared to those in the follicular phase. This effect is mediated by P4 addition in a concentration-dependent manner in A549 lung epithelial cells previously treated with TGFβ (68), in line with our own findings. In addition, P4 evokes an anti-inflammatory response under pathogenic stimuli by augmenting IL10 and decreasing IL1β, TNFα, and IL6 secretion in placental explants exposed to lipopolysaccharides prior to P4 stimulation (69). This action is exerted via the P4 nuclear receptor and membrane-bound receptors (*PR*) that inhibit NF-kb pathway activation (70, 71).

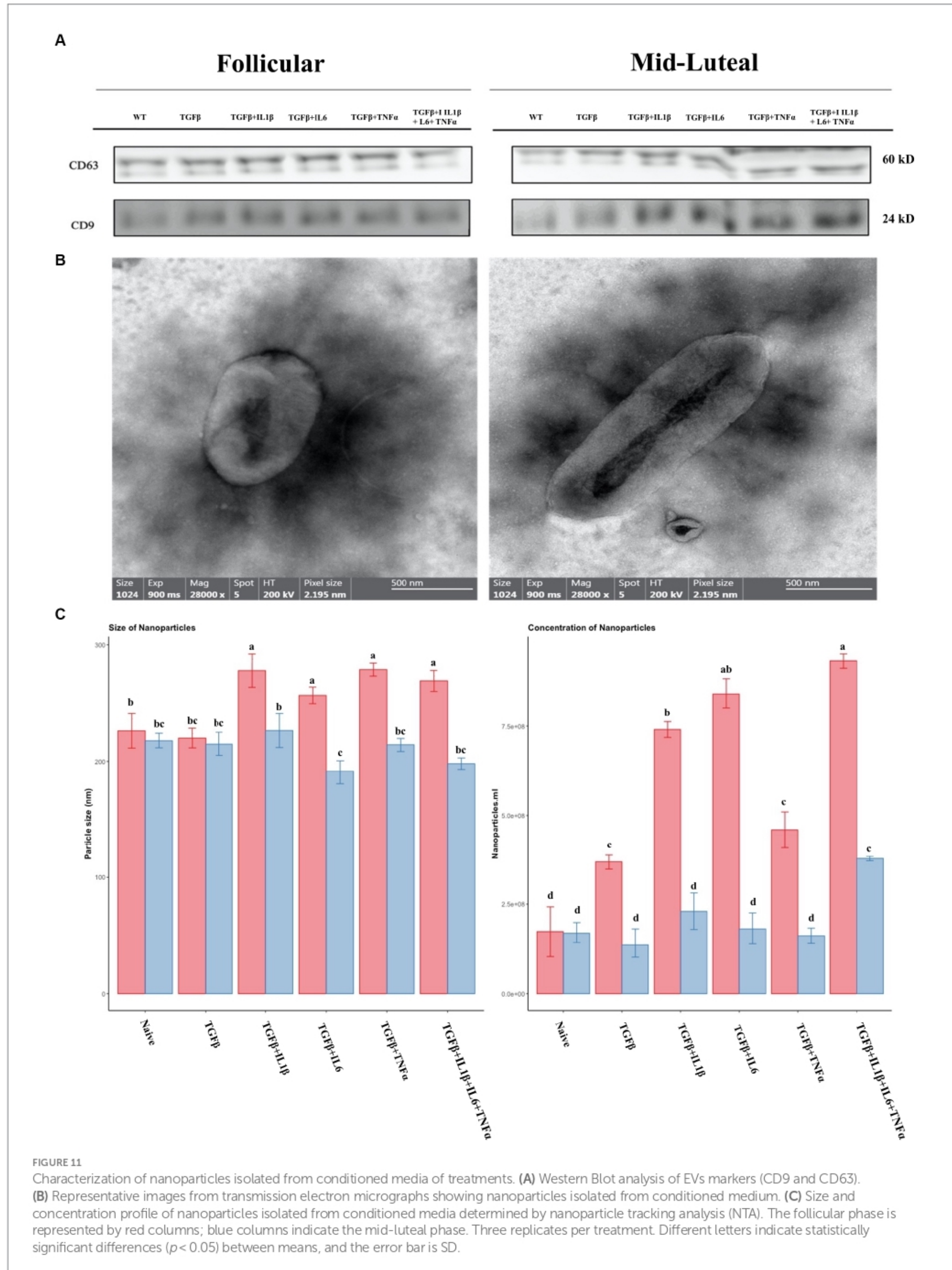

FIGURE 11
Characterization of nanoparticles isolated from conditioned media of treatments. **(A)** Western Blot analysis of EVs markers (CD9 and CD63). **(B)** Representative images from transmission electron micrographs showing nanoparticles isolated from conditioned medium. **(C)** Size and concentration profile of nanoparticles isolated from conditioned media determined by nanoparticle tracking analysis (NTA). The follicular phase is represented by red columns; blue columns indicate the mid-luteal phase. Three replicates per treatment. Different letters indicate statistically significant differences ($p < 0.05$) between means, and the error bar is SD.

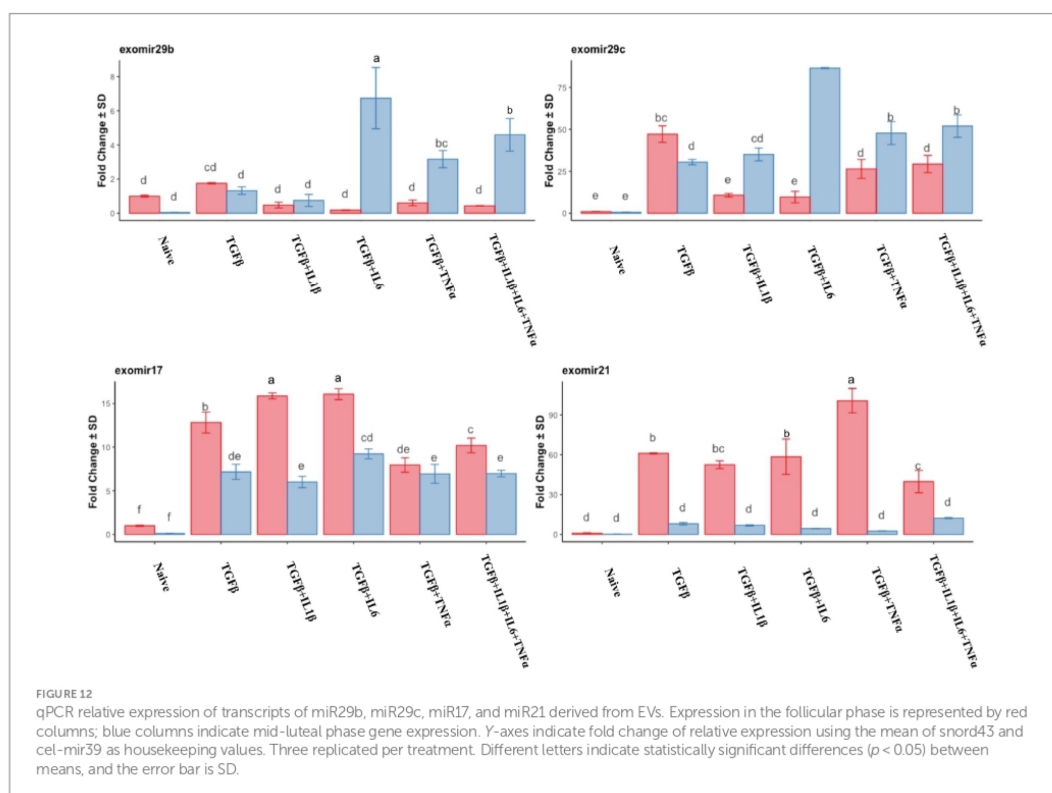

FIGURE 12
qPCR relative expression of transcripts of miR29b, miR29c, miR17, and miR21 derived from EVs. Expression in the follicular phase is represented by red columns; blue columns indicate mid-luteal phase gene expression. *Y*-axes indicate fold change of relative expression using the mean of snord43 and cel-mir39 as housekeeping values. Three replicated per treatment. Different letters indicate statistically significant differences ($p < 0.05$) between means, and the error bar is SD.

Recently, miRNAs were shown to be an alternative way of regulating the delicate axis of inflammation/fibrosis, particularly those acting on the TGFβ and NF-kb pathways (72, 73). We evaluated the expression of a set of fibrosis-related miRNA in our cellular model and found that in the follicular phase, there was an increased expression of mir17, mir21, and mir433, all with known pro-fibrotic action (74, 75). Meanwhile, in the mid-luteal phase, the anti-fibrotic miRNAs 29a, b, and c as well as mir145 were overexpressed. In both cases, expression was intensified when TGFβ was combined with pro-inflammatory cytokines compared to that with TGFβ alone. This miRNA profile is congruent with the pro-fibrotic and anti-fibrotic profiles of mRNAs in the follicular and mid-luteal phases, respectively, as discussed earlier. Previously, others reported that mir17 and mir21 are directly involved in pro-fibrotic progression in different cell lines and murine models by inhibiting SMAD7 and indirectly activating the NF-kb pathway, which would suggest the importance of miRNA regulation in prolonging inflammation favoring a fibrotic process (76–78).

The anti-fibrotic role of miRNAs in the mid-luteal phase lies primarily in the production of PGE_2 as well as in its direct effect in myofibroblasts and the NF-kb pathway in endometrial cells (79). However, in other cell types such as aortic smooth muscle cells (80) and hepatocytes (81), it has been solidly demonstrated that mir29 is a key modulator of tissue fibrosis targeting mainly *COL1A1, TGFβ, SMA,* and fibrillin transcripts that prevent excessive deposition of ECM and restore the sensitisation to apoptosis in myofibroblasts via the FAS ligand (82–84). Mir145 is directly mediated by P4/PGR signaling, which acts as an inhibitor of the epithelial endometrial cell proliferation process (85).

Another miRNA highly expressed in the mid-luteal phase is mir378. This miRNA is hosted in the first intron of the *PPARGC1-β* gene, a coactivator of *PPARG. PPARG* activation ameliorates TGFβ / COL1A1 synthesis in fibrotic tissue (86, 87). In addition, mir378 has shown an anti-fibrotic activity that inhibits the MAPK/ERK pathway in myocardial fibrosis (88, 89). Moreover, mir348 is a repressor of PGR and ER (90, 91), which can be a possible explanation for the reduction in oestrogens receptors observed in our results. Finally, mir488 was also upregulated in the mid-luteal phase in the presence of IL1β, IL6, TNFα, and TGFβ. Liu et al. (92) demonstrated the anti-inflammatory action in bovine uteri by inhibiting ROS production as well as the AKT/NF-kb pathway. Qui et al. (93) observed that this miRNA has an anti-fibrotic effect in hepatic stellate cells via its inhibition of TET 3, resulting in the inhibition of the TGFβ /SMAD2 pathway. Extracellular vesicles have recently gained prominence as players in the process of fibrosis as carriers of miRNA that promote epithelial-mesenchymal transition in neighboring cells (94, 95). We studied the EVs secreted by the cells of this study as potential tools for treating endometrial fibrosis. In cells in the follicular phase that were treated with TGFβ + IL1β + IL6 + TNFα, there was an increased secretion of EVs compared to that of cells in the mid-luteal phase. Previous works established a relationship between the increase of EVs released by injured tissue and the pro-inflammatory stimulus (96). In this scenario, the EVs from altered cells act as signal amplifiers and modifiers of immune innate

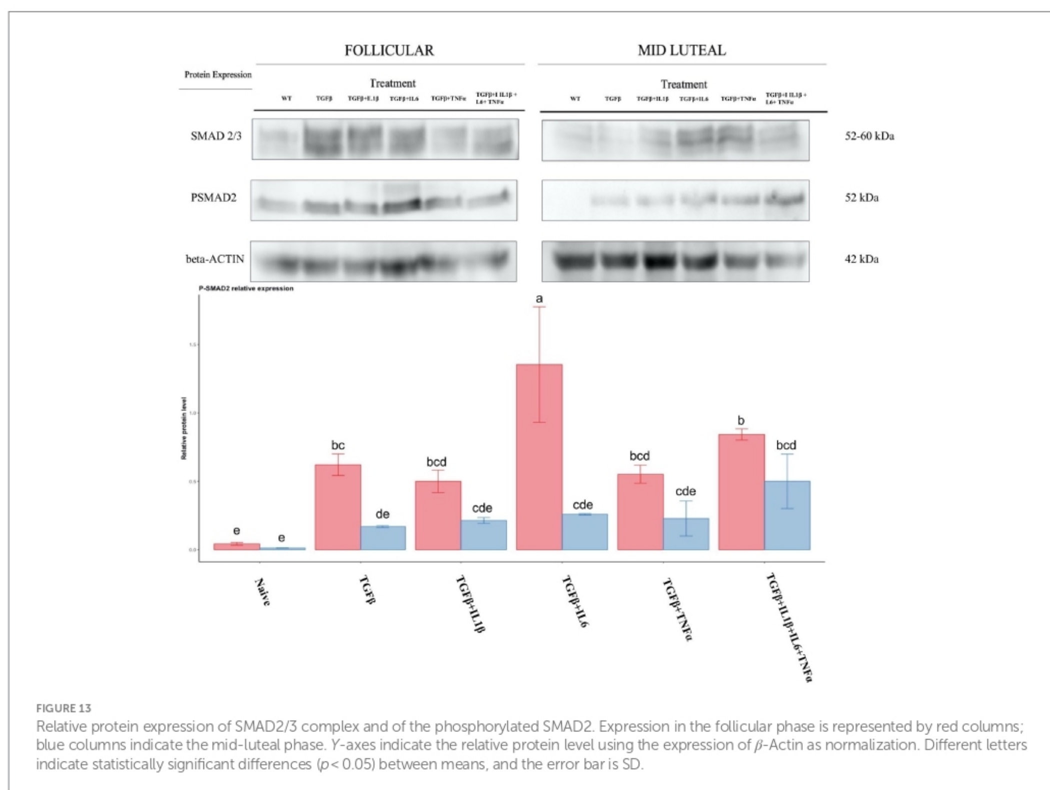

FIGURE 13
Relative protein expression of SMAD2/3 complex and of the phosphorylated SMAD2. Expression in the follicular phase is represented by red columns; blue columns indicate the mid-luteal phase. *Y*-axes indicate the relative protein level using the expression of *β*-Actin as normalization. Different letters indicate statistically significant differences ($p < 0.05$) between means, and the error bar is SD.

response (97, 98). Among their actions, they increase neutrophil recruitment due to its chemokine content, possibly paving the way for the massive release of neutrophil extracellular traps, characteristic of most fibrotic diseases (99). Likewise secreted EVs from inflamed tissue contribute to M1 macrophage polarization establishing a interaction loop with injured tissue and prolonging the inflammation (100, 101). Inflammatory signals not only modify the load of EVs released but also their size and distribution, reflecting the release of subpopulations that are likely to be enriched with inflammatory cytokines as observed by Yang et al. (96) and Hosseinkhani et al. (101). Furthermore, the miRNA content of said EVs correlated with the miRNAs found in the cells. In EVs from cells in the follicular phase, there was an upregulation of pro-fibrotic miRNAs, mir21, and mir17, whereas in cells from the mid-luteal phase, the anti-fibrotic miRNAs, mir29b, and mir29c, were upregulated. Other researchers have reported intercellular congruency of EV cargoes with the cellular environment (102, 103).

5. Conclusion

To the best of our knowledge, this is the first report showing a different response of mare endometrial fibroblasts under inflammatory conditions, marked by the presence of pro-inflammatory cytokines and TGFβ during the oestrous phase. This study suggests that pro-inflammatory cytokines might act as amplifiers of the signal of TGFβ in the follicular phase, and this is accompanied by: (1) significant upregulation of ECM-related genes (*CTGF* and *COL1A1*), (2) an imbalance in the metalloproteinase system (*MMP9*/*TIMP1*), (3) downregulation of oestrogen receptors, (4) upregulation of pro-fibrotic miRNA, and (5) the activation of the TGFβ /SMAD2 pathway. Conversely, during the mid-luteal phase, there is a protective role mediated essentially by PGE_2, which favors the upregulation of anti-fibrotic miRNAs, downregulation of SMAD2 phosphorylation, and as a result, a lower expression of fibrosis-related genes. These findings reassert the connection between the uncontrolled inflammatory mechanism in susceptible mares and the propensity for the establishment of endometrosis.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was approved by the Comite De Bioetica, Universidad De Concepcion, Chile. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YW: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. AM: Investigation, Methodology, Validation, Conceptualization, Writing – review & editing. FN: Methodology, Validation, Writing –review & editing. PP: Writing – review & editing, Investigation, Methodology. LM-P: Writing – review & editing, Investigation, Methodology. GF-D: Data curation, Methodology, Supervision, Writing – review & editing. LR-A: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. FC: Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – review & editing, Conceptualization, Data curation, Formal analysis.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1271240/full#supplementary-material

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2) Short preconditioning with TGF-β of equine adipose tissue-derived mesenchymal stem cells predisposes towards an anti-fibrotic secretory phenotype: a possible tool for treatment of endometrosis in mares. Accepted with minor changes in Veterinary Quarterly

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Abstract

Transforming growth factor beta (TGF-β) is crucial for establishment and progression of fibrosis in mare´s endometrosis. We aimed to develop regenerative approaches to treat endometrosis by using mesenchymal stem cells (MSC), for that, understanding the effect of TGF-β on exogenous MSC is crucial. We isolated and characterized equine adipose MSC from six donors, cells were pooled and exposed to 10 ng/mL of TGF-β for 0, 4, and 24h, after that, cells were analyzed for proliferation, migration, mesodermal differentiation, expression of fibrosis related mRNAs and for prostaglandin E2 secretion. At 24h of exposition to TGF-β, there was a progressive increase in the contraction of the monolayer leading to nodular structures while cell viability did not change. Exposure to TGF-β impaired adipogenic and osteogenic differentiation after 4h of treatment, which was more marked at 24h, represented by a decrease in Oil and Alizarin Red staining respectively, as well as in the significant drop ($p<0.05$) of the expression of key gene regulators of differentiation processes (*PPARG* for adipose and *RUNX2* for osteogenic differentiation). TGF-β increased chondrogenic differentiation as shown by upsurge in size of the resulting 3D cell pellet and intensity of Alcian Blue staining, as well as significant up-regulation of *SOX9* expression ($p<0.05$) at 4h which reached a maximum peak at 24h ($p<0.01$), indicative of up-regulation of glycosaminoglycans synthesis. Preconditioning MSC with TGF-β led to a significant increase ($p<0.05$) of expression of myofibroblast gene markers *aSMA, COL1A1* and *TGFβ* at 24h exposition time, while expression of *COL3A1* did not change respect to the control but registered a significant downregulation compared to 4h ($p<0.05$). TGF-β also affected the expression of genes involved in PGE_2 synthesis and function, *COX2*, *PTGES* and PGE_2 receptor, *EP4*, were all significantly upregulated early at 4h ($p<0.05$). Cells exposed to TGF-β showed a significative upregulation of PGE_2 secretion at 4h compared to untreated cells ($p<0.05$), conversely at 24h, the PGE_2 values decreased significantly comparing to control cells ($p<0.05$). Preconditioning MSC for 4h led to an anti-fibrotic secretory phenotype, while longer period (24h), led to profibrotic one. It is tempting to propose 4h preconditioning of exogenous MSC with TGF-β, to drive them toward an anti-fibrotic phenotype for cellular and cell-free therapies in fibrotic diseases such as endometrosis of mares.

1. Introduction

Regenerative therapies based on mesenchymal stem cells (MSC) have become a cutting-edge treatment for equine pathologies including those of musculoskeletal tissues and endometrium [1,2]. One of the pathologies that could benefit from the therapeutic

capacity of MSC is endometrosis, a chronic and degenerative disease characterized by peri-glandular fibrosis in mares [3] accompanied by 4/5/24 3:02:00 PMexcessive deposition of the extracellular matrix which disturbs the architecture of tissue and compromises its functionality [4]. Endometrosis is multifactorial; however inflammation of endometrial fibroblasts is a common factor [4]. Transforming growth factor beta (TGF-β) is crucial for resolution of inflammation as it plays a key role in the transition between inflammation and tissue remodeling, by stimulating the migration of neutrophils, inducing macrophage polarization and transcription of ECM-related genes in myofibroblast [5,6]. In chronic inflammation as in endometrosis, persisting TGF-β signaling occurs, which leds to the differentiation of resident cells, including epithelial, mesenchymal, and fibroblast to a myofibroblast phenotype and to the dysregulation of the balance of TIMPS/MMP, interleukins, and prostaglandins. This in turns results in increased collagen I deposition compromising the functionality of endometrial glands [7,8]. The delicate balance between correct tissue restoration and pathological response of the TGF-β pathway depends on the inflammatory context and the persistence of signaling in time [9]. Expression of TGF-β and COX2 is co-regulated in various cell types, which results in the production of PGE_2 with anti-fibrotic action and key mediator of acute inflammation scenario [10,11].

MSC has been used to treat different inflammatory conditions in endometrial tissue with dissimilar results [12,13]. In a previous work [14], adipose allogenic MSC were infused into the uterus of mares with different degrees of endometrosis, and a limited cell engraftment in endometrium and change in the expression of fibrotic markers was found. Falomo et al., 2015 [15] using an endometrial explant model co-cultivated with adipose derived MSC showed a potent inhibition for proinflammatory cytokines (IL1,IL6,TNFa) but high levels of a profibrotic matrix metalloprotease, MMP9. The limited therapeutic effect could be attributed to the endometrial fibrotic microenvironment, rich in TGF-β and other proinflammatory cytokines on exogenous MSCs, which could not only limit their homing capacity but also influence towards a potential myofibroblast-phenotype conversion [16].

Previously it was hypothesized that the regenerative activity of exogenously added MSCs was mediated by migration to the damaged area and subsequent engraftment and differentiation to residents cells [17]. Currently, paracrine mechanisms have been named as responsible for the therapeutic action, including immunomodulatory and antifibrotic activities, among these factors prostaglandin E2 (PGE_2) is one of the most important paracrine actors [18;19]. To take advantage of their paracrine signals, MSC-derived products instead of MSC are leading the way for new approaches in regenerative medicine, and different strategies have been designed to increase the potency and effect of MSCs, this includes the challenge toward specific secretome pattern of cells, mainly by preconditioning or “licensing” with cytokines and growth factors including TGF-β. [20]. Previously, Gosh et al., 2017 [21], reported an improvement in functionality of bone marrow derived MSC preconditioned with TGF-β in murine chronic wounds model. Also, licensing of Wharton jelly derived MSC with low dose of TGF-β modulated the secretome of said cells toward antifibrotic properties capable to revert the activated hepatic stellate cells [22]. Human adipose tissue derived MSCs, stimulated with TGF-β can sense TGF-β concentrations in the wound and adopt an anti-inflammatory and antifibrotic phenotype [23]. The mechanism behind this biological response has not been fully elucidated yet, but has been postulated to be based on the time of TGF-β receptor excitation and the crosstalk with regulatory pathway [24]. We hypothesize that MSCs exposed to TGF-β will change their phenotype depending on the length of exposition,

leading to an anti-fibrotic phenotype with short exposition (4 hours) and to a profibrotic phenotype after prolonged exposition (24 hours). Here, we analyzed the changes on biological properties associated to mesenchymal stem cell phenotype, genes related to fibrosis, PGE_2 secretion and protein expression in response to TGF-β exposition.

2. Materials and Methods

2.1. Biological Samples

Six MSC isolated from fat of different animals were used in this study. The samples were obtained from local abbatoir (Frigosur Ltda, Chillan, XVI Region – Ñuble). The procedures were carried out under the approval of the Ethics Committee of the Faculty of Veterinary Sciences, University of Concepcion, Chile (CB-10-2019).

2.2. Adipose Tissue derived Mesenchymal Stem cells isolation and characterization

The cells described above were named equine adipose tissue MSC (eAT-MSC) and were isolated and cultured as previously reported by Cabezas et al., 2018 [25]. Briefly the fat pads were mechanically homogenized by mincing and enzymatically dissociated overnight at 38.5°C in 2 mg/ml collagenase type I (Gibco^{TM}, Grand Island, NY, USA), 2X antibiotic-antimycotic (Corning^{TM}, Steuben, NY, USA), 5% Fetal Bovine Serum (FBS; Sigma-Aldrich^{TM}, Saint Louis, MO, USA) in Dulbecco's Modified Eagle Medium (DMEM HG; Sigma-Aldrich^{TM} ,Saint Louis, MO, USA). The resulting cell suspension was filtered through 40 µm cell strainers to remove undigested tissue. The filtrate was washed with DMEM HG + 1x antibiotic-antimycotic and centrifuged at 500 g for 10 minutes. The cellular pellets were split into two halves, the first was resuspended in MSC culture media (DMEM HG, 1x antibiotic-antimycotic, 1x Glutamax (ThermoFisher Scientific^{TM}, Waltham, MA, USA) and 10% FBS, and seeded in T25 flask vessel. The second half was used to evaluate the expression of surface markers (CD90, CD44, MHCI and MHCII) using Flow Cytometry in an acoustic cell cytometer (Attune NXT Acoustic Focusing Cytometer^{TM}, Thermo Fisher Scientific^{TM}, Waltham, MA, USA). Cell pellets were incubated in 0.5% BSA (Sigma-Aldrich^{TM}, Saint Louis, MO, USA) in PBS for 1 hour, washed twice in PBS and finally incubated with the antibodies diluted in Attune focusing^{TM} (ThermoFisher Scientific^{TM}, Waltham, MA, USA) (1:50) for 1 hour at 4°C in the dark. Antibodies used are specified in the Supplementary table 1.

The inclusion criteria for cells were: positivity to CD90, CD44 and MHCI and negative to MHCII, according to [26]. After expansion, cells were pooled and seeded in T175 flask vessels and at 80% of confluency, cell were cryopreserved until the experimental procedures.

For the analysis of biological activity, cells were subjected to multilineage differentiation assay. For that, 100,000 cells were plated per well in 12 wells plates and subjected to lineage specific differentiation using adipose, chondrogenic and osteogenic StemPro^{TM} kits of Gibco (Gibco^{TM} ,Grand Island,NY,USA) for 21 days. After that, the monolayers were fixed and stained in Alcian blue to visualize the glycosaminoglycan synthesis in chondrogenic differentiation, Alizarin Red for mineralization deposition in osteogenic differentiation, and Oil Red O for fatty acid vacuoles visualization in adipogenic differentiation.

2.3. Effects of TGF-β preconditioning on eAT-MSC properties: experimental design

For licensing with TGF-β, the pool of eAT-MSC was seeded in T175-flask plate at 100,000 cell/well in MSC culture medium. At 80% confluency, the monolayer was washed with PBS and replaced with induction medium (DMEM HG, 1x antibiotic-antimycotic,1x Glutamax, 1% Fetal Bovine Serum) for 12 hours. Subsequently, the eAT-MSC were exposed to 10 ng/mL of TGF-β (Peprotech™, Cranbury, NJ, USA) added to induction medium for 0, 4, and 24 h. At the indicated time points the monolayer was detached using 1X trypsin-eDTA (Sigma-Aldrich™, Saint Louis, MO, USA), and the single cell solution was split for: a) cell proliferation, b) migration assay, c) mesodermal differentiation, d) mRNA related to fibrosis and e) protein expression of TGF-β/SMAD pathway activation. The experimental design is shown (figure 1).

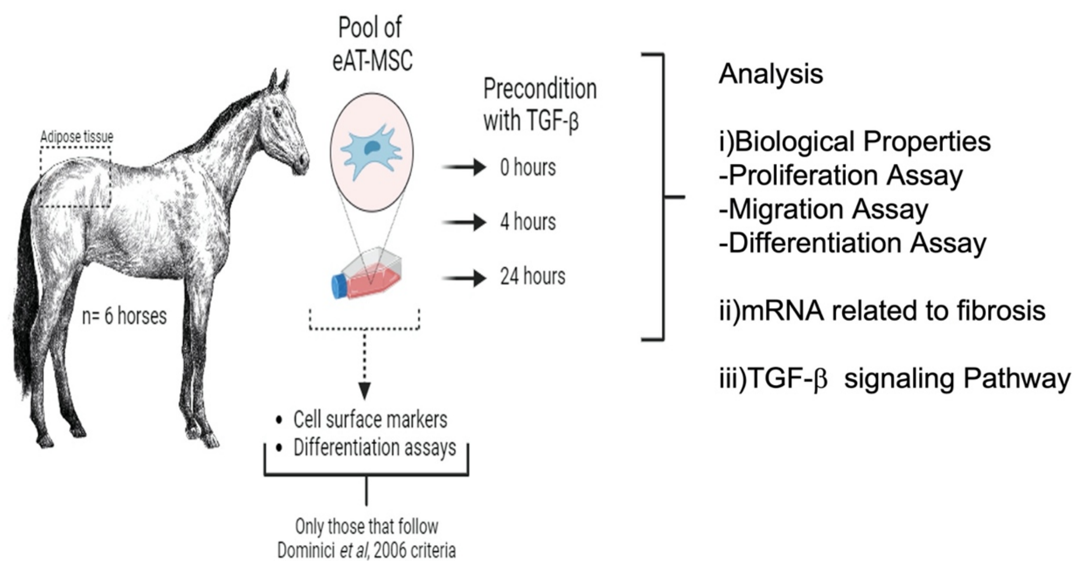

Figure 1. Diagram of the experimental design diagram. Six fat pads from lower back region excised post-mortem were used to isolate eAT-MSC. Resulting primary cultures that meet Domenici's criteria[26] were pooled and used for the experimental procedures.

2.4. Cell proliferation assay

Cell pellets corresponding to each treatment were seeded in triplicated in 96-well plates (5,000 cells/well) in MSC culture medium. After 24 hours, the monolayers were washed with PBS and replaced with 100 µL of DMEM HG without phenol red (Gibco™,Grand Island, NY, USA), 10% FBS and 0.5 mg/ml MTT (Invitrogen™, Carlsbad, CA, USA), and incubated for 4 hours at 38.5 °C in a humidified atmosphere with 5% CO_2. Finally, 100 µL of solubilization solution (5% SDS in DMSO) was added to each well and the formazan product was read at 562 nm in an ELISA reader (Biotek EPOCH, Agilent Technologies™, Santa Clara, CA, USA).

2.5. Migration assay using Transwells

The migration ability of MSCs was tested using transwell chambers (8 μm pore size) in triplicate for each condition. Preconditioned MSC pellets were suspended in DMEM HG + 1X antibiotic-antimycotic solution, and 100,000 cells were seeded in the transwell insert and incubated 10 min at room temperature to let the cells settle down. The lower chamber was filled with 600 μL of DMEM HG, 1X antibiotic-antimycotic solution supplemented with 50 ng/mL PDGF-BB (Peprotech™,Cranbury, NJ, USA), and then the plate was incubated for 4 hours at 38.5°C and 5% CO2. The inserts were carefully removed using a cotton-tipped applicator, the non-migrated cells were gently removed from the apical side. To fix the migrated cells, the inserts were immersed in 5% glutaraldehyde for 10 minutes and stained with 1% crystal violet in 2% ethanol for an additional 30 minutes. Excess stain was removed by washing the insert several times with PBS and the inserts were then allowed to dry at RT. The number of migrated cells were counted in inverted microscope in 4 randomly selected fields at 20X.

2.6. Mesodermal differentiation assay

The impact of preconditioning on MSC capacity was assessed by subjecting eAT-MSCs to a three-lineage differentiation assay for 21 days. Cell pellet from each condition was seeded in triplicate in a 12-well plate with an initial concentration of 100,000 per well in MSC culture media. When the monolayers reached maximum confluence, the MSC medium was changed to the respective differentiation medium (adipogenic, chondrogenic and osteogenic kit). The experiment was performed in duplicate, using the first group to quantify transcription factors (PPARG, RUX2 and SOX9) by qPCR and the second group to visualize differentiation using Oil red, Alcian blue and Alizarin red respectively. The primers used for gene expression analysis are listed in the supplementary table 2.

2.7. Analysis of the expression of mRNA related to fibrosis

It was analyzed the expression of mRNAs related to fibrosis. For this purpose, we selected typical myofibroblasts marker genes (*SMA*, *COL1A1* and *COL3A1, TGFB*) and prostanoid system genes (*COX2*, *PTGES*, *EP2*, *EP4* and *AKR1B1*). From the cell pellet, the whole RNA was isolated using E.Z.N.A Total RNA kit I (OMEGA™, Omega, GA, USA) following the manufacturer instruction and resuspended in 30 μL of molecular grade water. The RNA purity was checked using 260/280 nm ratio in microplate spectrophotometer (Biotek EPOCH, Agilent Technologies™, Santa Clara, CA, USA). The cDNA was transcribed using high-capacity cDNA Reverse Transcription kit (ThermoFisher Scientific™, Waltham, MA, USA) using 500 ng of template RNA. The qPCR reaction was performed in real time PCR System (MxPro, Agilent Technologies™, Santa Clara, CA, USA) with 5 μL of KiCqStart SYBRGreen qPCR Ready Mix with ROX (Sigma-Aldrich™, Saint Louis, MO, USA), 2.5 μL molecular grade water, 0.5 mix of forward and reverse primers at 10 μM and 2 μL of cDNA. Each reaction was performed in triplicate and the resulting CT were collected, the data were processed using delta delta CT method [27] and the relative gene expression was expressed in Fold Change using *GAPDH* and *B2M* as housekeeping genes. The 0 hours (control) group was used as calibrator and the resulting geometric mean of CT was used to normalise the gene expression. All the primers are listed in table 2 of supplementary section and the mRNA primers were designed in-house using AmplifX™ software.

2.8. Quantification of secreted PGE_2

Three T75-flasks for each condition were used as described above. After preconditioning, the monolayer was washed twice with PBS and changed to DMEM HG,1X antibiotic-antimycotic supplemented with 1% of FBS. After 24 hours, the medium was collected and PGE_2 determined using the commercial ELISA kit (Prostagladin E2 Express ELISA kit, Cayman^{TM}, Ann Arbor, MI, USA) following the manufacturer´s instruction.

2.9. Analysis of proteins involved in TGF-β signaling pathway

Cellular pellets were obtained from cultured cells as described above, and lysed using RIPA buffer (Thermo Fisher ScientificTM, Waltham, MA, USA) supplemented with protease inhibitor cocktail (Cell Signalling TechnologyTM, Danver, MA, USA), vortexed for 10 minutes and centrifuged for 30 minutes at 10,000 g. The resulting supernatants were collected, and the protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Fisher ScientificTM ,Waltham, MA, USA). For SDS-PAGE, 30 μg of protein were loaded in sample buffer 4x (InvitrogenTM, Carlsbad, CA, USA) with 2% of β-mercaptoethanol, heated to 95°C for 10 minutes and separated in 10% SDS-PAGE. The resolved gels were electroblotted using a semi-dry method onto 0.45 μm PVDF membranes with the Trans-Blot Turbo kit, according to the manufacturer (BioradTM, Hercules, CA, USA). The membranes were blocked using Super Block Blocking Buffer (Thermo Fisher ScientificTM ,Waltham, MA, USA) over night at 4°C and washed twice with 1X TBS-T buffer (Cayman, Ann Arbour, MI, USA) to continue with the primary antibody incubation at 4°C overnight. The antibodies probed were: anti-rabbit phospho-Smad2, anti-rabbit phospo-Smad3, anti-rabbit Smad2/3, anti-rabbit β-Catenin, anti-rabbit SMA, anti-mouse GAPDH and are listed and specified in supplementary table 1.
For detection, the membranes were incubated for 1 hour at room temperature with polyclonal anti-rabbit IgG-HRP conjugated (Cell Signaling TechnologyTM, Danver, MA, USA) or polyclonal anti-mouse IgG-HRP conjugated (Cell Signaling TechnologyTM, Danver, MA, USA). The membranes were then washed two times with TBS-T buffer and the signal detected using ECL substrate (Westar Antares, CyanagenTM, Bologna, Italy) in Gene Gnome XRQ system (SyngeneTM, Cambrigde, UK). Band intensities were quantified using ImageJ software and the relative protein expression was calculated according to Heidebretch et al., 2009 [28] using GAPDH protein expression as normalizer.

2.10. Statistical Analysis

Each experiment was performed in triplicated and the data were presented as mean ± standard deviation. One-way ANOVA with Tukey test was used to determinate the statistical significance ($p<0.05$). The obtained data was analyzed in R platform and plotted using the ggplot2 package.

3. Results

eAT-MSC were successfully isolated and kept in culture for five passages. The MSC phenotype was assessed by cell cytometry and all the six lines were $(CD90/CD44/MHC1)^{+}$ and $MHC2^{-}$ after the fifth passage, also cells differentiate into

osteogenic, chondrogenic and adipogenic lineages (data not shown). For the assays below, cells were pooled to minimize individual variability.

3.1. Effect of exposition to TGF-β on the MSC phenotype (morphology, migration and tri-lineage differentiation)

The morphology of the eAT-MSC cells varied as a function of time of exposure to TGF-β, there was a progressive increase in the contraction of the monolayer leading to nodular structures (figure 2A) which was more marked at 24 hours of exposition, however, cell viability did not change as demonstrated in the MTT assays (figure 2B). Conversely, the migration of MSC toward a potent chemoattractant such as PDGF added to the culture medium, increased with longer exposition to TGF-β (figure 2 C and D).

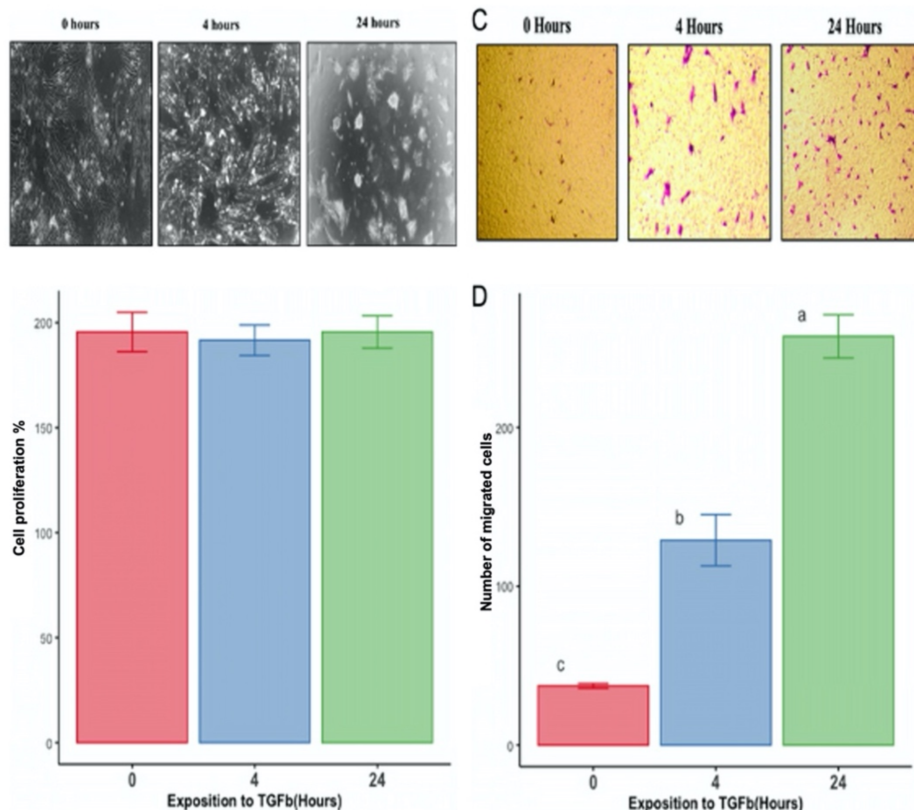

Figure 2. Effects of preconditioning with TGF-β on morphology, proliferation and migration of eAT-MSC. (A) A monolayer contraction is observed; the number of cell nodules increases with the extension of exposition to TGF-β.B) Cell proliferation was measured using MTT assay. (C) Migrated cells trapped on the Transwell matrix after staining with Crystal violet in representative fields at 10X from. (D) Quantification of cell migration assay. The data are expressed in mean ± SD of migrated cells. The different letters indicate statistically significant differences between groups ($p<0.05$)

Exposure of eAT-MSC to TGF-β impacts on the tri-lineage differentiation ability of the cells. We found an impairment of adipogenic and osteogenic differentiation as early as after 4h of TGF-β treatment, which was more marked at 24h, represented by a decrease in the staining assays (Oil and Alizarin Red respectively) as well as in the significant drop

($p<0.05$) of the expression of key gene regulators of differentiation processes (*PPARG* for adipose and *RUNX2* for osteogenic differentiation (figure 3A and 3B and figure 4A and 4B).

Conversely, TGF-β treatment provoked an increase in chondrogenic differentiation apparent in the increase in size of the resulting three-dimensional cell pellet and the intensity of Alcian Blue staining indicating the up-regulation of glycosaminoglycans synthesis (figure 3C), as well as in the significant up-regulation of *SOX9* expression ($p<0.05$) at 4 hours which reached a maximum peak at 24 hours of exposition ($p<0.01$; figure 4C).

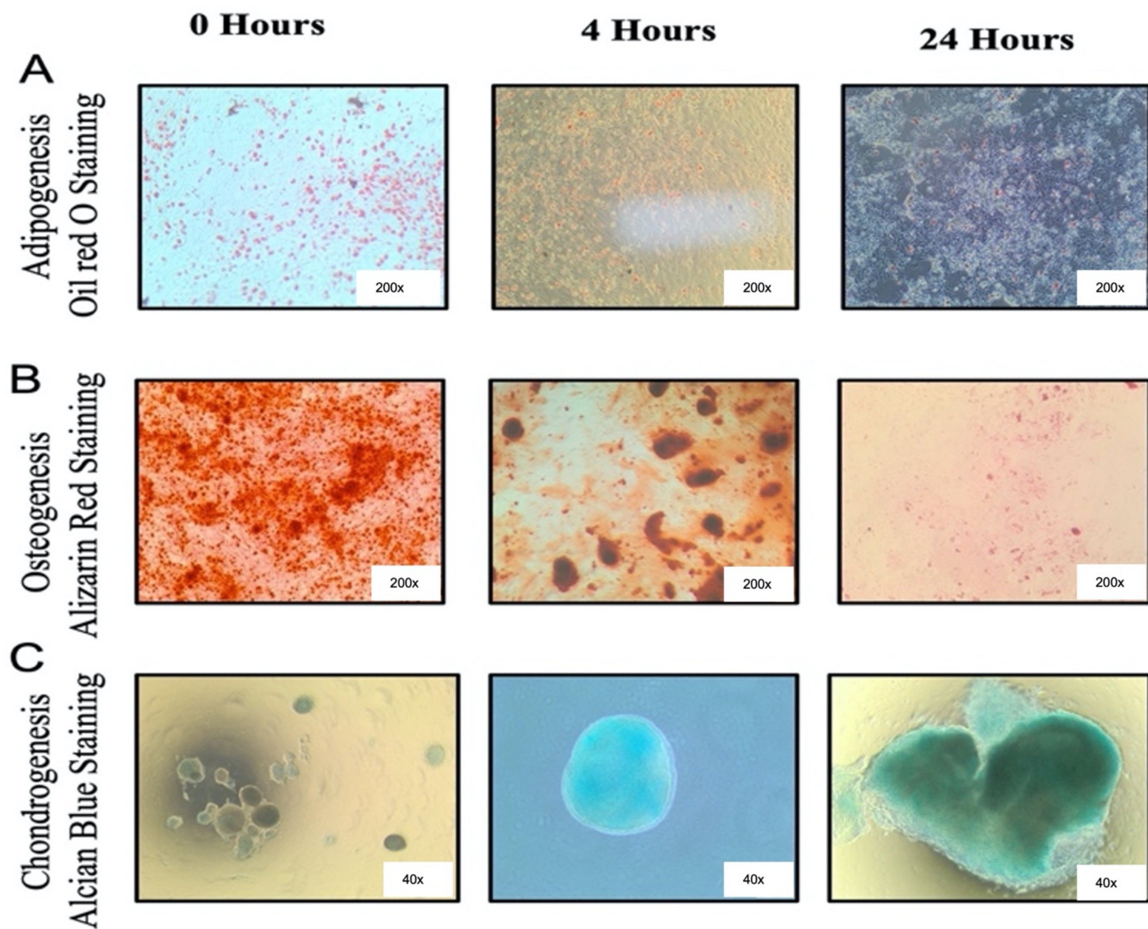

Figure 3. Representative images of specific staining for trilineage differentiation of eAT-MSC at different time points of TGF-β preconditioning. A) The intensity of adipogenic differentiation is denoted by the numbers of stained lipid vacuoles stained by Oil Red, magnification 200x. (B) The degree of the osteogenic differentiation is demonstrated by the amount of mineralized matrix staining by Alizarin Red, magnification 200x. (C) The degree of chondrogenesis differentiation is evidenced by the size of micro masses and the synthesis of glucoaminoglycans stained with Alcian Blue, magnification 40x.

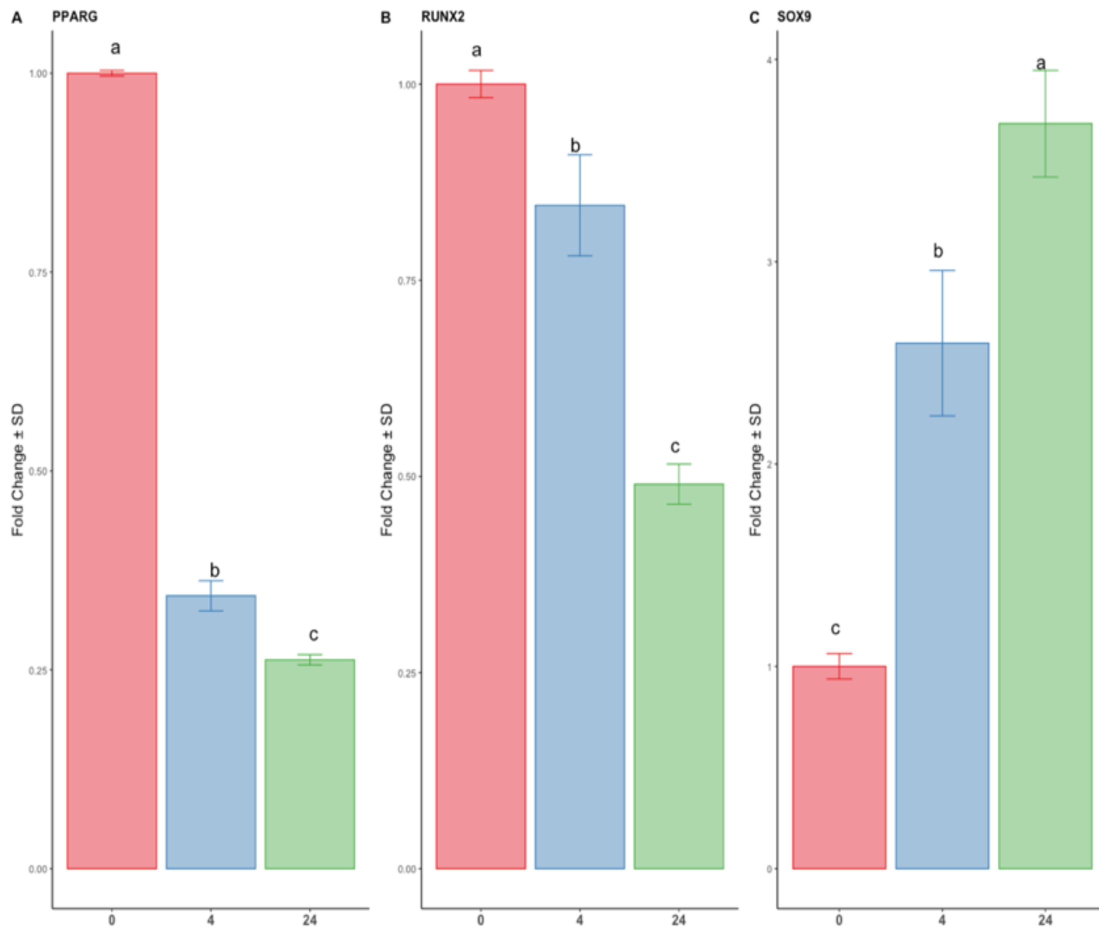

Figure 4. Relative expression by real time qPCR of transcripts related to trilineage differentiation of the samples in figure 3. Red bars: no exposition to TGF-β; blue bars: 4 hours by blue bars and green bars: 24 hours. Y-axes indicate Fold change of relative expression using the geometric mean *GAPDH* and *B2M* as housekeeping value. The different letters indicate statistically significant differences between groups ($p<0.05$) and the error bar is standard deviation. Three replicated per treatment.

3.2. Effect of exposition to TGF-β on the expression of mRNA related to fibrosis and the PGE_2 expression in eAT-MSC

Preconditioning eAT-MSC with TGF-β led to a significant increase ($p<0.05$) of expression of myofibroblast gene markers *aSMA, COL1A1* and *TGFβ* at 24h exposition time, while expression of COL3A1 did not change respect to the control but registered a significant downregulation compared to 4h ($p<0.05$; figure 5).

The exposition to TGF-β of eAT-MSC cells also affected the expression of genes involved in PGE_2 synthesis and function, *COX2*, Prostaglandin E_2 synthase (*PTGES*) and PGE_2 receptor, *EP4*, were all significantly upregulated early at 4h ($p<0.05$; figure 6) and their expression was either restored to basal levels (*COX 2* and *PTGE2*) or significant downregulated (*EP4*; $p<0.05$) after 24h treatment. Expression of PGE_2 receptor, *EP2*, did not change, while prostaglandin F2a synthase (*AKR1B1*) expression was significantly increased at 24h ($p<0.05$).

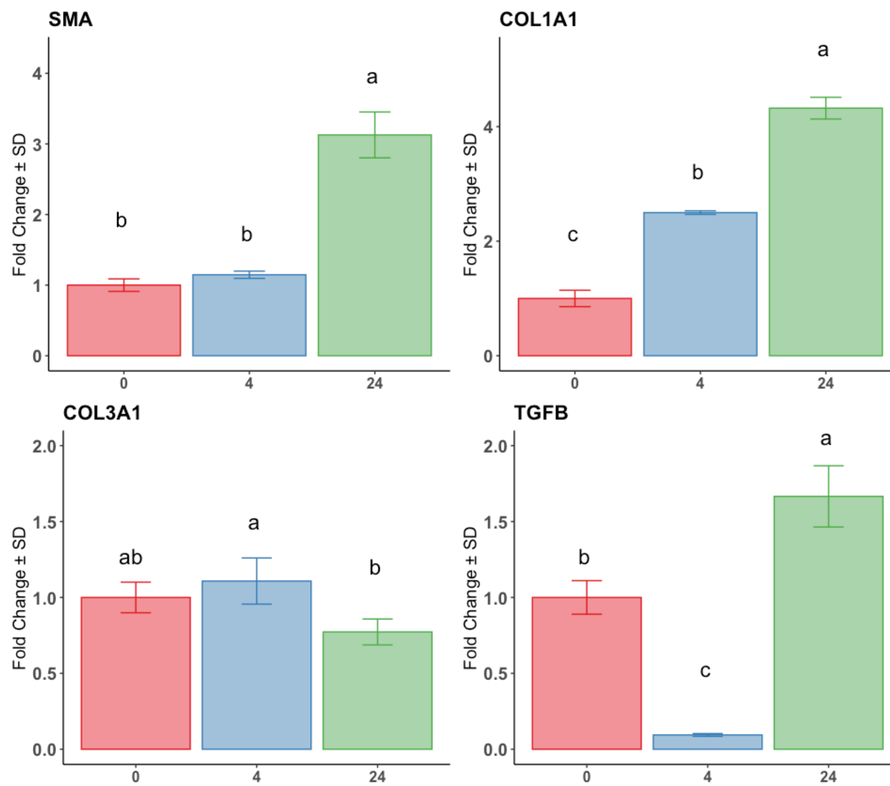

Figure 5. Relative expression by real time qPCR of myofibroblast gene markers in eAT-MSC exposed to TGF-β for different time periods. Red bars: no exposition to TGF-β; blue bars: 4 hours by blue bars and green bars: 24 hours. Y-axes indicate Fold change of relative expression using the geometric mean *GAPDH* and *B2M* as housekeeping value. The different letters indicate statistically significant differences between groups ($p<0.05$) and the error bar is standard deviation. Three replicated per treatment.

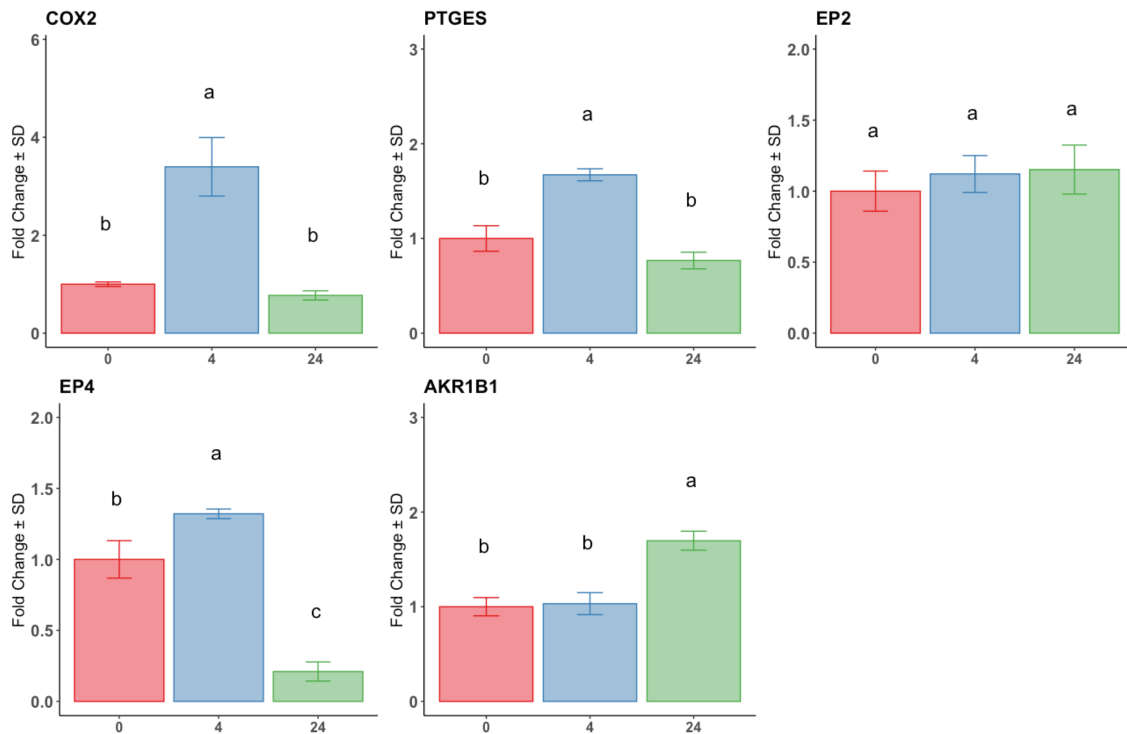

Figure 6. Relative expression by real time qPCR of genes related to prostaglandin pathway in eAT-MSC exposed to TGF-β for different time periods. Red bars: no exposition to TGF-β; blue bars: 4 hours by blue bars and green bars: 24 hours. Y-axes indicate Fold change of relative expression using the geometric mean *GAPDH* and *B2M* as housekeeping value. The different letters indicate statistically significant differences between groups ($p<0.05$) and the error bar is standard deviation. Three replicated per treatment.

3.3. Changes in PGE_2 secretion after preconditioning

Equine AT-MSC cells exposed to TGF-β showed a significative upregulation of PGE_2 secretion at 4 hours compared to untreated cells ($p<0.05$), conversely at 24 hours, the PGE_2 values decreased significantly comparing also to control cells ($p<0.05$; figure 7).

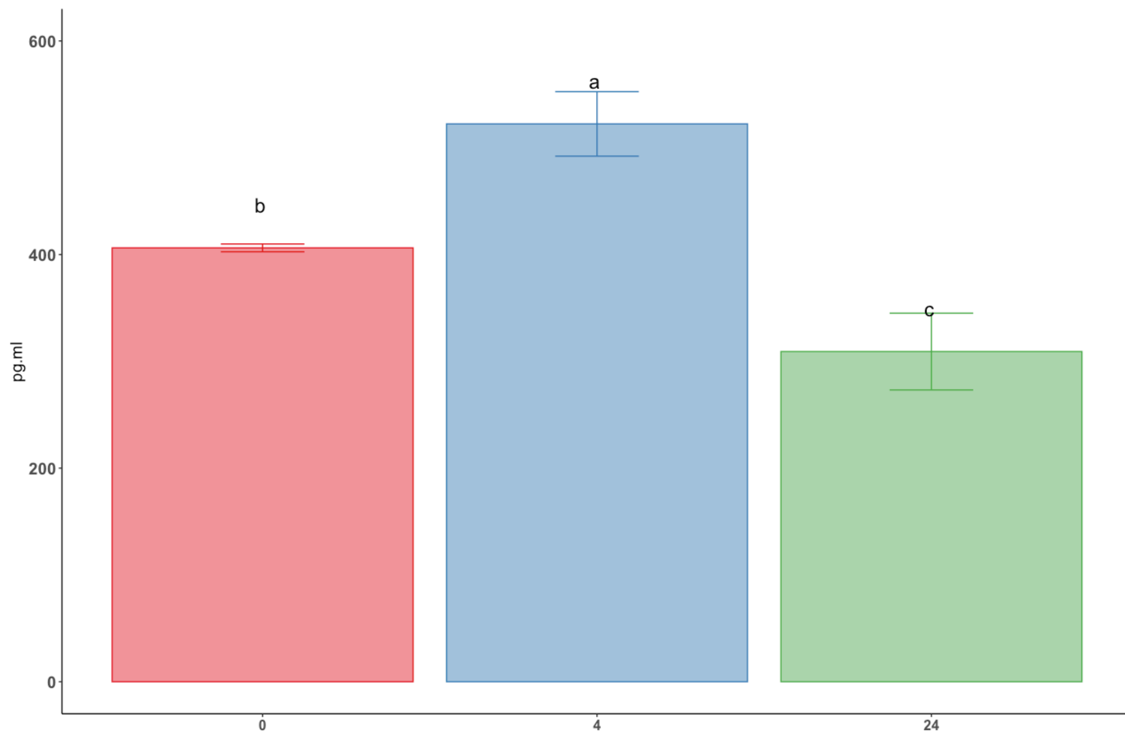

Figure 7. ELISA for quantification of PGE2 secretion into culture media of eAT-MSC exposed to the different times of TGF-β preconditioning. The secretion in 0 hours (control) of preconditioning is represented by red bars, 4 hours by blue bars and 24 hours by green bar. Y-axes indicate picograms per milliliter. The different letters indicate statistically significant differences between groups ($p<0.05$) and the error bar is standard deviation. Three replicated per treatment.

3.4. Analysis of the TGF-β pathway in preconditioned eAT-MSC

SMAD3 phosphorylation increased significantly at 24 hours ($p<0.05$), whereas SMAD2 phosphorylation did not change over time. β-catenin increased significantly ($p<0.05$) steadily from 4 hours and continued up-regulated after 24 hours of TGF-β exposure. SMA expression also increased significantly after 24 hours ($p<0.05$; Figure 8).

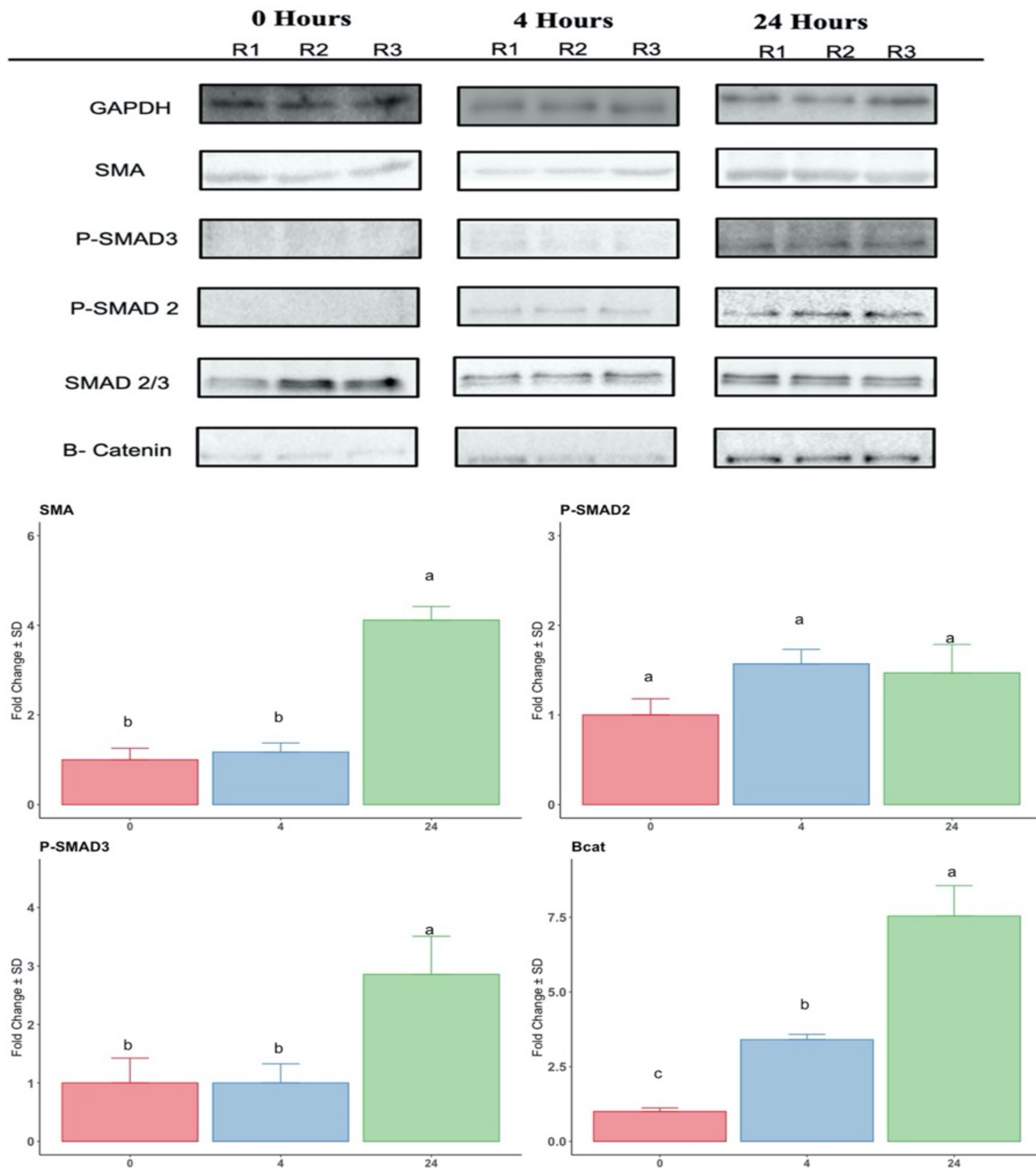

Figure 8. Relative protein expression of SMAD2/3 complex, phosphorylated SMAD2, phosphorylated SMAD3 and B-catenin. Red bars: no exposition to TGF-β; blue bars: 4 hours by blue bars and green bars: 24 hours. Y- axes indicate the relative protein level using expression of GAPDH as normalisation. Different letters indicate statistically significant differences ($p < 0.05$) between means, and the error bar is SD.

4.Discussion

This study was aimed to investigate the effect of TGF-β preconditioning on the properties of eAT-MSCs and to set a possible tuning for their use as an antifibrotic therapeutic tool in mare´s endometrosis. We demonstrated that preconditioning the cells for 4 hours led to an anti-fibrotic secretory phenotype, while if the period is extended to 24 hours, the phenotype on the contrary becomes profibrotic.

TGF-β participates in diverse processes related to wound healing, including cell proliferation and migration toward injured site; however, under pathological conditions it might led to fibrosis of a particular tissue. To exert its action, it binds to cellular receptors TGFβRI and TGFβRII which are present in most cells [29]. Upon receptor activation, proteins SMAD2 and SMAD3 are phosphorylated as key players in the transduction of the signal [30]. Upon phosphorylation, the SMAD2/3 complex translocates to the nucleus and activates transcription factors and genes related to extracellular matrix synthesis, immune response, cell proliferation and chemotaxis [31].

Proliferative action of TGF-β on cells depends on the cell type and the tissue microenvironment, in wounded tissue it favors the proliferation of fibroblast by inducing FGF2 expression and phosphorylation of p38 MAPK [32,33], conversely in hematopoietic cells and endothelial cells it induces quiescence via c-Myc repression [34,35]. In our work, we did not find induction of proliferation of eAT-MSC, what is coincident with others [36], working with bone marrow derived MSC. A possible explanation could be the fact that MSC are pericyte-derived cells of vascular progenitor origin, but at different stages of differentiation [37].

Here we found that TGF-β exerted chemoattractant properties toward eAT-MSC cells which was markedly observed at 24h of exposition in the transwell assays. This featured was observed also in stromal cells and bone marrow MSC recruitment to the injured site through SDF1/CXR4 pathway and upregulation of genes with promigratory roles [38,39]. At 24 hours formation of nodules in the monolayer is observed. Xu et al.,2007 [40], observed a similar phenomenon in a wide range of cell lines exposed to TGF-β, such nodules were formed as a consequence of cell migration and extracellular matrix production enriched with collagen I and fibronectin.

Preconditioning of eAT-MSC with TGF-β altered the capacity of the cells to differentiate. Adipogenic differentiation was impaired at 24h as judged by Oil red staining and drop of expression of *PPARG.* It has been described that TGF-β potently inhibits adipogenic differentiation through the association of SMAD2/3 with C/EBPβ resulting in abolishment of *PPARG* expression [41]. We observed a steady decay of *RUNX2* expression and osteogenic differentiation over the exposure times. Runt-related transcription factor (*RUNX2*) is the master gene of osteogenic differentiation and promotes the pre-osteoblast differentiation of MSC [42]. TGF-β has been identified as a repressor of osteogenic differentiation by recruiting HDCA (Histone deacetylase) 4 and 5 and interaction with SMAD3/RUNX2 complex, which results in transcriptional repression and downregulation of osteocalcin and other genes related to osteogenesis [43]. Conversely, TGF-β has been reported as promoter of chondrogenic differentiation of MSC through SMAD3 phosphorylation and interaction with SOX9/CPB/p300 complex [44] that leads to increment of *SOX9* expression and synthesis of glycoaminoglycans during. This is in agreement with our results, of intense Alcian blue staining and upregulation of chondrogenic gene markers (*SOX9* and *Col 1A1)* at 24 hours of treatment.

TGF-β is considered the major inducer of myofibroblast differentiation in stromal cells including MSC [45,46]. Marriot et al.,2014 [47] reported that mouse lung MSCs treated *in vitro* with TGF-β changed their global transcriptome to a myofibroblast-like one with marked expression of *SMA* and collagen. Our results showed an up-regulation of *SMA*,

$TGF\text{-}\beta$ and *COL1A1* at 24 hours of treatment, evidencing a myofibroblast phenotype. This finding might have clinical implications and could partially explain the therapeutic results obtained when treating endometrosis with MSCs.

Previously we established a cellular model in which healthy endometrial fibroblasts are converted to fibrotic ones, dependent on the phase of estrus with a marked tendency to develop fibrosis in the follicular phase, while anti-fibrotic activity is clearly displayed during mid-luteal phase [48]. In physiological endometrium of mares, the correct balance of the prostaglandin system is pivotal to maintain homeostasis and to control the remodeling of the tissue throughout the phases of oestrous [49]. In idiopathic pulmonary fibrosis and equine endometrosis, PGF2α signaling facilitates myofibroblast proliferation and collagen deposition [50,51], whereas PGE_2 acts as anti-fibrotic agent by acting as a TGF-β receptor antagonist [52]. This is in agreement with our observation, where the highest PGE_2 secretion coincided with the lowest expression of TGF-β at 4 hours of preconditioning, concomitantly with a surge in *COX2* and *PTGES* expression as well as the specific PGE_2 receptor, *EP4*. Earlier it was reported that murine bone marrow MSC treated with TGF-β secreted increased levels of PGE_2 through COX2/EP4 upregulation [53].

Conversely, the expression of prostaglandin F2α synthase (*AKR1B1*) was upregulated at 24 hours, alongside with the drop in *EP4* expression. This shift from an antifibrotic to a profibrotic pattern over time may be explained by negative autocrine regulation of PGE_2 secretion mediated by the desensitization of *EP4* receptor due to intracellular cAMP accumulation [54]. This is in concordance with our results at the mRNA level and ELISA detection of PGE_2. In our work, there was a progressive phosphorylation of SMAD2/3 over time resulting in increased stabilization of the SMAD2/3 complex, which is consistent with the increased myofibroblast phenotype observed in MSCs [55]. The cross-talk of TGF-β via phosphorylation of SMAD2/3 with the Wnt/β-Catenin pathway led to accumulation of β-catenin and facilitates the MSC-myofibroblast differentiation resulting in progression of fibrosis [56,57].

5. Conclusions

In this work, we showed that short-time preconditioning (4 hours) of equine adipose MSC with TGF-β favored an anti-fibrotric phenotype of said cells, while longer treatment (24 hours) conversely led to a profibrotic phenotype with a decay of antifibrotic prostanoid PGE_2. It is tempting to propose 4 hours preconditioning of exogenous MSC with TGF-β, to drive them toward an anti-fibrotic phenotyope for both cellular and cell-free therapies in fibrotic diseases such as endometrosis of mares. Of interest also is the fact that long treatment (24 h) led to an increase in chondrogenic capacity of MSC, which can be of value for regenerative therapies in which de novo chondrogenesis is required.

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7. Declaration of interest

Authors declare not to have any conflict of interests of any type.

8. References

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Supplementary material

Short preconditioning with TGF-β of equine adipose tissue-derived mesenchymal stem cells predisposes towards an anti-fibrotic secretory phenotype: a possible tool for treatment of endometrosis in mares

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Table 1. List of antibodies used for analysis of surface markers of MSC and used for TGF-β pathway in preconditioned eAT-MSC

Antibody	**Catalog or clone number**	**Company**
CD90 PE conjugated (mouse anti-human)	555596	BD Pharmingen
CD44 RPE conjugated (mouse anti-horse)	MCA1082PE	Bio-Rad
MHC I RPE conjugated (mouse anti-horse)	CVS22	Bio-Rad
MHC II RPE conjugated	MCA1085PE	Bio-Rad
p-SMAD2 (rabbit anti-human)	3108T	Cell Signaling Technology
p-SMAD3 (rabbit anti-human)	9520T	Cell Signaling Technology
Alpha SMA (rabbit anti-human)	AB5694	ABCAM
SMAD2/3 (rabbit anti-human)	8685T	Cell Signaling Technology
β-catenin (rabbit anti-human)	9582T	Cell Signaling Technology
GAPDH (mouse anti-human)	sc-166574	Santa Cruz
Anti Mouse IGG HRP conjugated	7076P2	Cell Signaling Technology
Anti Rabbit IGG HRP conjugated	7074P2	Cell Signaling Technology

Table2. List of primers used for the analysis of the effect of TGF-β on the expression of expression of mRNAs related to fibrosis and the PGE_2 system in eAT-MSC

Accesion Number Gene	**Forward**	**Reverse**	**Amplicon bp**
XM_001492411.5 **PPARG**	TCTCCAGCATTTCTGCTCCACA	AGGCTCCACTTTGATCGCACTT	**97**
XM_005603968 **RUN2**	ACTTCCTGTTGCTCCGTGCTGC	TCCCATCTGGTACCTCTCCGAG	**100**
XM_014736619.1 **SOX9**	GCTCTGGAGACTGCTGAACGAG	GTACTTGTAGTCCGGGTGGTCC	**100**
XM_001503035.6 **SMA**	TTTCATCGGGATGGAATCTGCT	CCGGAGAGGACGTTGTTAGC	**97**
XM_023652710.1 **COL1A1**	TAAGGGTGACAGAGGCGATG	GGACCGCTAGGACCAGTTTC	**85**
NM_000090.4 **COL3A1**	GCTCCCATCTTGGTCAGTCC	GATCCTGAGTCACAGACGCAT	**93**
NM_001081849.1 **TGFb**	AGCAGTGCCCGATCCCA	GCTGTATTTCTGGTACAGCTCCA	**85**
NM_001081775.2 **COX2**	CCCTTCCTGCGAAATGCAGTTA	GGATTCCCAGCTCTTATAGCC	**90**
NM_001081935.1 **PTGES**	GAAGAAGGCTTTCGCCAACC	GAAGAAGGCTTTCGCCAACC	**90**
NM_00112735.1 **EP2**	ACACTGCTCGAGAGCCACCATGGTGAGCGC	ACTGCTACCGGTCTGACACTGGGAGCGGGT	**100**
XM_001499068 **EP4**	CCGAGATCCAGATGGTCATCTT	TTGCTGACGTCTTGCTCTGAAC	**100**
XM_001500732.6 **AKR1B1**	ACCCATGTCGTGCTCAACACC	TGGCGGTACCCAAGGTCAATTG	**95**

3) Extracellular vesicles secreted by equine adipose mesenchymal stem cells preconditioned with transforming growth factor β-1 are enriched in anti-fibrotic miRNAs and inhibit the expression of fibrotic genes in an in vitro system of endometrial stromal cells fibrosis.

In second revision in Theriogenology

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Abstract:

Mare endometrosis is a major reproductive problem in mares associated with low fertility and is characterized by persistent inflammation, TGF-β signaling, and consequently, extracellular matrix deposition, which compromises endometrial glands. Mesenchymal stem cell-based products (MSCs), such as extracellular vesicles (EVs), have gained attention due to the regulatory effects exerted by their miRNA cargo. Here, we evaluated the effect of preconditioning equine adipose mesenchymal stem cells with TGF-β for short or long periods on the anti-fibrotic properties of secreted extracellular vesicles. MSCs were isolated from six healthy horses and exposed to TGF-β for 4 h, 24 h, and 0 h. The expression of anti-fibrotic and profibrotic miRNAs and mRNAs in treated cells and miRNAs in the cargo of secreted extracellular vesicles was measured. The resulting EVs were added for 48 h to endometrial stromal cells previously induced to a fibrotic status. The expression of anti-fibrotic and profibrotic genes and miRNAs was evaluated in said cells using qPCR and next-generation sequencing. Preconditioning MSCs with TGF-β for 4 h enriched the anti-fibrotic miRNAs (mir-29c, mir-145, and mir-200) in cells and EVs. Conversely, preconditioning the cells for 24 h leads to a profibrotic phenotype overexpressing mir-192 and mir-433. Treatment with preconditioned MSC-derived EVs for 4 hours significantly decreased the expression of fibrosis-related messenger RNAs (*SMA*, *CTGF, COL1A1, COL3A1*) in the endometrial stromal cell model and sequencing results indicate that they have an impact on the activation of the type I interferon response, which is an antagonist of the TGF-β pathway. This finding could have implications for the development of an EV-based protocol to treat endometrial fibrosis in mares. This finding might have implications for developing an EV-based protocol to treat endometrial fibrosis in mares.

Keywords: Endometrosis, miRNA, TGF-β, Mesenchymal Stem Cells

Introduction:

Endometrosis is a major reproductive problem in mares, defined as peri-glandular fibrosis of the endometrium (Katila and Ferreira-Dias 2022) with a loss of tissue architecture, impaired cellular hemostasis, and persistence of inflammation (Buczkowska et al. 2014). Endometrosis is multifactorial, with persistent inflammation being one of its main causes (Rebordão et al. 2014). TGF-β is pivotal for the transition between inflammation and tissue remodeling by stimulating the migration of neutrophils and inducing macrophage polarization and transcription of ECM-related genes in myofibroblasts (Sanjabi et al. 2009; Klingberg et al. 2013). However, in chronic inflammation, as in endometrosis,

persisting signaling occurs, leading to massive differentiation of other resident cells, provoking the conversion of fibroblasts to myofibroblasts and dysregulation of TIMPS/MMP balance, interleukin, and prostaglandin overexpression, leading to the deposition of collagen type I, which compromises endometrium gland plasticity (Hoffmann et al. 2009).

Endometrosis has no cure since the compromise of tissue functionality is usually irreversible (Trundell 2022). Regenerative therapies using mesenchymal stem cells (MSCs) are a potential tool to treat it, owing to their immunomodulatory and regenerative capacity (Gugjoo et al. 2019). Previously, it was hypothesized that the regenerative activity of MSCs was mediated through migration to the damaged area and subsequent homing and differentiation in the resident tissue (Balaji et al. 2012). Paracrine mechanisms have been identified as responsible for therapeutic activity, including soluble factors with immunomodulatory (Huang et al. 2022) and anti-fibrotic activity (Huang and Yang 2021). MSCs produce a broad range of biomolecules and extracellular vesicles (EVs), which are important tools in tissue repair and homeostasis due to their intrinsic molecular cargoes (Hou et al. 2021). EVs are anucleate lipidic bilayer structures with myriad cargoes, including miRNAs, capable of generating phenotypic changes in target cells via post-transcriptional regulation (Makarova et al. 2021).

Preconditioning MSCs with cytokines and growth factors, including TGF-β, have been used to increase their immunomodulatory potency (Saparov et al. 2016; Schäfer et al. 2016; Sarsenova et al. 2022; Le et al. 2024). The resulting phenotype is: activated MSCs with enhanced immunomodulatory and immunosuppressive features (Noronha et al. 2019). MSCs in a fibrotic environment, characterized by persistent TGF-β signaling, are an important source of active myofibroblasts, which participate in ECM deposition (Qin et al. 2023). To the best of our knowledge there are not previous reports on short exposition (4h) of cells in vitro to TGF-β1, others tried concentrations from 0.1 to 10 ng/ml for longer periods (24h; Li et al., 2016 and Qin et al., 2023). We hypothesized that short chase with TGF-β1 would mimic the effect of low concentration exposition as in Li et al., 2016 by shifting cell´s secretory phenotype to an anti-fibrotic one, owed to the miRNA cargo of secreted EVs. Here, we analyzed (i) the changes in the expression of miRNAs related to fibrosis in MSCs exposed to different times of TGF-β preconditioning, (ii) the presence and expression of such miRNAs in EVs, and (iii) the effects of EVs derived from preconditioned MSCs in reverting fibrosis in an in vitro system of fibrotic endometrium stromal cells.

Materials & Methods

The experimental design is displayed in supplementary Figure 1

Biological samples, isolation and characterization of adipose MSCs

The animal study was approved by the Ethics Committee of the Faculty of Veterinary Sciences, University of Concepcion, Chile (CB-10-2019). The study was conducted in accordance with the local legislation and institutional requirements.

MSCs positive for CD90/CD44/MHCI and negative for MHCII were isolated in the laboratory from adipose tissue (AT-MSCs) of Chilean Thoroughbred horses (n = 6) and

characterized as described by Cabezas et al. (2018), following the criteria stablished by Dominici et al. 2006.

TGF-β preconditioning in AT-MSCs

AT-MSCs were pooled and seeded in T175-flask plates at 20,000 cells/cm^2 in DMEM High Glucose (HG), 1x Antibiotic-Antimycotic, 1x Glutamax, and 10% Fetal Bovine Serum (FBS). At 80% confluency, serum was reduced to 1% for 12 h. After this, 10 ng/mL of TGF-β type I **(**PeproTech, USA**)** was supplemented with culture medium for 4 and 24 h. The control group was not treated with TGF-β (T0). At the indicated time points, the monolayer was detached with 1X Trypsin-EDTA (Sigma-Aldrich, USA), pelleted, and total RNA was extracted. Expression of miRNAs related to fibrosis—anti-fibrotic (mir-19, mir-29b, mir-29c, mir-145, mir-200, and mir-486) and profibrotic (mir-17, mir-192, mir-199, and mir-433)—was studied using 2ΔΔCT-qPCR.

Harvest and characterization of EVs

Monolayers of cells coming from 5 T175 flasks for each time point were used to generate EVs. After treatment as above, monolayers were washed twice with PBS and changed to culture medium with 1% FBS, previously depleted of EVs using ultracentrifugation protocol of Shelke et al. (2014). After an additional 48 h, the medium was collected and clarified using a 10 kDa Amicon filter (UFC901024, Merck-Millipore, Germany). As per the manufacturer's instructions, the retentate was used to isolate the EVs using the ExoLutE Exosome Isolation Kit (Rosetta Exosome, Korea). The EVs were resuspended in 100 µl of 0.22 µm-filtered commercial PBS and subjected to nanoparticle tracking analysis at 10 to 100 particles per frame using a Nano Sight NS300 (Malvern Instruments, Malvern, UK). PBS was used as a negative control, and samples were diluted 1:1000 in PBS and injected into a 5 µl/min continuous flow. Results were analyzed according to Gerritzen et al. (2017) using the built-in software NTA 3.2 (Malvern Instruments, UK).
For transmission electron microscopy (TEM), five µl of EVs in PBS were fixed with 4% paraformaldehyde (1:1). The solution was placed on carbon-formvar-coated copper electron microscopy grids for 20 minutes and washed with PBS. Then, 1.5% glutaraldehyde was applied to the grid for 5 minutes and then washed thrice with molecular-grade water. Finally, 0.5% uranyl oxalate pH 7.0 (Electron Microscopy Sciences, USA) was applied for 5 minutes to facilitate contrast. The grid was dried, placed, and images were taken at 40,000× to 80,000× magnification on the Talos™ F200C transmission electron microscope (Thermo Fisher Scientific, USA).
For the evaluation of miRNA cargo in EVs, 20 µL of EV suspension was mixed with 20 µl of cell lysis buffer from the Cells-to-cDNA kit (Thermo Fisher Scientific™,USA), and 25 fmol of synthetic spike (cel-mir39, Norgen™, Canada) was added, heated at 75 °C, and finally frozen at -80 °C until qPCR analysis. The miRNAs measured were: mir-29c, mir-145, and mir-200 (anti-fibrotic), and mir-192 and mir-433 (profibrotic).

Exposition of EVs to endometrial stromal cells in fibrosis in vitro system

To measure the anti-fibrotic capacity of preconditioned AT-MSCs medium, we used an in vitro endometrial stromal cell (EndSCs) fibrosis system previously validated in our laboratory (Wong et al. 2023). Briefly, 800,000 endometrial stromal cells/well from mares in the follicular phase of the estrus cycle were seeded in 12-well plates in DMEM HG supplemented with 1% AAM, 10% FBS, 0.5 ng/mL progesterone, and 30 pg/mL

estradiol. After attaching, FBS was reduced to 1%, and the medium was supplemented or not (naïve cells) with a fibrotic cocktail of 10 ng/ml each of IL1β, IL6, TNFα, and TGF-β for 24 h. The monolayers were then gently washed with PBS, and the serum was changed to 1% EV-free FBS. Culture was prolonged for another 48 h. The following experimental design was used: endometrial cells primed or not with the fibrotic cocktail were exposed to 10^{10} EVs from AT-MSCs challenged with TGF-β for 0 h, 4 h, or 24 h. The exposure of endometrial cells to the EVs coming from the AT-MSCs was allowed for 48 h, after that, the monolayers were washed with PBS, tripsinized, pelleted, and subjected to mRNA extraction, followed by conversion to cDNA and qPCR gene expression analysis of fibrotic gene markers (*SMA*, *COL1A1*, *COL3A1,* and *CTGF*). Samples from the best anti-fibrotic conditions were used for a global scan of the transcriptome using next-generation sequencing (NGS). To track the internalization of EVs, they were labeled with PKH-67 fluorescent dye (PKH67LG, Sigma-Aldrich, USA) according to the manufacturer's instructions and added for 24 h to EndSCs cultured in medium supplemented with 1% FBS-depleted. At that point, the nuclei of the cells were stained with 1 µl of NucBlue dye (Thermo Fisher Scientific™,USA), and internalization was analyzed in the EVOS FL Imaging System (Thermo Fisher Scientific™,USA).

qPCR analysis for gene expression

Total RNA was isolated from cells using EZNA Total RNA Kit I (OMEGA™, USA) and resuspended in 30 µl of molecular-grade water. The quality was assessed by a 260/280 nm ratio using a spectrophotometer (BioTek EPOCH, Agilent Technologies™,USA). For cellular miRNA analysis, cDNA was synthesized from 500 ng of RNA incubated with 2 µl of 0.1mM ATP (New England Biolabs™, USA), 2 µl of 10 µM RT primer, 2 µl of 10x poly (A) polymerase buffer (New England Biolabs™), 0.2 µl of 1 mM of dNTP mix (Thermo Fisher Scientific™,USA), 1 µl of SuperScript IV™ Reverse transcriptase (Thermo Fisher Scientific™,USA), 0.4 µl of poly(A) polymerase (New England Biolabs™,USA), and 0.6 µl of RiboLock (Thermo Fisher Scientific™,USA) at 37 °C for 30 minutes, then 52 °C for 10 minutes, and finally 95 °C for 5 minutes for inactivation of enzymes. For miRNA cargo in EVs, 20 µl of EV suspension was mixed 1:1 with 20 cell lysis buffer (Cells-to-cDNA kit, Thermo Fisher Scientific™,USA), and 25 fmol of synthetic spike (cel-mir39, Norgen™, Canada) was added and heated at 75 °C. The cDNA was synthesized as described above.

To analyze mRNA gene expression, total RNA from cells was converted to cDNA using a high-capacity reverse transcription kit (Thermo Fisher Scientific™,USA). Briefly, 500 ng of cDNA were mixed with 2 µl of 10x RT Buffer, 0.8 µl 25x dNTP mix, 10x RT Random primers, 1 µl MultiScribe Reverse Transcriptase, 1 µl RiboLock™, and 3.2 µl of molecular-grade water. The reverse transcription was performed at 25 °C for 10 minutes, 37 °C for 120 minutes, and 85 °C for 5 minutes.

The qPCR reaction was performed in a Real-Time PCR System (MxPro 3000, Thermo Fisher Scientific™,USA) with 5 µL of KiCqStart SYBR Green qPCR Ready Mix with ROX (Sigma-Aldrich,USA), 2.5 µL molecular-grade water, 0.5 mix of forward and reverse primers at 10 nM, and 2 µL of cDNA. Each reaction was performed in triplicate, and the resulting CT was collected. The data were processed using the 2ΔΔCT method (Livak and Schmittgen 2001), and the relative gene expression was expressed in fold change using *SNORD43* (for miRNA related to fibrosis in MSCs), the geometric mean of SNORD43 and cel-mir39 for miRNA cargo evaluation in EVs, and the mRNA gene

expression of *GAPDH* and *B2M* housekeeping genes. The time-zero group was used as a normalizer.
All the primers are listed in Table 1 of the Supplementary Section, and the mRNA primers were designed in-house using AmplifX^{TM} software. The miRNA primer set was designed with miRprimer^{TM} software.

Next-Generation Sequencing

EndSCs displaying the best anti-fibrotic profile were subjected to global transcriptomic analysis (NGS), alongside with EndSCs induced to fibrosis or not (naïve) non-treated with EVs.The isolated RNA was quantified, and RNA integrity was checked using an RNA pico-chip kit (Agilent^{TM},USA). The mRNA was isolated using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (NEB^{TM},USA). The libraries were created using the Ultra™ II Directional RNA Library Prep Kit (NEB^{TM},USA) and sequenced using the Illumina NextSeq 500 platform (Illumina^{TM}, USA). The results were paired-end (PE) reads with 150 bp of length each. Subsequently, using FASTP software, the quality of the resulting raw reads was checked, the remaining adapters were trimmed, and low-quality reads (Phreads < 25 or length < 50 bp) were cleaned out. The filtered reads were mapped using HISAT2 software against the *Equus caballus* reference genome (EquCab3.0), and the produced SAM files were converted into BAM files using SAM tools. The individual gene quantification was performed using feature counts (R-subread package), contrasting the BAM files to the EquCab3.0 GTF annotation file (Ensembl version 104). The differential expression analysis was performed on the R platform using the EdgeR package, the results (PCA, Heatmap, and Volcano plots) were visualized using the ggplot2 package, and gene ontology prediction was realized using the clusterProfiler package.

Statistical analysis

Data were analyzed using R platform and expressed as means ± standard deviation. ANOVA was used to compare different conditions and post-hoc Tukey was used for pairwise comparisons. A p value < 0.05 was considered significant.

Results

Characterization of AT-MSC

The isolated cells fulfilled the criteria of Dominici et al., 2006, i.e: differentiation towards three mesodermal lineages. Adipogenic differentiation was evidenced by the formation of intracellular lipid droplets, which were stained with Oil Red. The formation of calcium mineralized deposits showed a differentiation towards the osteogenic lineage and the formation of a micro mass rich in glycoaminoglycans showed a capacity of differentiation towards the chondrogenic lineage. In addition, the immune phenotyping showed positivity for CD44, CD90 and MHCI and almost no expression of MHCII (supplementary figure 2).

Expression of miRNAs related to fibrosis in AT-MSCs

Expression of cellular miRNAs with anti-fibrotic or profibrotic action was studied by qPCR after TGF-β challenge.
Anti-fibrotic miRNAs: mir-19: downregulated at 4 h of TGF-β exposition, its expression abolished at 24 h; mir-29, mir-29c, and mir-486: upregulated at 4 h, downregulated at 24 h. Micro-RNAs 145 and 200 were highly (8-fold) upregulated at 4 h, then decreased at 24 h, but still higher than T0. The expression of profibrotic miRNAs (mir-17, mir-192, mir-199, and mir-433) throughout the exposure time compared to the control showed a significant increase only at 24 h, with mir-433 being the most expressed. In contrast, mir-214 peaked at 4 h of exposure. All differences were statistically significant ($p < 0.05$; Figure 1).

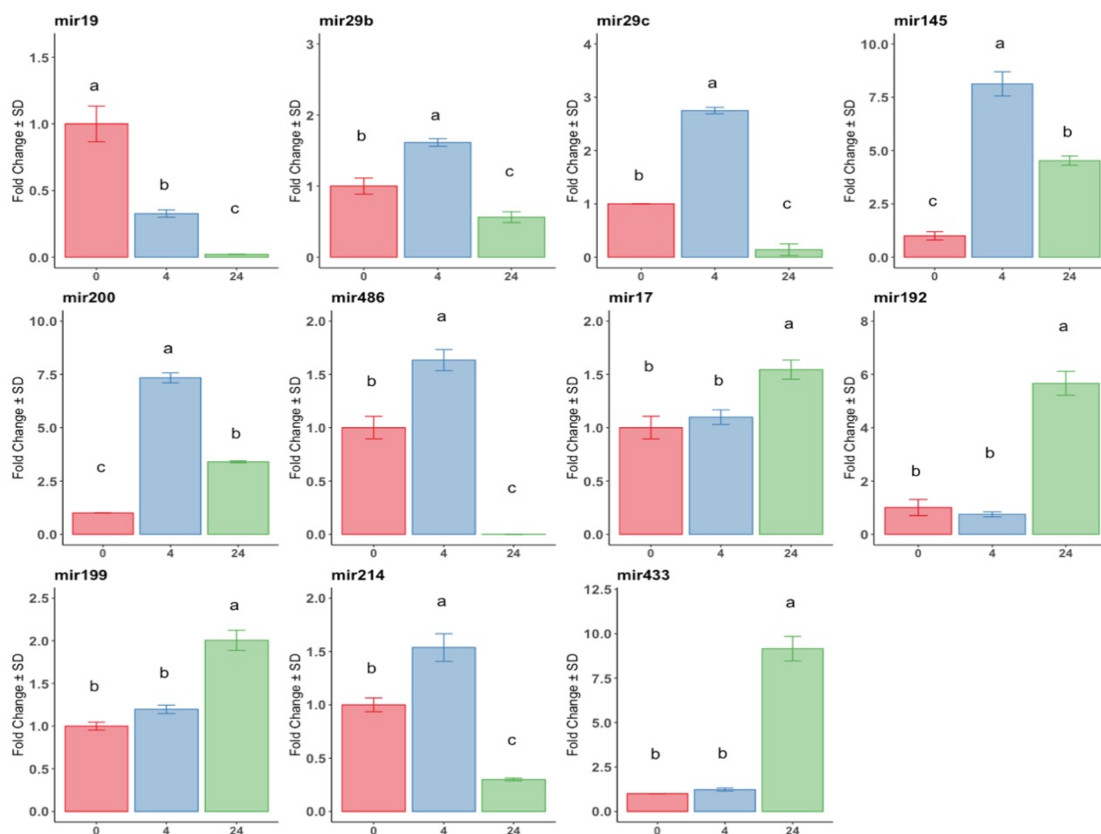

Figure 1. Relative gene expression analysis by RT-qPCR of anti and profibrotic miRNAs of AT-MSC preconditioned with TGF-β at time points 0h: red bars; 4h: blue bars; 24h hours: green bars.Y-axes: fold change of the relative expression, the error bar is standard deviation. Different letters indicate statistically significant differences between groups ($p<0.05$). Three replicates per treatment. Housekeeping: SNORD43.

Changes in EVs and their cargo after preconditioning

We analyzed the vesicle cargo of AT-MSCs exposed to the treatments, and EVs were characterized (Figure 2). TEM showed the classical cup-shaped morphology of EVs, and NTA analysis displayed a change in size of EVs, with 166 nm at T0 and a single peak in the histogram. At 4 h and 24 h, there was an increment in size (195.5 and 210 nm, respectively) with multiple peaks. Expression of EV protein markers were the same at all tested times.

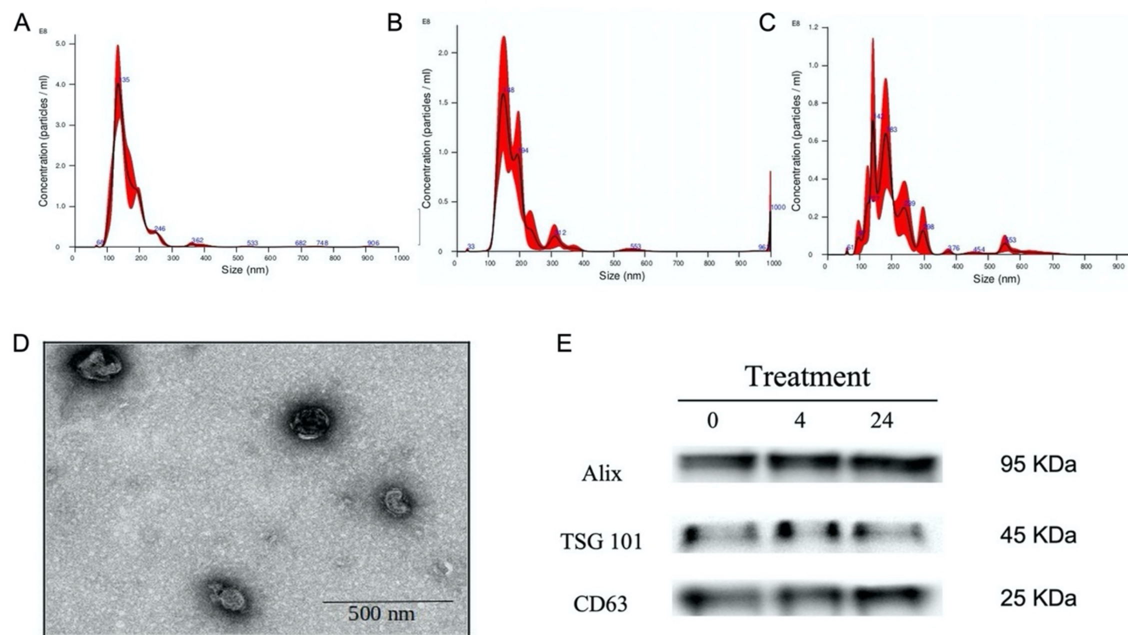

Figure 2. Characterization of extracellular vesicles secreted by AT-MSC and isolated from culture media. A to C, histograms of nanoparticle tracking analysis at time zero, 4 and 24h respectively, size (X-axis) and concentration (Y-axis). D: representative image from TEM showing the morphology of EV. E: Bottom right: western blot analysis of selected EV markers, molecular weight is displayed.

At the miRNA level, the anti-fibrotic miRNAs (mir-29c, mir-145, and mir-200) were significantly highly expressed at 4 h compared to the control and 24-h groups; mir-145 was the most upregulated. Conversely, the profibrotic miRNAs mir-192 and mir-433 were significantly upregulated at 24 h of exposition compared to 0 h, and no difference was registered between 0 h and 4 h (Figure 3).

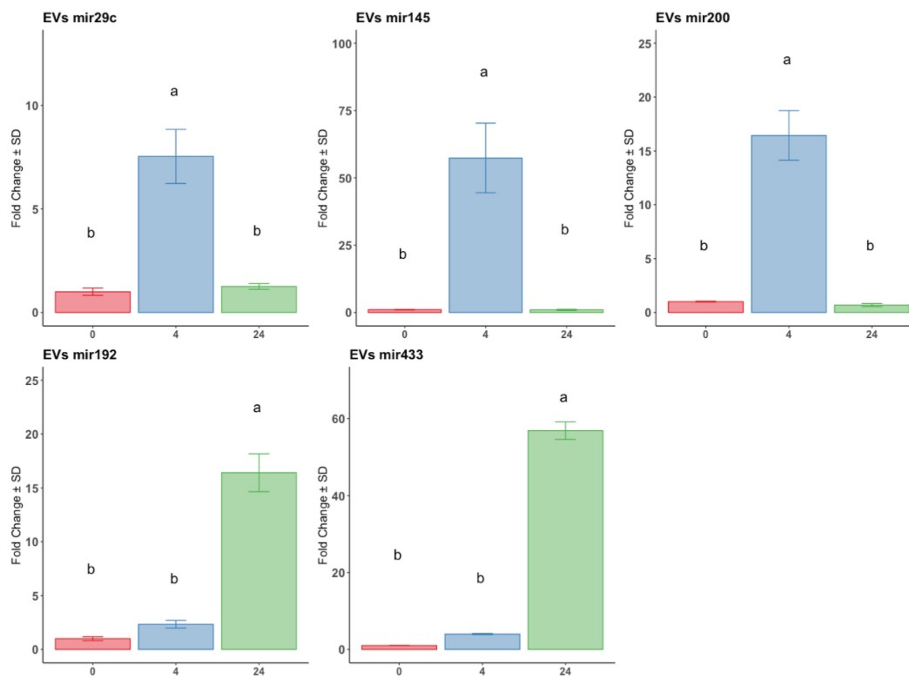

Figure 3. Relative gene expression analysis by RT-qPCR of anti and profibrotic miRNAs of EV, derived from AT-MSC preconditioned with TGFβ at time points 0h: red bars; 4h: blue bars; 24h hours: green bars. Y-axes: fold change of the relative expression, the error bar is standard deviation. Different letters indicate statistically significant differences between groups ($p<0.05$). Three replicates per treatment. Housekeeping: geometric mean of *cel-mir39* and *SNORD43*.

Evaluation of the anti-fibrotic properties of EVs in a cellular model

To evaluate the anti-fibrotic properties of EVs triggered by preconditioning MSCs with TGF-β for 0 h, 4 h, and 24 h, we exposed EndSCs to EVs derived from AT-MSCs. The uptake was visualized using PKH-67-labeled EVs; stain was detected at all time points, while no staining was detected in control cells (Figure 4). EndSCs were subjected to the fibrosing cocktail described earlier and exposed to EVs derived from AT-MSCs treated with TGF-β for 0 h, 4 h, or 24 h. EVs from AT-MSCs pretreated for 4 h induced a decrease in the expression of *SMA*, *CTGF,* and *COL1A1* compared to Induced, T0 and non-treated EndSCs (naïve), ($p < 0.05$). Conversely, after 24 h of TGF-β-preconditioning, expression of *SMA* and *CTGF* increased ($p < 0.05$) compared to non-treated EndSCs (naïve), while expression of *COL1A1* and *COL3A1* did not change (Figure 5).

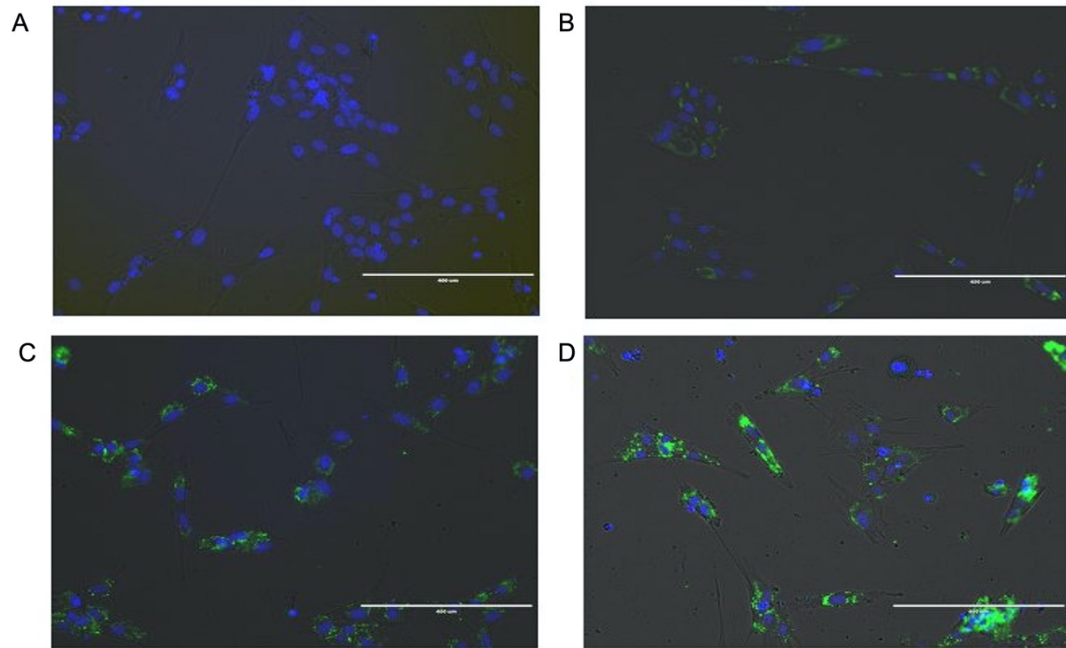

Figure 4. Uptake of PKH-labelled EV derived from AT-MSC preconditioned with TGF-β. Endometrial stromal cells were co-cultured with $1x10^{10}$ PKH-67 labelled EV for 48 hours at 38.5°C and visualized 24h later. Blue: nuclei stained with Nuc-Blue; green stained EVs with PKH-67 fluorescent dye. A) background control; B to D: internalization of EVs at time 0, 4 and 24h respectively.

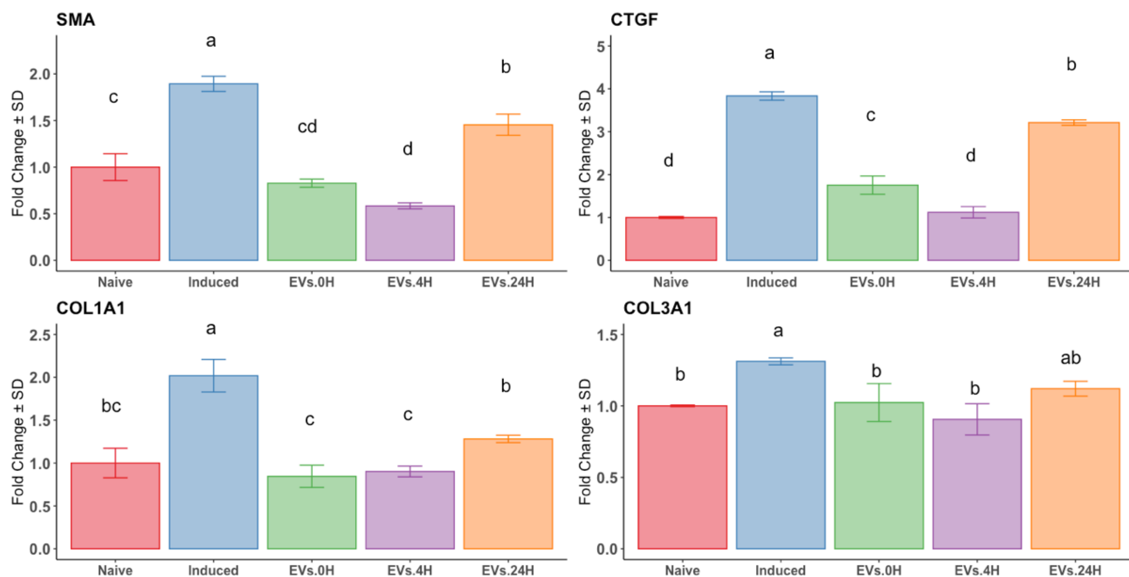

Figure 5. Relative gene expression analysis by RT-qPCR of fibrotic markers in an endometrial stromal in vitro fibrotic model. Endometrial stromal cells untreated (naïve) or primed with the fibrotic cocktail (induced),exposed to EV derived from AT-MSC preconditioned with TGF-β at time points 0h: green; 4h: purple and 24h hours: orange bars) as described in M&M. Y-axes: fold change of relative expression using the geometric mean of *GAPDH* and *B2M* as housekeeping. Different letters indicate statistically significant differences between groups ($p<0.05$) and the error bar is standard deviation. Three replicates per treatment.

Global transcriptome analysis

At the PCA plot, an evident grouping according to the treatments was formed (Figure 6). A total of 13394 genes were detected with a threshold > 0.5 counts per million (cpm) in at least two samples. For differentially expressed genes (DEG) analysis, we compared the following groups: (i) induced vs. naïve, (ii) EV.4H vs. induced group, and (iii) naïve vs. EV.4H and log2FC > 0.5 or log2FC < -0.5 and -Log10FDR < 0.05 as cut-off criteria (supplementary table 2). Volcano plots and gene ontology(GO) analysis associated with DEG are depicted in supplementary figures 3 and 4. In the first comparison, 1230 genes were upregulated in the induced condition compare to naïve condition. Gene onthology (GO) indicated an enrichment in SMAD-R binding and inflammatory response. In the second comparison,1526 genes were upregulated in EV.4H condition compared to induced condition. GO indicated Interferon type I response. The third comparison 225 genes were upregulated in EV.4H condition vs naïve.

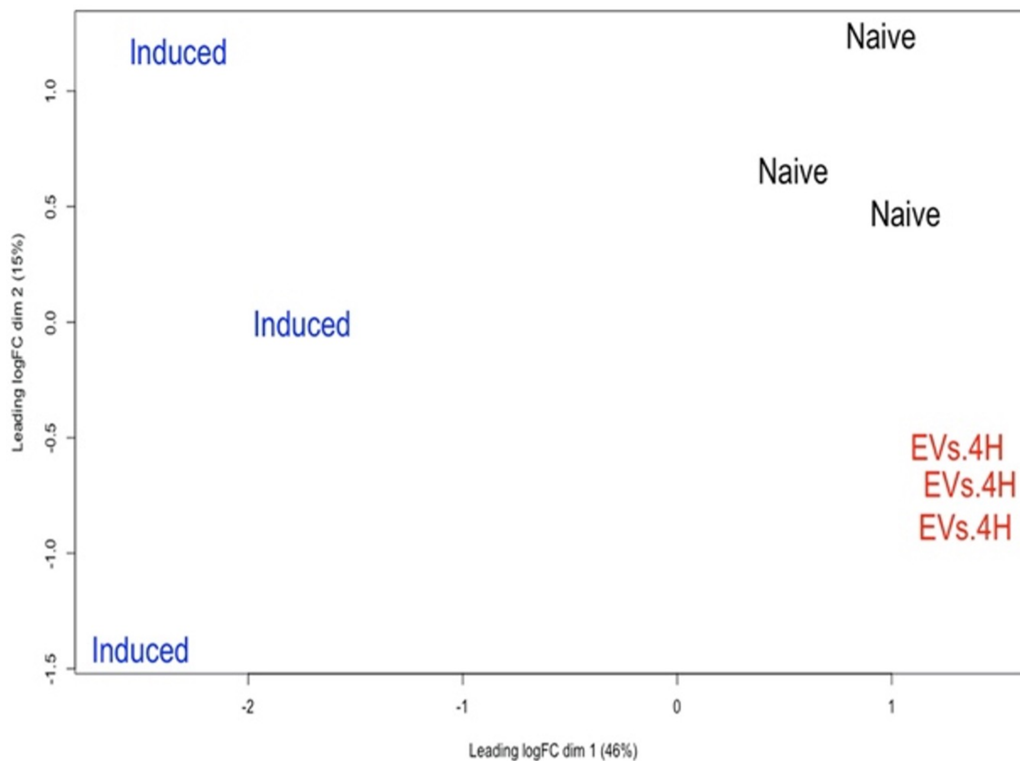

Figure 6. Principal component analysis (PCA) of naïve, induced and EVs.4H. PCA was executed in edgeR software with the normalized Log2-transformed count per million gene values.

To have a clearer view of genes involved in profibrotic or anti-fibrotic events in our experiments, we used an enrichment analysis based on the fibrosis-related gene list of Sun et al. (2022). The top 100 expressed transcripts in the different experimental conditions were analyzed using an assemblage algorithm, which yielded two branches: A (induced) and B (naïve and EV.4H), clearly separated (Figure 7). In addition, the algorithm grouped the pattern of genes into two clusters, where cluster 1 contained the most highly expressed genes in the induced group. The clusters of genes are listed in supplementary table 3. In the heat map, *SMA*, *COL1A1,* and *COL3A1* genes were

upregulated in the induced group and downregulated in the naive and EV.4H groups, confirming our PCR results.

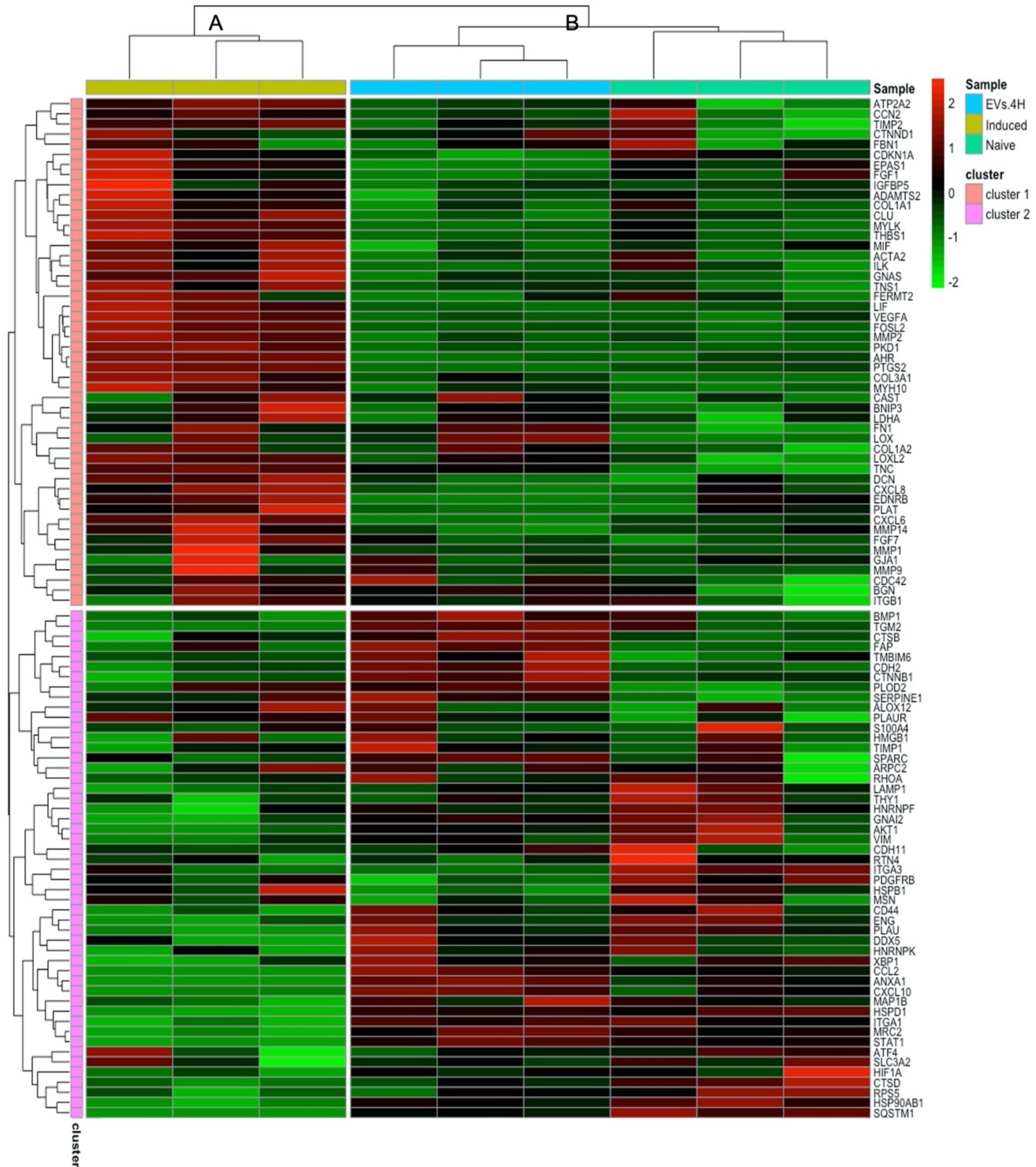

Figure 7**.** Heatmap of hierarchical clustering using z-score for normalized value (Log2-transformed count per million gene values) of fibrosis related genes. Scale of intensity of expression: the greenest the lowest, the reddest the highest

Discussion

This study aimed to investigate the impact of TGF-β preconditioning on AT-MSC's potential use as an anti-fibrotic therapeutic tool in mare endometrosis. In our experiments, a 4-h treatment of AT-MSCs with TGF-β resulted in a notable increase of anti-fibrotic miRNAs in EVs. Conversely, if cells are exposed for longer periods (24 h), there was a shift towards a fibrosis-related mRNA and miRNA pattern. These findings were substantiated by exposing endometrial stromal cells to EVs from preconditioned AT-MSCs, leading to decreased expression of main fibrosis markers.

In healthy tissues, the overproduction of extracellular matrix components (ECM) and the onset of fibrosis are limited by anti-fibrotic miRNAs, like the mir-29 family, which targets 16 genes related to ECM (Cushing et al. 2011). Others reported upregulation of mir29 expression in macrophages (Domingo-Gonzalez et al. 2015) via inhibition of H4K20me3 (Lyu et al. 2018). Other miRNAs, like mir-145, regulate TGFBRII and SMAD3 (Megiorni et al. 2013), and their downregulation is observed in various types of fibrosis; mir-200 plays a crucial role in regulating *ZEB1/SMA* expression (Yang et al. 2012); and mir-486 directly targets SMAD2 (Douvris et al. 2022). In this work, these miRNAs were upregulated at 4 h with a decline at 24 h, suggesting a negative feedback of TGF-β on its effectors to dampen the myofibroblast phenotype of AT-MSCs. Melling et al. (2018) propose a similar negative feedback loop as a regulator of the cancer-associated fibroblast phenotype.

In fibrotic tissues, differentiation into myofibroblasts is accompanied by changes in miRNA levels, which target genes controlling disease progression (Fu et al. 2021). In the case of endometrosis, a specific pattern of miRNA is found for each stage of the pathology, implicating that miRNA are active participants in the processes of focal adhesion and extracellular matrix-receptor interaction (Wójtowicz et al. 2023).

In our work, at 24 h, there was an upsurge of profibrotic miRNAs mir-17, mir-192, mir-199, and mir-433. mir-17 targets SMAD7, which negatively regulates the TGF-β/SMAD2/3 pathway and is linked to cystic and renal fibrosis (Oglesby et al. 2015; Fu et al. 2021). In turn, SMAD7 inhibition leads to increased expression of mir192 (Chung et al. 2010), which acts directly on the ZEB2-E-box repressor of *COL1A2* (Kato et al. 2007). High levels of expression of mir-199 are related to bleomycin-induced lung fibrosis in sheep and murine models and myofibroblast differentiation in oral submucous fibrosis with an increment of *COL1A1* and *COL3A1* (Yuan et al. 2019; Perera et al. 2021), and dysregulation of mir-433 results in inhibition of *AZIN1*, a regulator of ECM production, with subsequent amplification of the TGF-β signaling pathway (Li et al. 2013).

In our experiments, endometrial stromal cells exposed to EVs released by AT-MSCs pretreated with TGF-β for 4 h reduced the expression of fibrotic genes such as *SMA*, *CTGF,* and *COL1A1*. Notably, EVs coming from T0 preconditioning (basal levels, not exposed to TGF-β) led to a similar phenotype of fibrotic genes. Other reports support the anti-fibrotic properties of the AT-MSCs secretome and highlight the key action of miRNAs in reverting the myofibroblast phenotype (Liu et al. 2023). Basalova et al. 2020 note that human AT-MSCs significantly inhibited *SMA* expression in myofibroblasts by transferring anti-fibrotic mir-29 within secreted EVs. In endometrial fibrosis, EV-enclosed mir-145 ameliorated the disease in humans (Zhou et al. 2023). Noticeably, we found overexpression of profibrotic mir-199 in equine AT-MSCs (Navarrete et al. 2020).

Therefore, MSCs may promote or fight fibrosis, depending on their culture or niche conditions. In vitro, this may be attained by fine-tuning the TGF-β preconditioning of AT-MSCs, like in our experiments. At 4 h of TGF-β preconditioning, there is an anti-fibrotic cargo of miRNAs in EVs, while the opposite is observed if cells are treated for 24 h. These results are consistent with those of Salehipour-Bavarsad et al. (2022), who observed a reduction of *SMA* and *COL1A1* levels and inhibition of phosphorylated SMAD2/3 complex proteins in activated hepatic stellate cells using EVs derived from Wharton jelly MSCs pretreated with TGF-β at low doses.

In this research, NGS revealed a different transcriptomic pattern in cells induced with the fibrotic cocktail compared to the control. The DEG in these cells was related to TGF-β stimulation, ECM organization, and regulation of the inflammatory response. Of note was the high expression of *MMP13*, a proteolytic protein related to the progression of chronic inflammation pathologies, and *POSTN*, an ECM protein related to TGF-β stimulus, which has been involved in the induction of the production of collagen type I and myofibroblast differentiation in lung fibrosis Alzobaidi et al. 2022). Other relevant genes in fibrosis overexpressed in the induced condition were *CCN2* or *CTGF*, *ACTA2*, *COL1A2,* and *COL3A1*. Our results are in agreement with those of (Szóstek-Mioduchowska et al. 2023), who showed a differential expression of genes related to inflammation, abnormal metabolism and production of connective tissue in advanced degree of endometrosis. These results validate our endometrial in vitro model, characterized by chronic inflammation and ECM deposition in stromal cells (Trundell 2022).

When these cells were exposed to EVs from TGF-β-pretreated AT-MSCs, several interferon I-related genes were upregulated. Type I interferons are stimulators of antiviral genes, act as regulators of the immune response, and might antagonize TGFβ action (Okada et al. 2018). Activation of the *TL3* gene, a specific inducer of type-I interferons, mitigates the profibrotic phenotype of skin myofibroblasts through autocrine interferon signaling, with disruption of SMAD2/3 phosphorylation and upregulation of their inhibitor, SMAD 7 (Fang et al. 2013). A similar mechanism is observed in interferon-gamma interference with TGF-β response through JAK/STAT in hepatic stellate cells, leading to a reduction of *SMA* and *COL1A1* expression via inhibition of the SMAD3/4 complex and overactivation of SMAD 7 (Weng et al. 2007). Also, the failure of inflammation control in fibrotic diseases has been related to the axis COX2/IFN-lambda, which is controlled by mir-29 via its direct target of the DNA methyltransferase 3a gene, a *COX2* inhibitor (Fang et al. 2012). Accordingly, we found an increase in mir-29 expression in the 4 h EV-treated group and high expression of SMAD7, suggesting a possible mechanism of anti-fibrotic regulation caused by this miRNA cargo.

Conclusions

TGF-β preconditioning of AT-MSCs for 4 h allowed for an increase of anti-fibrotic miRNAs in the cells, reflected in the cargo of their EVs. These vesicles could inhibit the expression of genes related to the myofibroblast phenotype and ECM proteins while increasing the expression of interferon-related genes in an in vitro system of endometrial stromal cells fibrosis. These EVs could become a potential strategy for treating fibrotic diseases such as mare endometrosis.

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Supplementary Section

Extracellular vesicles secreted by equine adipose mesenchymal stem cells preconditioned with transforming growth factor β-1 are enriched in anti-fibrotic miRNAs and inhibit the expression of fibrotic genes in an in vitro system of endometrial stromal cells fibrosis

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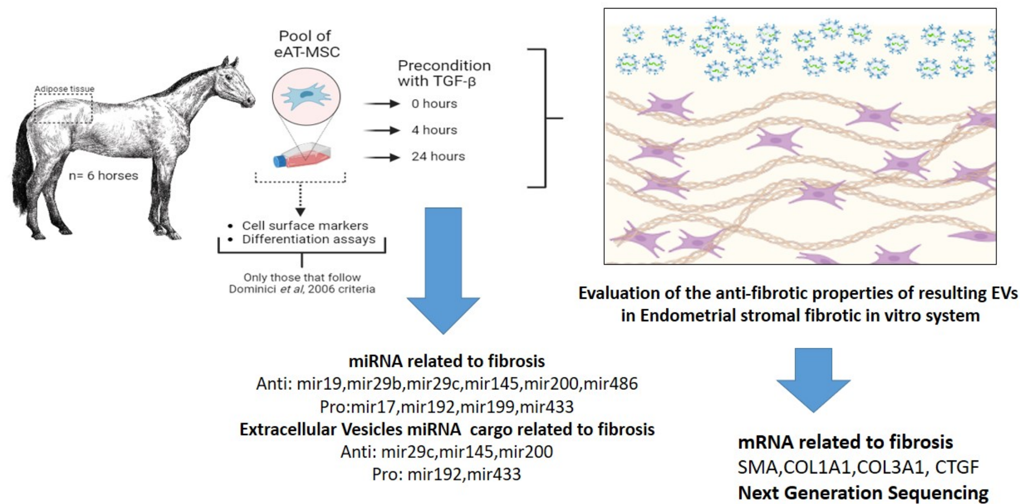

Figure 1. Schematic representation of experimental design. Pooled AT-MSC primary cell line were isolated and pre-conditioned with TGFβ at 0,4 and 24 hours. miRNA related to fibrosis was evaluated using qPCR in cells and EVs cargo. Resulting extracellular vesicles were applied to endometrial stromal fibrotic in vitro system and mRNA related to fibrosis were analyzed and the best condition was subjected to next generation sequencing.

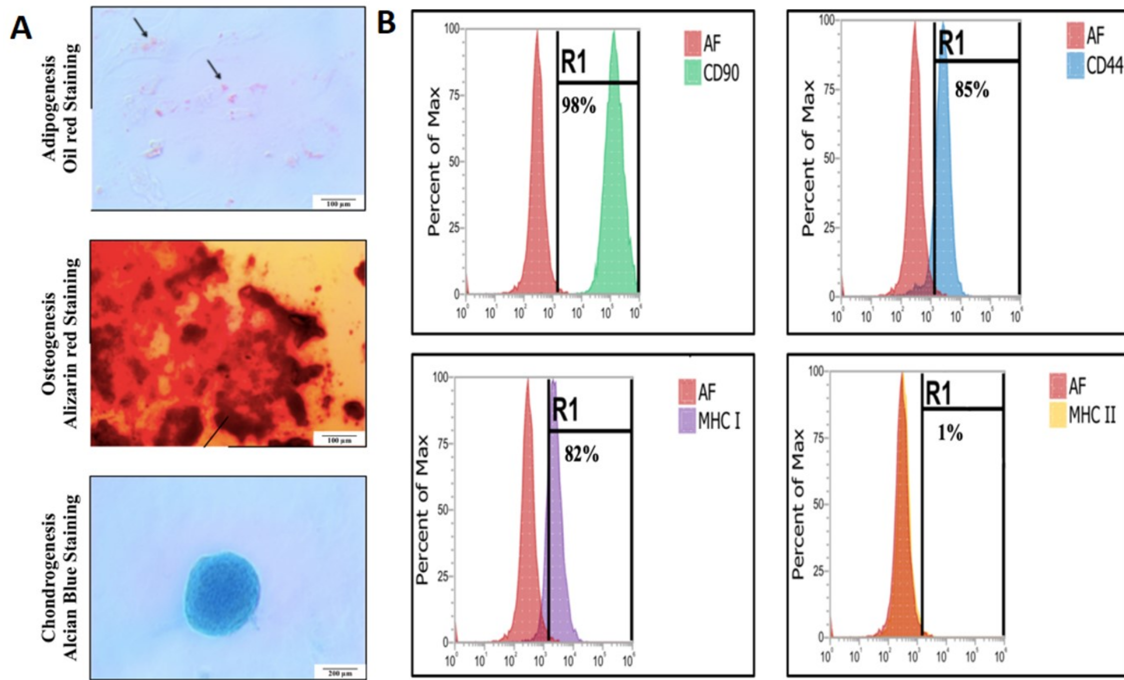

Figure 2. Characterization of Adipose tissue derived Mesenchymal Stem Cell (AT-MSC). (A) Three lineage assay, Adipogenesis is denoted with staining of lipid droplets by Oil red Staining marked with black arrows; Osteogenesis with staining of mineralization of calcium deposit by Alizarin red and Chondrogensis is observed with staining of micromass rich in glycoaminoglycans. (B) Immunophenotyping by flow cytometry of pooled AT-MSC. The cells expressed the classical MSC pattern, positive to CD90, CD44, MHC I and negative to MHC II.

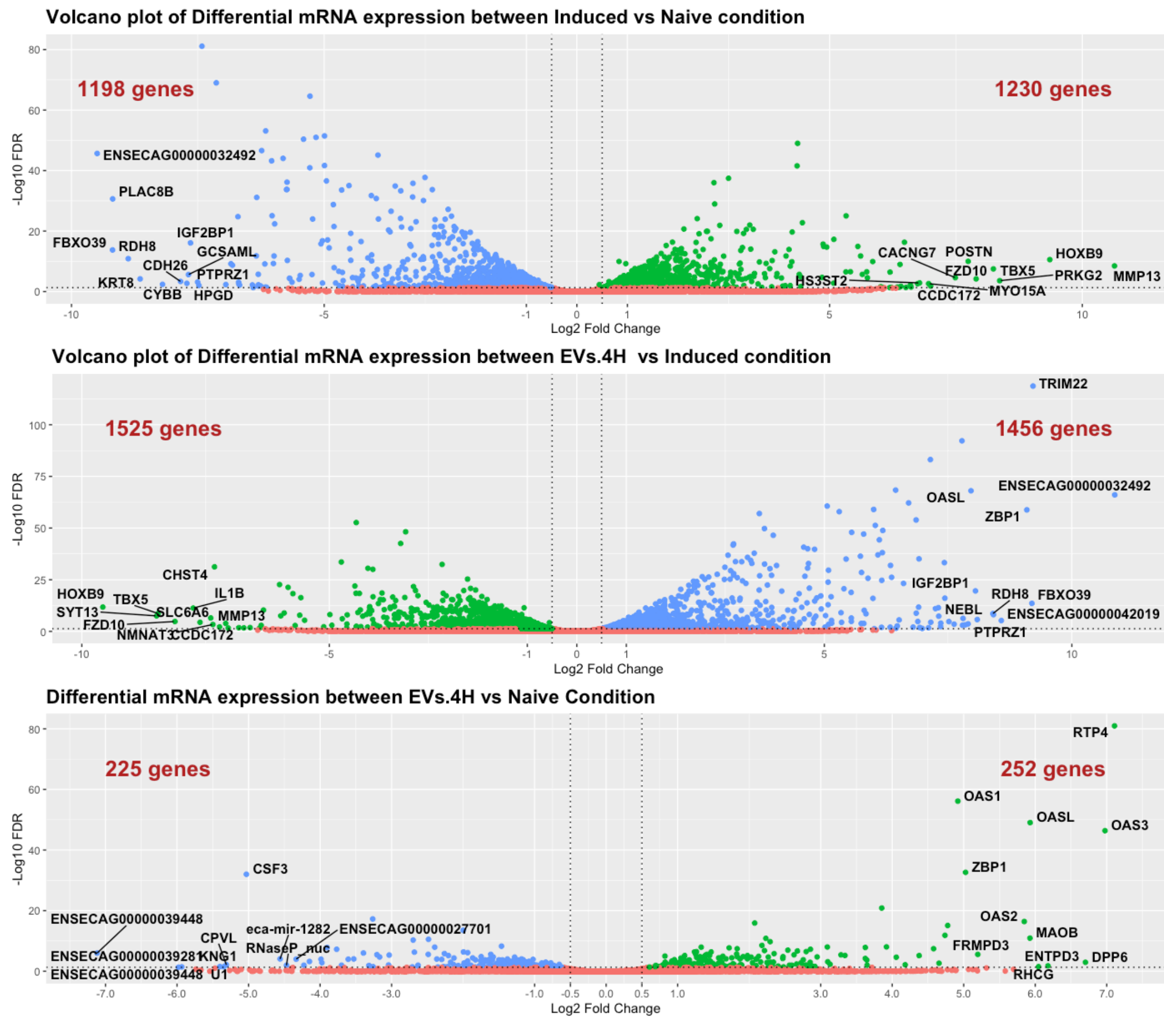

Figure 3. Volcano Plots of the comparative analysis of differentially expressed genes. The number of differentials expressed genes is marked and Top10 expressed genes in each condition are denoted in the plot. The positive grid is representing the first term of the comparation and the second term is represented in the negative grid of the plot.

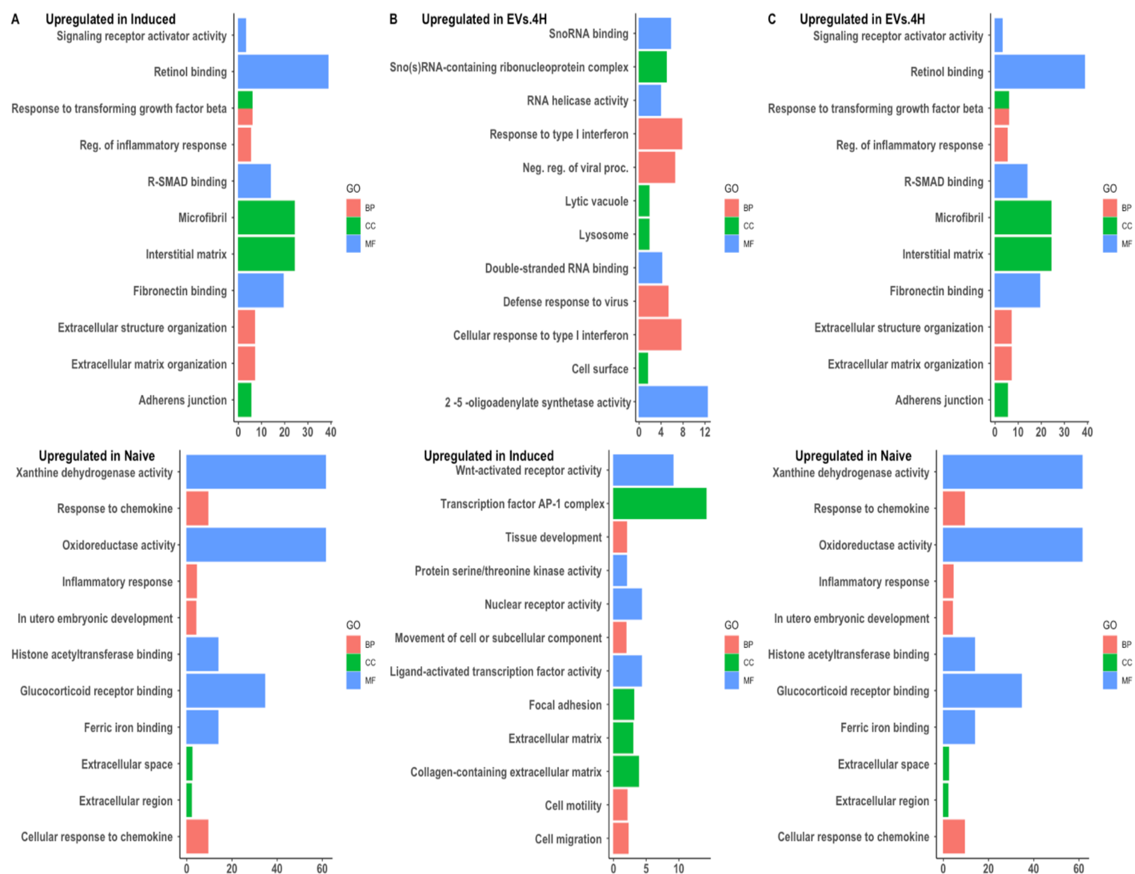

Figure 4. Gene onthology graphs of upregulated genes in each condition. (A) Induced vs Naive, (B) EVs.4H (C) EVs.4H vs Naive. X-axis indicates fold enrichment of Gene Ontology. The Gene ontology domain is represented in red bar for Biological process, green bar for Cellular component and blue for Molecular function.

Supplementary Table 1. List of primers used for the quantification of miRNA relative expression

ID	Forward Primer	Reverse Primer	Mirbase ID	Amplicon pb
Mir 19	GCAGTGTGCAAATCTATGCAAA	GGTCCAGTTTTTT TTTTTTTTTCAGT	MIMAT 0013086	48
mir 29b	CAGTAGCACCATTTGAAATCAG	GGTCCAGTTTTTT TTTTTTTTTAACAC	MIMAT 0012941	48
mir 29c	CAGTAGCACCATTTGAAATCG	GGTCCAGTTTTTT TTTTTTTTTAACC	MIMAT 0012964	48
mir 145	GTCCAGTTTTCCCAGGAATC	AGGTCCAGTTTTT TTTTTTTTTTAGG	MIMAT 0013064	48
mir 200	CAGTAACACTGTCTGGTAACG	GGTCCAGTTTTTT TTTTTTTTTACATC	MIMAT 0012909	48

mir 486	GCAGTCCTGTACTGAGCTG	GTCCAGTTTTTTTTTTTTTTCTCG	MIMAT0013186	48
mir 17	GCAAAGTGCTTACAGTGCAG	GGTCCAGTTTTTTTTTTTTTCTAC	MIMAT0013084	48
mir 192	CAGCTGACCTATGAATTGACA	TCCAGTTTTTTTTTTTTTGGCT	MIMAT0013049	48
mir 199	GCCCAGTGTTCAGACTAC	GTCCAGTTTTTTTTTTTTTGAACAG	MIMAT0012960	48
mir 214	ACAGCAGGCACAGACA	CAGGTCCAGTTTTTTTTTTTTTTACT	MIMAT0012963	48
mir 433	CATGATGGGCTCCTCG	GGTCCAGTTTTTTTTTTTTTTACAC	MIMAT0013158	48
Snord43	GAGCTTATTGACGGGCGGACAGAGACTCTGTGCTGATTGTCACGTTCT	GGTCCAGTTTTTTTTTTTTTTCACAT	XR_002804811.1	60
Cel-mir 39	GTCACCGGGTGTAAATCAG	GGTCCAGTTTTTTTTTTTTTCAAG	MIMAT0000010	60

Supplementary Table 2. Differential expressed genes among experimental groups compared

Comparison	Differential expressed genes	
	Upregulated	Down Regulated
Induced vs Naive	1230	1198
EVs.4H vs Induced	1526	1456
EVs.4H vs Naive	225	252

Supplementary Table 3. Top 100 fibrosis-related genes represented in the heatmap and their respective cluster of aggrupation

Gene	Cluster
ACTA2	1
ADAMTS2	1
AHR	1
AKT1	2
ALOX12	2
ANXA1	2
ARPC2	2
ATF4	2

ATP2A2	1
BGN	1
BMP1	2
BNIP3	1
CAST	1
CCL2	2
CCN2	1
CD44	2
CDC42	1
CDH11	2
CDH2	2
CDKN1A	1
CLU	1
COL1A1	1
COL1A2	1
COL3A1	1
CTNNB1	2
CTNND1	1
CTSB	2
CTSD	2
CXCL10	2
CXCL6	1
CXCL8	1
DCN	1
DDX5	2
EDNRB	1
ENG	2
EPAS1	1
FAP	2
FBN1	1
FERMT2	1
FGF1	1
FGF7	1
FN1	1
FOSL2	1
GJA1	1
GNAI2	2
GNAS	1
HIF1A	2
HMGB1	2
HNRNPF	2
HNRNPK	2
HSP90AB1	2

HSPB1	2
HSPD1	2
IGFBP5	1
ILK	1
ITGA1	2
ITGA3	2
ITGB1	1
LAMP1	2
LDHA	1
LIF	1
LOX	1
LOXL2	1
MAP1B	2
MIF	1
MMP1	1
MMP14	1
MMP2	1
MMP9	1
MRC2	2
MSN	2
MYH10	1
MYLK	1
PDGFRB	2
PKD1	1
PLAT	1
PLAU	2
PLAUR	2
PLOD2	2
PTGS2	1
RHOA	2
RPS5	2
RTN4	2
S100A4	2
SERPINE1	2
SLC3A2	2
SPARC	2
SQSTM1	2
STAT1	2
THBS1	1
THY1	2
TIMP1	2
TIMP2	1
TMBIM6	2

TNC	1
TNS1	1
VEGFA	1
VIM	2
XBP1	2

Supplementary Table 4. Top 30 fibrosis-related genes differentially expressed in Treated vs Induced condition

ID	logFC	FDR	diffexpressed
KRT8	7.791112551	0.001082406	UpRegulated in Treated Condition
SPP1	6.337185771	1.23E-32	UpRegulated in Treated Condition
CD274	4.938398695	1.44E-11	UpRegulated in Treated Condition
KIF1A	4.928823078	6.97E-18	UpRegulated in Treated Condition
TGM2	4.805735951	2.05E-15	UpRegulated in Treated Condition
WNT4	4.592937324	0.000000865	UpRegulated in Treated Condition
AREG	3.944098796	7.52E-10	UpRegulated in Treated Condition
DKK1	3.777014726	0.000157654	UpRegulated in Treated Condition
ENPP1	3.712297829	0.0000281	UpRegulated in Treated Condition
SLC26A5	3.281675782	0.000186271	UpRegulated in Treated Condition
PTGER4	3.211775745	0.000000112	UpRegulated in Treated Condition
IL15	3.02663195	1.52E-10	UpRegulated in Treated Condition
AIM2	2.95033567	2.27E-08	UpRegulated in Treated Condition
FGF19	2.872151716	0.00000582	UpRegulated in Treated Condition
PTGS2	2.853316412	0.00000788	UpRegulated in Treated Condition
TBX5	-8.45861914	2.29E-09	UpRegulated in Induced Condition
IL1B	-7.749401572	5.06E-12	UpRegulated in Induced Condition
MMP13	-7.395377065	3.20E-07	UpRegulated in Induced Condition

BCHE	-5.832774533	4.83E-22	UpRegulated in Induced Condition
POSTN	-5.597908322	1.47E-08	UpRegulated in Induced Condition
MMP12	-4.738207948	7.73E-07	UpRegulated in Induced Condition
ID1	-4.46135263	3.73E-19	UpRegulated in Induced Condition
NPR1	-4.422324953	7.52E-10	UpRegulated in Induced Condition
IL6	-4.220673366	2.91E-31	UpRegulated in Induced Condition
ALOX15	-4.102824675	0.000943808	UpRegulated in Induced Condition
ADRB1	-4.090274999	0.000819479	UpRegulated in Induced Condition
MMP9	-3.910597862	5.10E-16	UpRegulated in Induced Condition
ADIPOQ	-3.802737325	0.001752555	UpRegulated in Induced Condition
PTGDR	-3.786327035	0.000419962	UpRegulated in Induced Condition
JAG1	-3.682943239	9.75E-13	UpRegulated in Induced Condition

Supplementary Table 5. Top 30 fibrosis-related genes differentially expressed in Induced vs Wildtype condition

ID	logFC	FDR	diffexpressed
MMP13	10.6370521	3.32E-09	UpRegulated in Induced Condition
TBX5	8.24042321	3.23E-08	UpRegulated in Induced Condition
POSTN	7.74165808	1.03E-10	UpRegulated in Induced Condition
MMP12	6.3936809	1.09E-09	UpRegulated in Induced Condition
ADIPOQ	5.74865676	0.0000273	UpRegulated in Induced Condition
BMP7	5.41252932	2.71E-07	UpRegulated in Induced Condition
MMP9	5.32644871	9.5E-26	UpRegulated in Induced Condition
ITGB6	5.3224382	3.06E-07	UpRegulated in Induced Condition
BCHE	4.88247323	1.18E-15	UpRegulated in Induced Condition
IL1B	4.42806349	0.0000136	UpRegulated in Induced Condition
IL33	3.61134531	1.63E-10	UpRegulated in Induced Condition
NPR1	3.44367316	0.00000946	UpRegulated in Induced Condition
S100A12	3.20808255	0.00000134	UpRegulated in Induced Condition
IL1A	2.83866645	9.36E-10	UpRegulated in Induced Condition
COL4A3	2.71966151	0.00666639	UpRegulated in Induced Condition

KRT8	-8.6419137	0.000065	UpRegulated in Wildtype Condition
CYBB	-8.1987535	0.00415001	UpRegulated in Wildtype Condition
SPP1	-6.3344737	7.61E-32	UpRegulated in Wildtype Condition
PTX3	-5.2411713	0.00000228	UpRegulated in Wildtype Condition
NKX2-5	-3.8082575	0.00888408	UpRegulated in Wildtype Condition
CD274	-3.7699064	0.00000172	UpRegulated in Wildtype Condition
TGM2	-3.4582229	6E-09	UpRegulated in Wildtype Condition
CAPN5	-3.3898697	4.49E-08	UpRegulated in Wildtype Condition
HMOX1	-2.8636138	5.25E-16	UpRegulated in Wildtype Condition
IL13RA2	-2.7871623	0.00297246	UpRegulated in Wildtype Condition
AIM2	-2.747034	0.00000124	UpRegulated in Wildtype Condition
TNFRSF8	-2.7384918	1.49E-18	UpRegulated in Wildtype Condition
SLC26A5	-2.7076254	0.00548891	UpRegulated in Wildtype Condition
ERG	-2.5627801	8.03E-08	UpRegulated in Wildtype Condition
SFRP2	-2.4505027	0.02748794	UpRegulated in Wildtype Condition

Authors contribution:
YW: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. AM: Investigation, Methodology, Validation, Conceptualization, Writing – review & editing. FN: Methodology, Validation, Writing –review & editing. PP: Writing – review & editing, Investigation, Methodology. LM-P: Writing – review & editing, Investigation, Methodology.CJ: Writing – review & editing, Investigation, GR: bioinformatics, cluster supervision. LR-A: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. FOC: Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – review & editing, Conceptualization, Data curation, Formal analysis.